Reactions of Horseradish Peroxidase with Azide. Evidence for a Methionine Residue at the Active Site*

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ABSTRACT: Sodium azide, a reagent known to react with methionine and methionine sulfoxide groups, has been shown to inactivate horseradish peroxidase (HRP). The rate and specificity of inactivation of HRP by azide is greatly enhanced by the presence of small amounts of H_2O_2. Azide also acts as an inhibitor in the HRP–H_2O_2–hydrogen donor system, and a kinetic analysis of the inhibition is proposed which explains the experimental data and is consistent with the probable chemistry of the azide–methionine reaction. It is demonstrated that inactivation and inhibition effects cannot arise from the competition of azide with peroxide for the HRP heme group in these experiments. A *chemical* mechanism for HRP action which includes direct participation of a methionine residue is proposed and discussed.

While hemoproteins have been the object of a great number of investigations in recent years, almost all of the work reported has dealt with the reactivity and spectral characteristics of the heme prosthetic group. Even in the case of heme enzymes such as catalases and peroxidases, the kinetics of catalysis have been interpreted largely in terms of changes in the iron oxidation state and in the integrity of the porphyrin conjugated system. Where a role for the protein has been suggested, for example, in peroxidase action, it has generally been presented as an influence on either heme group accessibility (Chance, 1951; George and Lyster, 1958) or reactivity (Kurozumi *et al.*, 1961; Nicholls, 1962) rather than as a matter of direct involvement in the catalytic process. Such proposals, however, do not satisfactorily explain the inhibition of peroxidases by compounds which apparently do not react at the heme locus (Saunders *et al.*, 1964). A kinetic study of the peroxide inactivation of horseradish peroxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) has suggested that a nonheme enzymic site may exist, and possible amino acid residues constituting this site were proposed (Weinryb, 1966). A subsequent study of the alkylation of HRP[1] apoprotein has focused attention upon the possibility that histidine and/or methionine residues may be involved at the active site (Weinryb, 1967a,b). Further chemical studies were considered in order to distinguish between the roles of these two kinds of residues. Reagents which reacted with methionine rather than histidine were of primary interest because evidence for an essential methionine residue in HRP action would be novel and interesting while histidine involvement would be expected if the latter residue were bound to the heme iron in HRP, as suggested by Brill and Sandberg (1967).

Azide is known to react with both methionine and methionine sulfoxide to give methionine sufoximine (Misani *et al.*, 1951; Whitehead and Bentley, 1952). The study of the reaction of azide with methionine groups in a hemoprotein would be of special interest since azide has been long known to associate reversibly with the heme group of hemoproteins to give complexes with characteristic visible absorption spectra (see, for example, Brill, 1966), and this phenomenon has been extensively studied without regard to the possible effects of azide on the particular protein moiety.

In this report we present an analysis of the reaction of HRP with azide which strongly supports the idea of protein as well as heme participation in the catalytic site and indicates that methionine is involved in the active site.

Experimental Procedures

Materials

HRP was an electrophoretically purified preparation (Code HPOFF) from Worthington Biochemical Corp. (Freehold, N. J.). A Worthington assay with *o*-dianisidine as hydrogen donor indicated an activity of about 4000 units/mg of protein for most of the preparations used (see Worthington Handbook for further details). Enzyme concentrations were determined spectrophotometrically at 403 mμ, where the extinction coefficient is 91 mM^{-1} cm^{-1} (Keilin and Hartree, 1951).

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[1] Abbreviations used: HRP, horseradish peroxidase; OPD, *o*-phenylenediamine.

Mesidine (2,4,6-trimethylaniline) and hydrogen peroxide were purchased and used as described previously (Weinryb, 1966). *o*-Phenylenediamine dihydrochloride (Eastman Organic Chemicals, Rochester, N. Y.) and sodium azide (purified grade, Fisher Scientific Co., Fairlawn, N. J.) were used as purchased. Other reagents were of highest purity commercially available.

