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Chemical and Kinetic Evidence for an Essential Histidine Residue in the Electron Transfer from Aromatic Donor to Horseradish Peroxidase Compound I*

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Dipak Kr. Bhattacharyya, Uday Bandyopadhyay, and Ranajit K. Banerjee‡

From the Department of Physiology, Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Road, Calcutta 700032, India

Horseradish peroxidase, when incubated with diethyl pyrocarbonate (DEPC), a histidine-specific reagent, shows time-dependent inactivation to oxidize aromatic electron donor, guaiacol. The inactivation follows pseudo-first order kinetics with a second order rate constant of 0.67 min^{-1} M^{-1}. The pH dependence of inactivation shows an inflection point at 6.02, indicating histidine derivatization by DEPC. A difference spectrum of modified *versus* native enzyme shows a peak at 244 nm for *N*-carbethoxyhistidine that is diminished by hydroxylamine. Stoichiometric studies indicate that out of 2 histidine residues modified, one is responsible for inactivation. A plot of log reciprocal half-time of inactivation against log DEPC concentration suggests that only 1 histidine is essential. From the computer-stimulated structure of horseradish peroxidase, we tentatively suggest that this critical histidine is most likely distal histidine 42.

Binding studies show that this histidine is not involved in guaiacol binding. Modified enzyme forms compound I with H_2O_2 but not compound II, suggesting a block of electron transfer process. Modified compound I cannot oxidize guaiacol as evidenced by the absence of donor-induced spectral shift from 408 nm, suggesting a block of electron transfer from bound donor to compound I. We suggest that this tentatively identified distal histidine controls aromatic donor oxidation by regulating electron transport without affecting donor binding or compound I formation.

Horseradish peroxidase (HRP[1]; EC 1.11.1.7; donor-H_2O_2 oxidoreductase) catalyzes the oxidation of aromatic donors, halides, and pseudohalides by H_2O_2 (Saunders *et al.*, 1964; Björkstein, 1970; Morrison and Schonbaum, 1976; Dunford and Stillman, 1976; Dunford, 1982). The reaction mechanism has been explored (see Yamazaki *et al.* (1981) for a review), and distinct catalytic intermediates are formed during catalysis (Chance, 1949; George, 1952; Yamazaki *et al.*, 1960). HRP, a ferric protoporphyrin IX, reacts with H_2O_2 to form compound I, which is 2 oxidation above the resting enzyme and contains an oxoferryl porphyrin-π cation radical (Dolphin *et al.*, 1971; Penner-Hahn *et al.*, 1986). During oxidation of aromatic donors, a one-electron reduction of compound I produces a second intermediate, compound II, containing the oxoferryl imidazole, but its porphyrin is reduced to the neutral state (Dunford, 1982; Frew and Jones, 1984). Compound II is further reduced by one-electron reduction from the donor to the native enzyme to complete the catalytic cycle. Very recently, Baek and Van Wart (1990) have reported the evidence of "compound 0" likely to be produced as an intermediate preceding compound I.

Hemoproteins show diversity in biological functions while retaining an essentially unaltered heme prosthetic group containing iron protoporphyrin IX. The fine structural difference in the folded polypeptide chain around the heme cavity and their interaction with the heme and the substrate appear to dictate the specificity of the catalytic function of the heme iron center. Recently, much attention is being paid to explore the mechanism of protein control of heme reactivity in a variety of hemoproteins including cytochrome P-450, hemoglobin, myoglobin, catalase, and peroxidase. Site-directed mutagenesis has been applied in cytochrome *c* peroxidase to know the requirement of fine structure in catalysis (Fishel *et al.*, 1987; Smulevich *et al.*, 1991). Chemical modification studies have also furnished valuable information on this aspect. Evidence for the essential role of histidine in cytochrome *c* peroxidase-catalyzed cytochrome *c* oxidation (Bosshard *et al.*, 1984), chloroperoxidase-catalyzed chlorination (Blanke and Hager, 1990), and HRP-catalyzed iodide oxidation (Bhattacharyya *et al.*, 1992) has been reported by chemical modification studies. However, our knowledge is still immature on the role of heme-peripheral fine structure involved in the binding of various electron donors, formation of catalytic intermediates, and the transfer of electrons from donor binding site to the heme ferryl group during oxidation.

HRP catalyzes the oxidation of various phenolic substrates such as guaiacol, commonly used for the assay of its activity (Hosoya, 1960; Saunders *et al.*, 1964; Santimone, 1975). On the basis of optical difference spectroscopy (Critchlow and Dunford, 1972a, 1972b; Schonbaum, 1973; Schejter *et al.*, 1976; Ricard and Job, 1974; Paul and Ohlsson, 1978), hyperfine-shifted NMR spectroscopy (Morishima and Ogawa, 1978), and nuclear magnetic relaxation studies (Burns *et al.*, 1975; Leigh *et al.*, 1975; Schejter *et al.*, 1976), a number of workers have reported that the enzyme binds aromatic donors to form a 1:1 enzyme-donor complex. The binding presumably occurs at some aromatic amino acid residue with hydrophobic bonds (Paul and Ohlsson, 1978) at a distance of 5.8–11.2 Å from the heme iron center (Burns *et al.*, 1975; Leigh *et al.*, 1975; Schejter *et al.*, 1976). Sakurada *et al.* (1986), using NMR relaxation methods and transferred nuclear Overhauser effect techniques, have suggested that aromatic donors bind in the vicinity of heme-peripheral 8-methyl group by hydrophobic

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‡ To whom all correspondence should be addressed. Tel.: 91-33-4733491; Fax: 91-33-4730284.