Methods

The Inactivation of HRP by Azide. Sodium azide (0.25 M) was mixed with approximately 10 μM HRP at 42 ± 1° in 0.25 M phosphate buffer. In each experiment, a control sample of untreated HRP was incubated at the same temperature, pH, and buffer concentration. Aliquots were withdrawn from both samples and their activities were compared by assay in duplicate. The assay was conducted as reported previously (Weinryb, 1966), except that the assay reaction was initiated by the addition of hydrogen peroxide to the enzyme–hydrogen donor system. The assay reaction mixture contained 2.3 ml of 18 mM mesidine-HCl (in 0.15 M sodium acetate buffer), 0.1 ml of 0.12 M H_2O_2 (in distilled water), and 0.01 ml of HRP–azide (or HRP) solution; assay pH, 4.9.

The azide concentration present during the assay of the treated HRP was approximately 1 mM. It is important to determine whether this concentration is high enough to cause a significant decrease in activity through competition with hydrogen peroxide for the heme site of the enzyme. The ratio of heme-bound azide to heme-bound peroxide in the absence of hydrogen donor can be calculated from eq 1.

$$r \equiv \left\{\frac{[\text{heme–}N_3]}{[\text{heme–}H_2O_2]}\right\}_{[AH_2]=0} = \frac{[N_3]K_{d(H_2O_2)}}{[H_2O_2]K_{d(N_3)}} \qquad (1)$$

For the concentrations of azide (1 mM) and hydrogen peroxide (5 mM) in the assay, and with the dissociation constants for azide ($K_{d(N_3)} \simeq 2 \times 10^{-2}$ at pH 4.9, Sandberg (1967)) and hydrogen peroxide ($K_{d(H_2O_2)} \simeq 10^{-7}$, Chance (1949b)), it can be calculated that 10^6 times more heme binds peroxide than binds azide ($r = 10^{-6}$). In the presence of hydrogen donor, the fraction (f) of HRP heme complexed with azide is increased. One can derive an expression for f from steady-state analysis of the mechanism used later in the paper (eq 4–6).

$$f \equiv \frac{[\text{heme–}N_3]}{[\text{total heme}]} = r\left\{\frac{1 + \frac{k_2}{k_{-1}}[AH_2]}{1 + \frac{k_2}{k_3}[AH_2] + \left(\frac{K_{d(H_2O_2)}}{[H_2O_2]} + \frac{k_2}{k_1}\frac{[AH_2]}{[H_2O_2]}\right)\left(1 + \frac{[N_3]}{K_{d(N_3)}}\right)}\right\} \qquad (2)$$

For the catalysis of mesidine oxidation, $k_2 = 2.7 \times 10^4$ M^{-1} sec^{-1} and $k_3 = 3.5 \times 10^2$ sec^{-1} (Weinryb, 1967a). Under the conditions of the assay, then, $f = 2 \times 10^{-4}$. Thus, competition between azide and peroxide for the heme site of HRP during the assay cannot be a reason for any apparent decreases in activity. An inhibitory effect due to a possible azide interaction at a nonheme site must also be considered. This effect will be described and discussed in detail below. Under the conditions of the assay of the inactivated enzyme, such an effect has been determined not to exceed 5% of the activity of the untreated enzyme.

HRP Inhibition by Azide. The inhibition studies were run in a manner similar to that used for the assay described above except that the pH was 4.3 ± 0.1 (0.55 M sodium acetate buffer) for mesidine as hydrogen donor and 5.5 ± 0.1 (0.48 M sodium phosphate buffer) for *o*-phenylenediamine as hydrogen donor. The rate of formation of the oxidation product of OPD was followed at 450 mμ (an absorption maximum). Small amounts of solid sodium azide were added to the hydrogen donor stock solution, which was then employed as usual in the assay.

For the catalysis of OPD oxidation, $k_2 = 4 \times 10^5$ M^{-1} sec^{-1} and $k_3 = 1.2 \times 10^3$ sec^{-1} (Weinryb, 1967a). The largest value of f (eq 2) for all the inhibition experiments reported in this paper, corresponding to the open square ($[OPD]_0 = 6.6$ mM) of Figure 3 at $[N_3] = 30$ mM is 8×10^{-3}, a negligibly small occupation of the heme site.

Amino Acid Chromatography. Amino acid analyses were performed as described by Spackman *et al.* (1958) on automatic recording equipment in Professor F. M. Richards' laboratory. Protein samples were hydrolyzed with 6 N HCl in sealed, degassed, evacuated tubes for 24 hr at 110°. Analyses of native HRP were in good agreement with previously published results (Klapper and Hackett, 1965).

Optical Rotatory Dispersion Spectra. Optical rotatory dispersion measurements were carried out on a Cary Model 60 recording spectropolarimeter in the laboratory of Professor J. M. Sturtevant. Cells (1 cm) were used, and a base line was determined (with blank solution). In order to facilitate the achievement of reproducible spectra, the exteriors of the cells were routinely washed with 95% ethanol and dried in a stream of pure, dry nitrogen gas. Touching the optical surfaces with lens tissue, etc., was avoided.

The optical rotatory dispersion spectra here presented are intended for comparison purposes only and are plotted in terms of the recorded rotation. Spectra are compared at equal protein concentration as determined by the absorbance at 275 mμ.

Spectrophotometric Studies. All spectrophotometric measurements and experiments (as well as the assays) were carried out with a Cary Model 14 recording spectrophotometer. In general a base line would first be obtained over the wavelength region of interest by recording with blank solutions in both the sample and reference cuvets.

Results

The Inactivation of HRP by Azide. The results of the

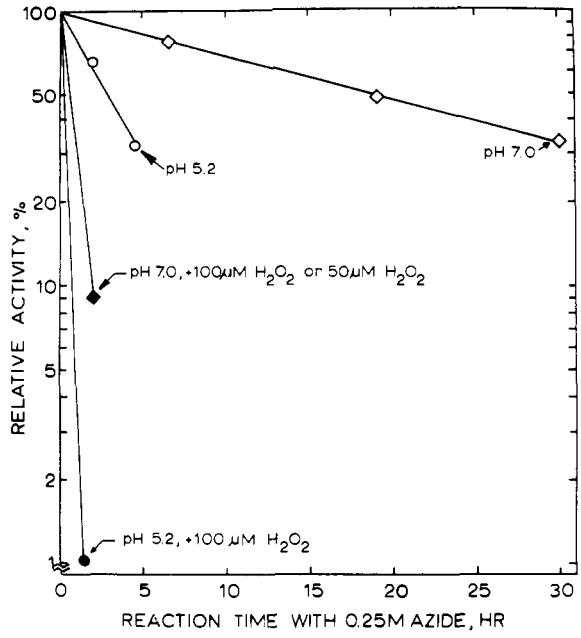


FIGURE 1: The inactivation of HRP by 0.25 M azide. Conditions as noted. HRP incubated with 100 μM H_2O_2 at pH 7.0 for 2 hr showed no loss of activity.