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

interaction probably with Tyr-185 and by hydrogen bonding with adjacent Arg-183. Recently, a model based on heme alkylation studies has been proposed by Ortiz de Montellano (1987) on the active site topology of the HRP. These studies indicate that electrons from the substrates are transferred to the exposed heme edge in the vicinity of the δ-meso carbon before being transferred to the heme iron during oxidation (Ator and Ortiz de Montellano, 1987; Ator *et al.*, 1987). It is thus conceivable that due to the very high electron-attracting property of the compound I-π cation radical (Yamazaki *et al.*, 1981), the electron may be transferred from the bound donor to the δ-meso carbon (Ortiz de Montellano, 1987) before reacting with compound I. The interaction of donor with compound II is slower than its reaction with compound I (Chance, 1952) and thus forms a rate-limiting step in catalysis (Dunford and Stillman, 1976). Therefore, a route may be formed between the aromatic donor binding site to the heme ferryl group for electron flow, and Schonbaum *et al.* (1971) have suggested the plausible role of distal histidine in this process. The sequence homology studies on horseradish peroxidase, cytochrome *c* peroxidase, lignin peroxidase, four isozymes of turnip peroxidase, and chloroperoxidase have shown the presence of highly conserved histidine residue in the heme distal pocket (Tien and Tu, 1987; Blanke and Hager, 1990). This invariant histidine residue in concert with arginine residue may take part in compound I formation (Finzel *et al.*, 1984; Poulos and Kraut, 1980; Poulos, 1982). Kinetic studies on the reaction of HRP compound I with a variety of electron donors also suggest the involvement of distal histidine (Dunford, 1982). Although the role of distal histidine in HRP-catalyzed oxidation of aromatic donors has been suggested, direct evidence for its involvement is still lacking. It is not clear yet whether this histidine in HRP is involved in the binding of aromatic donor, in the formation of catalytic intermediates, or in the electron transfer during donor oxidation.

In this communication, we present direct evidence by chemical modification of HRP with histidine-specific reagent, diethyl pyrocarbonate (Miles, 1977; Takeuchi *et al.*, 1986; Sams and Mathews, 1988; Blanke and Hager, 1990), that out of a total of 3 histidine residues of HRP, two are modified and only one is essential for oxidation of aromatic electron donor, guaiacol. This histidine, tentatively identified as histidine 42 from the computer-simulated structure of HRP (Sakurada *et al.*, 1986), is neither involved in guaiacol binding nor is it obligatory in the formation of compound I, but it takes part in controlling the electron flow from the bound donor to the catalytic intermediates of HRP.

EXPERIMENTAL PROCEDURES

Horseradish peroxidase (A_{403}/A_{278} = 3), diethyl pyrocarbonate, hydroxylamine, and guaiacol 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-visible recording spectrophotometer at 30 ± 2 °C. Peroxidase-catalyzed guaiacol oxidation was monitored by following the formation of tetraguaiacol at 470 nm as described earlier (De and Banerjee, 1986). The reaction mixture contained in a final volume of 3 ml: 50 mM sodium phosphate buffer, pH 7.0, 10 mM guaiacol, a suitable amount of enzyme, and 0.3 mM H_2O_2 added last to start the reaction. Spectral studies were carried out in a Shimadzu UV-2201 computerized spectrophotometer at 30 ± 2 °C using quartz cuvettes of 1-cm light path. CD spectral studies were done in a Jasco CD spectrometer, model J20.

Modification of Horseradish Peroxidase with DEPC—Fresh solutions of DEPC were prepared with absolute alcohol by dilution of the original DEPC stock just before use. The exact concentration of the stock solution was calculated from the increase in 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 guaiacol oxidation after incubation with varying millimolar concentration of DEPC was determined by the standard assay method described above. The incubation was performed at 30 ± 2 °C; aliquots were removed and immediately quenched with 500 mM potassium phosphate buffer, pH 4.5, at 4 °C, which prevents further inactivation at least for 1 h. For pK_a determination, 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} and assuming the molecular mass of HRP to be 40 kDa. The reaction was initiated by the addition of DEPC and 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.

Difference Spectrum Measurement—For measurement of the difference spectrum of enzyme-guaiacol *versus* enzyme, both the reference and sample cuvettes were filled up with 1 ml of enzyme solution (10 μM) for base-line tracing. This is followed by the addition of a small volume of the ligand to the sample cuvette with concomitant addition of alcohol into the reference cuvette (as guaiacol was diluted in absolute alcohol). The contents were stirred with a plastic rod before recording the spectrum. Equilibrium dissociation constant (K_D) for the complex formation was calculated from the following expression (Schejter *et al.*, 1976),

$$1/\Delta A = (K_D/\Delta A_a)1/S + 1\Delta A_a \quad (1)$$

where ΔA is the change in absorbance, S is the substrate concentration, and ΔA_a is the change in absorbance at saturating concentration of the substrate.

RESULTS

Time Course of Inactivation of HRP by DEPC—Diethyl pyrocarbonate is hydrolyzed in aqueous solution. To calculate the kinetics of inactivation by DEPC, this hydrolysis must be considered. The rate of inactivation of HRP-catalyzed guaiacol oxidation is expressed by the following equation,

$$\ln A/A_0 = -(k/k')I_0(1 - e^{-k't}) \quad (2)$$

where (A/A_0) is the measure of activity remaining at time t, I_0 is the initial concentration of DEPC, k is the second order rate constant of inhibition of enzymatic activity by DEPC, and k' is the pseudo-first order rate constant of the hydrolysis of DEPC in buffer (Gomi and Fujioka, 1983). Phosphate buffer was best to retard the hydrolysis of DEPC. The value of k' for DEPC measured in 0.1 M phosphate buffer, pH 6.0, at 30 ± 2 °C was found to be 18.3×10^{-3} min^{-1} ($t_{1/2}$ = 30 min). The inactivation of HRP as measured by guaiacol oxidation follows a pseudo-first order kinetics with respect to DEPC concentration (Fig. 1*A*). The control enzyme, when incubated with alcohol (8%) required to dilute DEPC, showed no loss of activity. The k values obtained from the slope of the curves in Fig. 1*A* when plotted against DEPC concentration produce a straight line (Fig. 1*B*), indicating a bimolecular reaction between the enzyme and DEPC without reversible complex formation (Church *et al.*, 1985). From the slope of this curve, a second order rate constant was calculated to be 0.67 min^{-1} M^{-1}.

The pH Dependence of Inactivation—Diethyl pyrocarbonate modifies deprotonated amino acid residues only, and thus the pH dependence of inactivation provides valuable information about the nature of the modified residue resulting in inactivation of HRP to oxidize guaiacol. The extent of inactivation depends on the pH according to the following equation.

$$k = k_{max}/(1 + [H^+]/K_a) \quad (3)$$

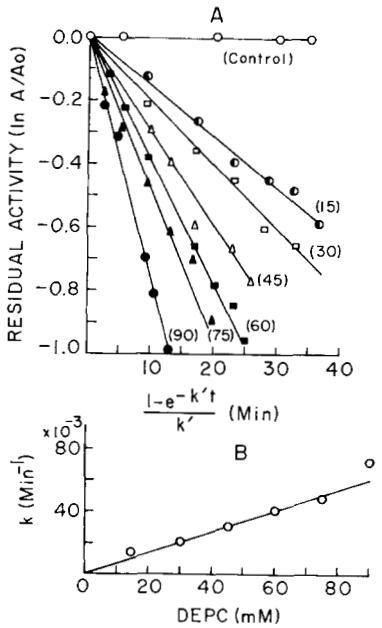


FIG. 1. **Kinetics of the inactivation of HRP by DEPC.** *A*, calculation of the pseudo-first order rate of inactivation for guaiacol oxidation. HRP (2.5 μM) was incubated with different concentrations (mM) of DEPC at 30 ± 2 °C in 100 mM potassium phosphate buffer, pH 6.0, and the data were plotted according to Equation 2. The curves were best fit by least square analysis. The mM concentrations of DEPC used are indicated in *parentheses*. *B*, determination of the second order rate constant of inactivation of guaiacol oxidation by HRP. The slopes of the straight lines obtained in *panel A* were plotted against concentration of DEPC. The slope of this curve indicates the second order rate constant of inactivation, which is 0.67 min^{-1} M^{-1}.

Equation 3 can be rearranged to the linear form as

$$k(H^+) = K_a(k_{max}) - K_a k \quad (4)$$

where K_a is the dissociation constant of the reacting group and k_{max} is the pseudo-first order rate constant of the unprotonated reacting group (Takeuchi *et al.*, 1986). The pseudo-first order rate constant of inactivation was plotted against pH (Fig. 2*A*) according to Equation 3. The *open circle* represents the experimental data, and the *solid line* indicates the theoretical curve. By plotting $k(H^+)$ against k (values taken from Fig. 2*A*) according to Equation 4, a *straight line* is obtained (Fig. 2*B*) that gives a value of $k_{max} = 55 \times 10^{-3}$ min^{-1} and pK_a = 6.02. The apparent pK_a value of 6.02 strongly suggests that inactivation of HRP by DEPC is due to modification of histidine residues.

Specificity of DEPC Modification—Diethyl pyrocarbonate can also react with other nucleophilic groups, particularly cysteine, lysine, and tyrosine residues of proteins (Miles, 1977). As all the cysteine residues in HRP are present in disulfide linkage (Welinder, 1979), the reaction of DEPC with the cysteine does not arise. The optical difference spectra of DEPC-modified HRP against native HRP shows a peak at 244 nm (*inset* of Fig. 3*A*) but no significant trough at 278 nm. This indicates derivatization of histidine only and not of tyrosine residue (Burstein *et al.*, 1974; Miles, 1977). On addition of hydroxylamine to the modified HRP, the absorbance at 244 nm decreased drastically (Table I), indicating reversibility of the DEPC derivatization of HRP. This is associated with reactivation of the enzyme also (Table I). DEPC can also form ethoxyformyllysine, but the reaction is not reversed by hydroxylamine (Miles, 1977), thereby eliminating the involvement of lysine in the inactivation of HRP. These results therefore strongly suggest that carbethoxyformylation of histidine residues is specifically responsible for the loss of enzyme activity to oxidize guaiacol.