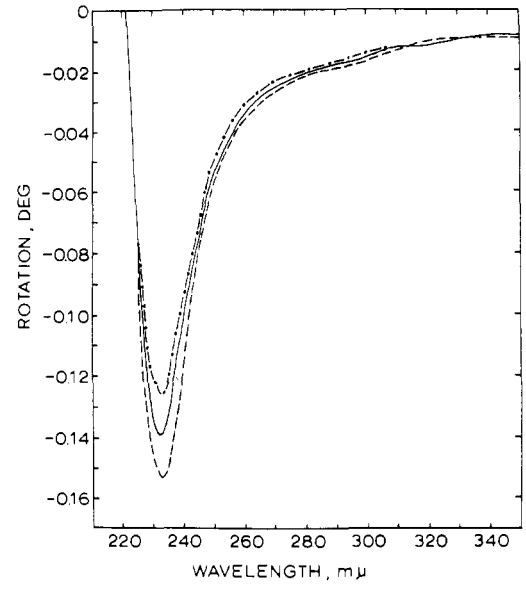


FIGURE 2: Optical rotatory dispersion spectra of azide-treated, inactivated HRP (215–350 mμ). Dashed curve is spectrum of control (untreated) HRP. Solid curve is spectrum of 99% inactivated HRP treated with 0.25 M azide and 100 μM H_2O_2 (pH 5.2). Dot-dashed curve is spectrum of 55% inactivated HRP treated with 0.25 M azide (pH 5.2). Spectra are normalized to equal absorbances at 275 mμ, and were run with 33 mM acetate buffer (pH 5.0) as solvent.

inactivation of HRP by azide are shown in Figure 1. Note that the rate of inactivation increases as the pH is lowered from 7.0 to 5.2, and that the addition of peroxide to the HRP–azide system causes a marked (about tenfold) increase in the rate of inactivation. The striking feature of this latter result is the low concentration of peroxide needed; as little as 50 μM (a fivefold excess over HRP) is sufficient. The incubation of HRP with 100 μM peroxide alone gave no inactivation over the same time periods at pH 7.0, demonstrating that the enhancement in the inactivation rate cannot be ascribed to inactivation by peroxide alone.

TABLE I: Increase in Activity of Azide-Treated HRP after Dialysis.

$[H_2O_2]$ (μM)	pH	Residual Rel Act. (before dialysis)	Reaction Velocity after Dialysis[a]/ Reaction Velocity before Dialysis
100	7.0	0.10	1.9
50	7.0	0.10	2.2
0	7.0	0.30	1.2
0	5.2	0.30	1.6

[a] Dilution corrected

The azide-treated, partially inactivated HRP samples were subjected to prolonged dialysis and then reassayed. The samples showed increased activity, suggesting that the chemistry of inactivation is at least partially reversible. These data are given in Table I. The Soret bands of these samples were broadened and diminished, as might be expected if a residue quite close to the heme were modified; nevertheless, the samples could bind cyanide in apparently normal fashion.

Amino acid analyses of the azide-treated, inactivated HRP preparations are shown in Table II. Results for the untreated (native) enzyme are included for comparison. Although the recovery of proline is somewhat low for the azide-treated enzyme, this result is of doubtful significance since, to the authors' knowledge, azide is not known to react with secondary amines. However, it is conceivable that free proline may be especially sensitive to decarboxylation and subsequent amination by residual azide in the presence of strong acid (Noller, 1958), as in the hydrolysates. None of the other amino acids in the azide-treated enzyme hydrolysate showed low recoveries relative to the untreated enzyme. Since methionine is recovered as several compounds, total recovery does not indicate absence of chemical attack (see Discussion).

In order to determine what effects, if any, the azide–HRP interaction has on the tertiary structure of HRP, optical rotatory dispersion spectra of azide-treated HRP

TABLE II: Amino Acid Analyses for Untreated and Azide-Treated HRP.

Amino Acid	Residues/MMWU (10,700)[a]			
	Untreated[b]	N_3 Treated, pH 7 (no H_2O_2), 70% Inactivation	N_3 Treated, pH 7 (+100 μM H_2O_2), 90% Inactivation	N_3 Treated, pH 7 (+50 μM H_2O_2), 90% Inactivation
Lysine	1.9 ± 0.1	1.7	1.7	1.8
Histidine	1.0 ± 0.1	1.1	0.9	1.0
Arginine	6.1 ± 0.9	4.7	6.2	6.5
Aspartic acid	16.1 ± 0.5	15.7	16.3	16.6
Threonine[c]	8.0 ± 0.4	8.0	8.2	8.4
Serine[c]	8.1 ± 0.3	8.1	8.3	8.3
Glutamic acid	6.7 ± 0.3	6.8	6.8	6.8
Proline	6.2 ± 0.5	5.3	5.1	5.2
Glycine	6.0	6.0	6.0	6.0
Alanine	7.7 ± 0.5	7.6	8.0	8.0
$CysSO_3H$ + half-cystine[c]	2.0 ± 0.4	2.4	2.6	2.6
Valine	5.2 ± 0.3	4.9	5.0	5.1
Methionine[d]	1.1 ± 0.3	1.0 (5)	1.2 (5)	1.2
Isoleucine	4.0 ± 0.4	3.8	3.9	3.9
Leucine	10.4 ± 0.6	10.3	10.5	10.7
Tyrosine[c]	1.6 ± 0.2 (5)	1.8	1.6 (5)	1.5
Phenylalanine	6.1 ± 0.4	5.9	6.3	6.3