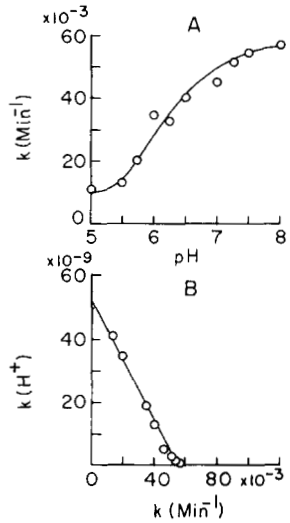


FIG. 2. **The pH dependence of the inactivation rate of HRP with DEPC.** *A*, HRP (2.5 μM) was incubated with 60 mM DEPC in 100 mM potassium phosphate buffer, pH 5–8. The pseudo-first order rate constants obtained were plotted against pH. The curve obtained was a theoretical one. *B*, the experimentally obtained pseudo-first order rate constants were plotted according to Equation 4. The pK_a value obtained from the slope was 6.02, and the $(K_{obs})_{max}$ value was 0.055 min^{-1}.

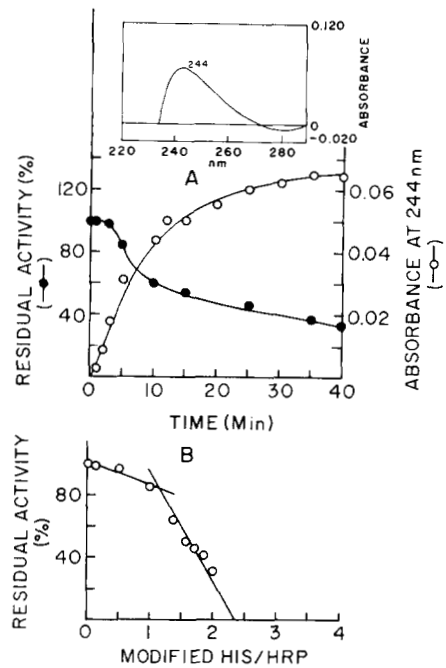


FIG. 3. **Correlation between inactivation and histidine modification.** *A*, HRP (10 μM) was incubated with 60 mM DEPC in 100 mM potassium phosphate buffer, pH 6. The percent of the residual activity and the increase in the absorbance at 244 nm as a measure of modification were plotted as a function of time as indicated. *Inset* shows the UV difference absorption spectrum of the native *versus* DEPC-modified HRP. *B*, shows the correlation between the number of histidine residues modified per HRP molecule and the extent of residual activity. 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.

Correlation between the Extent of Inactivation and the Number of Histidine Residues Modified—HRP contains 3 histidine

TABLE I

Reversal of DEPC modification by hydroxylamine

HRP (7.5 μM) was incubated with 60 mM DEPC in 100 mM potassium phosphate buffer, pH 6.0, and the change in absorbance against control enzyme was monitored up to 40 min. At the end of 40 min, hydroxylamine was added, and absorption was noted. The content of the cuvette was 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 for the assay of enzyme activity. Both the enzymes showed 80% of the original activity, which is now considered as 100%.

	Absorbance at 244 nm	Residual activity
		%
Control HRP	0	100
DEPC-modified HRP	0.065	32
DEPC-modified HRP treated with 0.6 M NH_2OH	0.008	91

residues in its primary structure (Welinder, 1979). Fig. 3*A* represents the correlation between the extent of enzyme inactivation by DEPC and the extent of histidine modification. The figure shows that when 50% of total histidine residues were modified after 5 min of incubation with DEPC, there was very little loss of guaiacol oxidation (activity remaining was 84%). On subsequent modification of the 2nd histidine residue as evidenced by a further increase in 244-nm absorption, the activity declined gradually. When almost 70% of the activity is lost, the modification of the 2nd histidine is almost complete. These data suggest that HRP contains two types of histidine residues. The first one reacts very quickly with DEPC but is not involved in guaiacol oxidation. The second one reacts slowly but appears to be essential to oxidize guaiacol. This has been confirmed in Fig. 3*B*, which shows a plot of residual activity against the number of histidines modified, calculated from the absorbance change at 240 nm ($\epsilon_{240} = 3200$ M^{-1} cm^{-1}) according to Ovadi *et al.* (1967). The data indicate that there is 1 nonessential histidine residue, the modification of which does not cause any significant loss of guaiacol oxidation. However, when the next 1.5 histidine residues are modified, the enzyme loses 50% of its activity. The enzyme is completely inactivated with the modification of 2.3 histidine residues (extrapolation of the line). These data suggest that the second slow reacting histidine residue is required for the oxidation of guaiacol by HRP.

Evidence for an Essential Histidine Residue—A kinetic analysis was further made to find out the number of essential histidine residues required for guaiacol oxidation. This could be determined from a plot of log (reciprocal of the half-time of inactivation) *versus* log (concentration of DEPC), assuming that DEPC would react with essential histidine residue to inactivate HRP (Blanke and Hager, 1990). The analysis also assumes that if there were more than 1 histidine, they react simultaneously, which, however, may not be true considering the complex tertiary structure of the protein. However, in both cases, such a plot should yield a straight line with slope n, where n is the number of inhibitor molecules reacting with the active site of the enzyme to form the enzyme-inhibitor complex (Levy *et al.*, 1963; Takeuchi *et al.*, 1986; Blanke and Hager, 1990). As shown in Fig. 4, the plot yields a straight line with a slope of 1.2 close to unity. This indicates that inactivation is due to reaction of a single histidine residue essential for guaiacol oxidation. The identity of this essential histidine residue has been clarified under "Discussion."

Structural Integrity of the DEPC-modified HRP—Circular dichroism (CD) spectra of the DEPC-modified HRP were compared with that of the native HRP (Fig. 5). The positive

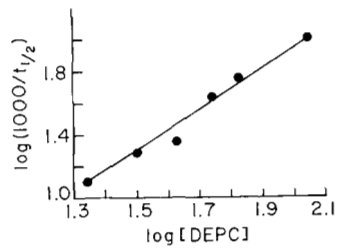


FIG. 4. **The apparent inactivation order of HRP in relation to DEPC concentration.** The conditions are the same as indicated in the legend of Fig. 1. The slope of the line, $n = 1.2$, indicates that modification of a single histidine residue is necessary for inactivation of the HRP.

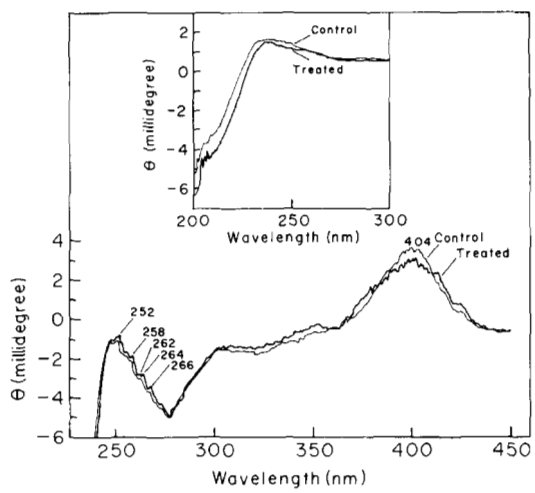


FIG. 5. **CD spectra of native and DEPC-modified HRP.** HRP (5 μM) was incubated with 60 mM DEPC in 100 mM potassium phosphate buffer, pH 6.0, for 40 min, and the excess DEPC was hydrolyzed by adding 50 mM Tris-HCl, pH 8. The control enzyme was treated with the same amount of alcohol (which was needed to dilute DEPC). The enzyme was transferred to a CD quartz cuvette before taking the spectra.

heme CD peak obtained at 404 nm did not alter, indicating no change in the heme site after DEPC modification. The far UV CD spectra, specifically the minima at 221 and 208 nm (Strickland *et al.*, 1968), are the characteristics of α-helical secondary structure (Shoemaker *et al.*, 1988) that showed no change after modification with DEPC (see Fig. 5, *inset*). This suggests that inactivation is not due to alteration of the α-helix. No significant change of the Soret spectrum of the native enzyme at 402 nm and visible spectrum at 500 nm and 640 nm (data not shown) was observed, providing further evidence for unaltered heme and protein structure after modification with DEPC.

Guaiacol Binding in Native and DEPC-modified HRP—In order to investigate the possibility that the loss of guaiacol oxidation following histidine modification is due to altered binding of guaiacol at its binding site, its binding constant (K_D) was therefore measured in native and DEPC-modified enzyme by optical difference spectroscopy (Paul and Ohlsson, 1978; Hosoya *et al.*, 1989). The difference spectra of enzyme-guaiacol complex for native HRP are comparable with those of the DEPC-modified HRP at increasing concentrations of guaiacol and have the same peak at 408 nm (not shown), characteristic of enzyme-guaiacol complex (Hosoya *et al.*, 1989). The K_D for the native HRP-guaiacol complex as calculated from the plot (Fig. 6*A*) and employing Equation 1 was found to be 7.6 mM, which is comparable to the K_D (7.2 mM) obtained for the guaiacol complex of DEPC-modified enzyme

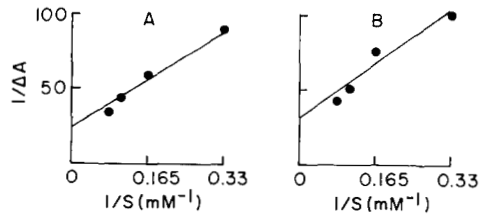


FIG. 6. **Binding of guaiacol with native and modified HRP as measured by optical difference spectroscopy.** HRP (10 μM) was treated with DEPC as described in the legend of Fig. 5. *A*, represents the plot from difference spectra of enzyme-guaiacol *versus* native enzyme, and *B*, represents the same for modified enzyme. The initial and final enzyme concentrations were 10 μM and 9.5 μM, whereas the final guaiacol concentration used was 12 mM. K_D was calculated from the plot of $1/\Delta A$ at 408 nm against 1/guaiacol concentration according to Equation 1 as described under "Experimental Procedures."