[a] The minimum molecular weight unit (MMWU) is one-third the molecular weight of the HRP protein moiety. Results are calculated on a basis of six glycine residues per MMWU. [b] Results are averages of seven determinations. Included with each result is the computed standard deviation, $\sigma = [\Sigma(\text{dev})^2/(n-1)]^{1/2}$. [c] The recoveries of cystine, tyrosine, and threonine were multiplied by 1.05, and those of serine by 1.1, to correct approximately for hydrolysis losses (see Moore and Stein, 1963). [d] Includes amounts recovered as methionine sulfoxide and methionine sulfone.

were compared with the corresponding control (Figure 2). The dashed curve represents the control. The solid curve is the optical rotatory dispersion spectrum of an HRP preparation which has been 99% inactivated by 0.25 M azide–100 μM H_2O_2 at pH 5.2, while the dashed-dotted curve represents HRP 55% inactivated with azide alone at pH 5.2. It may be seen that the negative lobe of the Cotton effect shows less change (relative to the control) for the HRP inactivated by azide–H_2O_2 than for HRP inactivated by azide alone, although the former preparation is more extensively inactivated. This inverse relationship between the magnitudes of the changes in activity and optical rotatory dispersion supports a site specific attack rather than a gross structural alteration as the mode of azide inactivation of HRP in the presence of peroxide. The larger change in tertiary structure of the HRP sample inactivated by azide alone is probably a reflection of more extensive attack by azide at residues not involved in the catalytic site of HRP.

HRP Inhibition by Azide. It was hoped that further insight into the mechanism of action of azide upon the enzymic properties of HRP might be gained through an examination of the inhibitory role of azide in HRP-catalyzed peroxidations. As in the case of the inactivation studies, the inhibition experiments were designed so that inhibition effects due to competition between azide and peroxide for the heme site were negligible (f always less than 10^{-2}).

The results of the inhibition studies with OPD as hydrogen donor are shown in Figure 3. The data are plotted so as to yield a straight line passing through the origin if simple competitive inhibition of hydrogen donor binding by azide were the case; *i.e.*, inhibition describable by a reversible reaction of azide with a peroxide compound of HRP. Clearly, the actual situation is more complex than this, since a pronounced curvature (perhaps quadratic) is introduced at higher azide concentrations. The data appear to fit a relation of the following general form

$$(v_0/v_i) - 1 \propto \frac{a[HN_3] + b[HN_3]^2}{\prod_j (K_j + [AH_2])} \qquad (3)$$

where v_0/v_i, as in Figure 3, represents the ratio of enzymic reaction velocities with azide absent and present, and a, b, and the K_j are constants. A kinetic scheme which agrees well with the data of Figure 3,