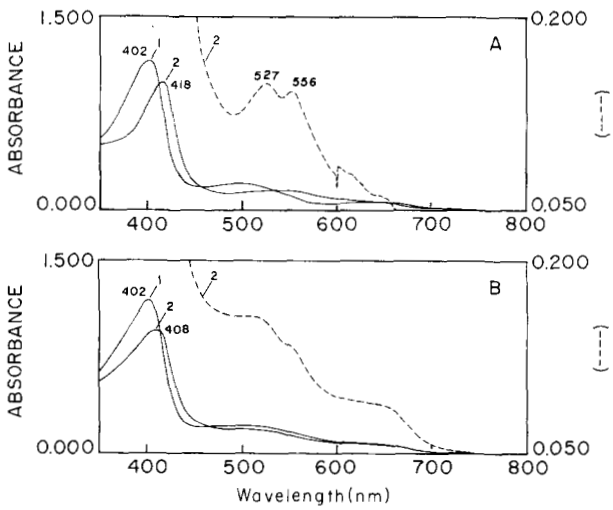


FIG. 7. **Soret and visible spectra of native and DEPC-modified HRP·H_2O_2 complex.** The native and modified enzymes were prepared as described in the legend of Fig. 5. *A*, represents the intermediate (compound II) formation by adding H_2O_2 on native HRP in 100 mM potassium phosphate buffer, pH 6.0. *B*, represents the effect of addition of the same amount of H_2O_2 on DEPC-modified HRP. Enzyme used in both cases was 10 μM (*tracing 1*), and H_2O_2 used was 20-fold over the enzyme concentration (*tracing 2*). *Dashed line* in *A* represents the visible spectrum of compound II (*tracing 2*) having characteristic peaks at 527 and 556 nm that are absent in the visible spectrum of the modified enzyme in *B*.

(Fig. 6*B*). This suggests that DEPC modification of histidine does not affect guaiacol binding.

Formation of Catalytic Intermediates in Native and DEPC-modified HRP—Since catalytic intermediates are essential for one-electron oxidation of guaiacol, the formation of compound I and compound II by H_2O_2 has been studied in native and DEPC-modified HRP. Fig. 7*A* shows compound II formation from native HRP with a 20-fold excess of H_2O_2. Compound II formation is evident from the appearance of the characteristic Soret peak at 418 nm and the visible peaks at 527 and 556 nm (Keilin and Hartree, 1951). DEPC-modified HRP (Fig. 7*B*), under identical conditions, does not show the Soret peak at 418 nm. Instead, it produces a peak at 408 nm, characteristic of compound I formation (Keilin and Hartree, 1951). No well defined peaks at 527 and 556 nm are evident, suggesting that compound II formation is affected by the modification of histidine by DEPC. Moreover, modified compound I, unlike native compound I, never forms compound II on standing for a few minutes by one-electron reduction from the endogenous electron source such as sugar moieties (data not shown). This suggests that this histidine is also involved in the electron transport from the endogenous source during native compound II formation. Fig. 8 shows the spectral evidence for one-electron oxidation of guaiacol by native and modified enzyme. Addition of guaiacol on the preformed native compound I caused its immediate reduction to native enzyme (Fig. 8*A*), whereas the same addition to the DEPC-modified compound I could not reduce it (Fig. 8*B*), suggesting a block of electron flow from the bound donor to compound I. Thus HRP is unable to oxidize guaiacol due to a block of electron transport from the bound substrate to compound I because of carbethoxyformylation of the essential histidine residue.

DISCUSSION

DEPC can selectively modify histidine residues at pH 5.5–8.0 (Miles, 1977; Melchior and Fahrney, 1970). In the present study, we have attempted to identify the functional role of histidine at the active site of HRP in the oxidation of aromatic electron donors such as guaiacol. Incubation of HRP with DEPC resulted in a time-dependent inactivation of HRP to oxidize guaiacol. Kinetic analyses reveal that the inactivation is due to a bimolecular reaction following pseudo-first order kinetics. The rate of inactivation exhibited a pH dependence indicating that modification of a titratable residue with a pK_a value of 6.02 is responsible for inactivation. This provides strong evidence for histidine derivatization by DEPC, and the value is consistent with the pK_a value for histidine derivatization (Takeuchi *et al.*, 1986; Blanke and Hager, 1990; Bhattacharyya *et al.*, 1992). The possibility of modification of other amino acids by DEPC has been excluded. Kinetic data indicate that more than 1 histidine residue is modified when HRP is incubated with DEPC. However, modification of histidine residue essential for activity could be differentiated from nonessential residues. Stoichiometric analyses have provided strong evidence that out of a total of 3 histidine residues of HRP (Welinder, 1979), only two are modified, and modification of a single histidine residue is critical for the loss of activity of the enzyme to oxidize guaiacol.

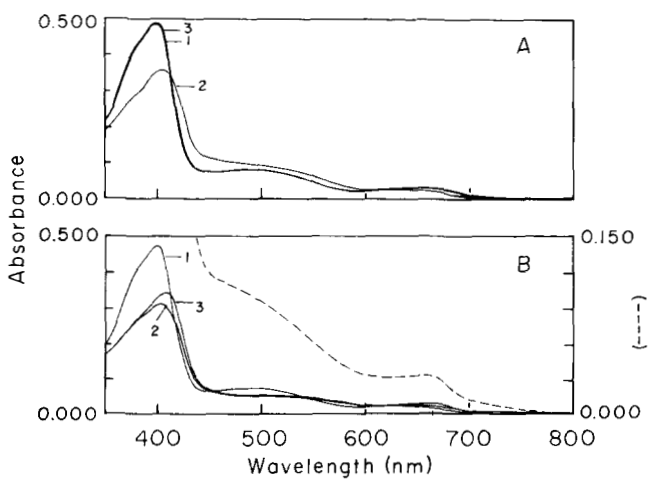


FIG. 8. **Effect of guaiacol on the spectra of native and DEPC-modified HRP compound I.** DEPC-modified enzyme was prepared as described in the legend of Fig. 5. *A*, represents the effect of 5 μM guaiacol on native compound I. *B*, represents the effect of 5 μM guaiacol on DEPC-modified compound I. Enzyme used in both cases was 5 μM (*tracing 1*), and H_2O_2 used was 20-fold over the enzyme concentration (*tracing 2*). *Tracing 3* represents the spectra obtained after adding 5 μM guaiacol on the incubation mixture of *tracing 2*. *Dashed line* in *B* represents the visible spectrum of compound I (*tracing 2*) in the absence or presence of guaiacol.

The primary amino acid sequence of HRP reveals the presence of His-40, His-42, and His-170 at the heme periphery (Welinder, 1979), similar to cytochrome *c* peroxidase (Poulos and Kraut, 1980). Sakurada *et al.* (1986) have provided computer-simulated secondary structure of HRP as shown in Fig. 9. His-42 is located at the heme distal pocket, and His-170 is ligated with the heme iron at the fifth coordination position of the heme proximal side. His-40 appears to be located away from the heme site. Since His-170 is not likely to be modified due to ligation with the heme iron (modification of this histidine would have significantly altered the heme CD band that is not observed), we propose that His-40 and His-42 have been modified under the conditions used. Since His-40 is away from the heme plane and probably more exposed, it shows faster reactivity with DEPC but no loss of activity. His-42 is hanging in the heme cavity perpendicular to the heme plane (Sakurada *et al.*, 1986) and hence is not easily approachable by DEPC. This may explain its slower reactivity, but carbethoxyformylation of this histidine leads to loss of activity. Thus, out of a total of 3 histidine residues, His-42 appears to be essential in catalyzing the oxidation of guaiacol. However, this proposition here and in the rest of the discussion is based on the assumption that the simulated structure is correct and His-42 is actually protected to some extent from DEPC relative to His-40. Thus, based on the simulated structure (Sakurada *et al.*, 1986), we can now tentatively identify distal His-42 as the critical histidine residue for guaiacol oxidation although definitive identification awaits peptide mapping using radiolabeled DEPC or site-directed mutagenesis. Since His-40 and His-42 are very close to each other, they are expected to come in the same peptide fragment in most of the protease digestions. This would not provide a definitive identification of the histidine residue. Site-directed mutagenesis is expected to provide conclusive evidence for the critical role of this His-42 in the future. The plausible involvement of this distal histidine has also been proposed earlier in HRP-catalyzed iodide oxidation (Bhattacharyya *et al.*, 1992), in chloroperoxidase-catalyzed chlorination reaction (Blanke and Hager, 1990), and in cytochrome *c* peroxidase-catalyzed cytochrome *c* oxidation (Bosshard *et al.*, 1984).

The distal histidine may play the following functions in HRP. It may be involved in the maintenance of the α-helicity of the distal pocket. Second, it may control the binding of aromatic donor at the distal pocket. Third, it may be involved in compound I or compound II formation, and last, it may regulate electron flow from the bound donor to these catalytic intermediates during oxidation. The first possibility is ruled out by our observation that the CD spectra of the native and DEPC-inactivated HRP in the far UV region do not change. The heme CD spectra (Strickland *et al.*, 1968) and Soret spectra also remain unaltered. Thus, inactivation is neither due to a change of α-helicity of the apoprotein nor due to any alteration in the heme site. The second possibility is also ruled out by the finding that the modified enzyme binds guaiacol with the K_D (7.2 mM) similar to the native HRP (K_D = 7.6 mM). This K_D value is comparable with the values obtained earlier by Paul and Ohlsson (1978) and by Hosoya *et al.* (1989). The third possibility is that the distal histidine is more likely involved in guaiacol oxidation by regulating the formation of catalytic intermediates of HRP with H_2O_2. Our studies suggest that modification of distal histidine does not affect the formation of compound I as revealed by the appearance of the characteristic peak at 408 nm, whereas compound II formation is blocked. This is in contrast to the suggestion of Poulos and Kraut (1980) that compound I formation in peroxidases is regulated by distal histidine in concert with arginine by a "push-pull" mechanism (Finzel *et al.*, 1984; Poulos, 1988). As this distal histidine is conserved in most of the peroxidases (Tien and Tu, 1987), it is plausible that this histidine may be involved in compound I formation. However, our studies indicate that this distal histidine in HRP is not absolutely required for compound I formation. When this histidine is modified, some other residues may take part in compound I formation. It is speculated that the carboxylate side chain of aspartate 43 may be involved in compound I formation as suggested earlier by Dunford and Ariso (1979). Bosshard *et al.* (1984) have shown that modification of cytochrome *c* peroxidase with DEPC results in modification of 5 histidine residues out of 6, and histidine 52 modification is associated with the loss of the Soret spectrum of compound I. Recently, Blanke and Hager (1990) have proposed from their modification studies that distal histidine 38 is involved in chloroperoxidase-catalyzed chlorination, but

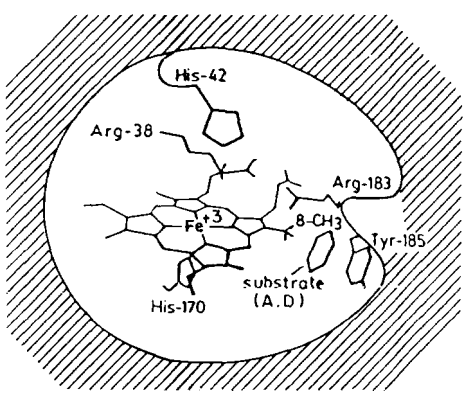
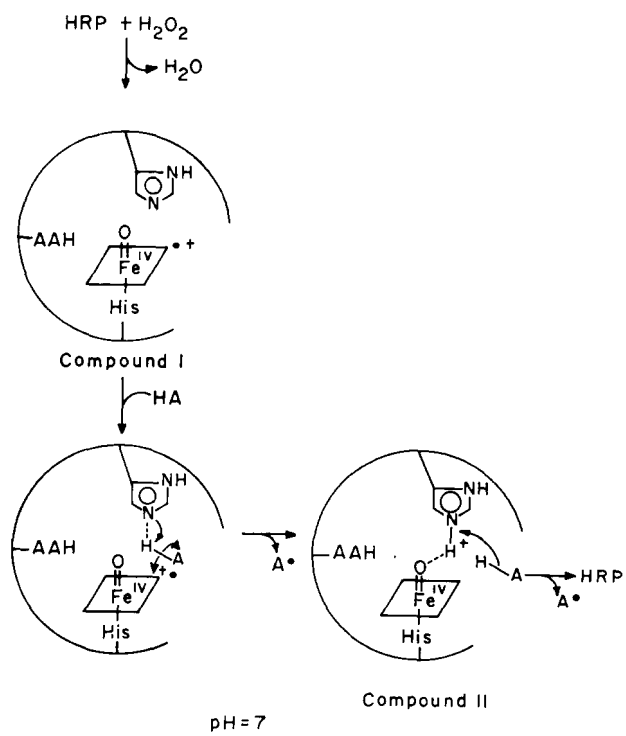


FIG. 9. **Computer-simulated secondary structure of the distal heme pocket of HRP.** The figure has been taken from Sakurada *et al.* (1986) with permission. *A.D* represents aromatic donor that may bind in the plausible hydrophobic pocket formed by 8-CH_3, Tyr-185, and Arg-183 (Sakurada *et al.*, 1986).



SCHEME 1. **Schematic diagram of the active site of HRP-catalytic intermediates and their interaction with a phenolic substrate (*HA*) (Oertling and Babcock, 1988).**

its role in the formation of the catalytic intermediate remains to be investigated.

It is now generally believed that aromatic donor is hydrogen-bonded to the distal histidine 42 (Dunford, 1982; Oertling and Babcock, 1988) when it binds at the hydrophobic site of HRP (Sakurada *et al.*, 1986). When an electron is transferred from the donor (*HA*) to the porphyrin-π cation radical of compound I, a proton concomitantly passes to the distal histidine along the hydrogen bond, resulting in the homolytic cleavage of the H–O bond to form a free radical ($A^{\cdot}$) of the donor (Shiga and Imizumi, 1975) and compound II as shown in Scheme 1 (Oertling and Babcock, 1988). Since our studies indicate that modification of distal histidine does not produce compound II from compound I but leads to a block of guaiacol oxidation, it appears that the defect lies in the electron transfer mechanism from the bound donor to compound I. Presumably carbethoxyformylation of histidine 42 (Scheme 1) by DEPC results in the block of proton transport during electron flow to compound I. We therefore suggest that this tentatively identified distal histidine 42 in HRP plays an important role in aromatic donor oxidation by taking part in the electron transfer mechanism that is coupled with the concomitant flow of proton from the bound donor to the distal histidine through the hydrogen bond (Oertling and Babcock, 1988). Since the modified enzyme is unable to form compound II from compound I by endogenous one-electron reduction, we can also suggest that this distal histidine may form a route for normal electron flow presumably from the endogenous sugar moieties to compound I during compound II formation.

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