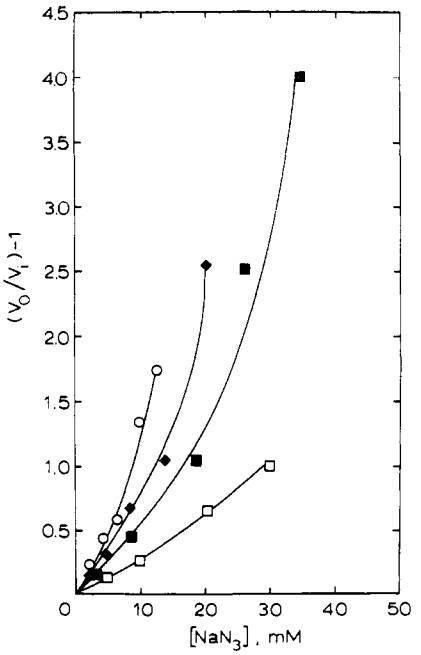


FIGURE 3. Effect of azide on rate of HRP-catalyzed oxidation of OPD. [HRP] = 1.2 mμM, $[H_2O_2]_0$ = 5 mM, pH 5.5 ± 0.1, (○) $[OPD]_0$ = 2.1 mM, (◆) $[OPD]_0$ = 3.4 mM, (■) $[OPD]_0$ = 5.3 mM, and (□) $[OPD]_0$ = 6.6 mM.

especially in the region of low azide concentrations, is given below.

$$E + H_2O_2 \xrightarrow{k_1} E_I \quad (4)$$

$$E_I + AH_2 \xrightarrow{k_2} E_{II}\cdot AH \quad (5)$$

$$E_{II}\cdot AH \xrightarrow{k_3} E + A + H_2O \quad (6)$$

$$E_I + HN_3 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E_I' \quad (7)$$

$$E_I' + HN_3 \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E_I'' \quad (8)$$

$$E_I' + AH_2 \xrightarrow{k_6} E_{II}\cdot AH \quad (9)$$

In contrast to many other hydrogen donors from each of which several products are formed, mesidine and OPD yield single products upon peroxidation catalyzed by HRP (Mason, 1957; Saunders *et al.*, 1964; Weinryb, 1967a). Single *vs.* multiple-product formation has been explained on the basis of two- *vs.* one-electron-transfer reactions (Brill, 1966). The oxidation of hydrogen donor by E_I is represented as essentially a two-electron transfer (eq 5 and 6). The intermediate $E_{II}\cdot AH$ may be viewed as the ternary complex first postulated by Mann (1931) from experiments with HRP, and later used by Hosoya (1960) to explain the kinetics of certain turnip peroxidase catalyzed reactions. Kinetic studies of mesidine and OPD peroxidation catalyzed by HRP are in accord with the scheme given; in particular, these studies require the first-order reaction described by eq 6. The postulated effects of azide addition on the enzymic activity are summarized in eq 7–9. Two azide molecules are postulated to react successively with E_I. The product (E_I') of the reaction of E_I with the first azide molecule is still capable of binding hydrogen donor. Reaction with the second azide molecule, however, yields an inactive form of the enzyme (E_I'') and is responsible for the quadratic deviation of $(v_0/v_i) - 1$.

Steady-state analysis of the scheme of eq 4–9 yields the following expression

$$(v_0/v_i) - 1 = \frac{K_M}{(K_M + [AH_2])(K + [AH_2])} \times \left\{\frac{k_4[HN_3](1/k_6 - 1/k_2) + (k_4k_5/k_6k_{-5})[HN_3]^2}{1 + k_4[HN_3]/k_2(K + [AH_2])}\right\} \quad (10)$$

where $K_M \equiv k_3/k_2$ and $K \equiv k_{-4}/k_7$. At low $[HN_3]$, eq 10 becomes

$$(v_0/v_i) - 1 = \frac{K_M}{(K_M + [AH_2])(K + [AH_2])} \times k_4(1/k_6 - 1/k_2)[HN_3] \quad (11)$$

i.e., the initial slopes of the curves in Figure 3 should be inversely proportional to $(K_M + [AH_2])(K + [AH_2])$. With a value for K_M of 3 mM for OPD (Weinryb, 1967a), the data of Figure 3 are best fit for K about 10 mM. The use of mesidine as hydrogen donor in the inhibition studies resulted in the same kind of inhibition plot behavior. With a value for K_M for 13 mM (Weinryb, 1967a), a value for K of about 10 mM was again obtained for the best fit of the data, an indication that K is primarily a reflection of the interaction of azide with HRP. These data are summarized in Table III. The analysis of the inhibition data for mesidine as hydrogen donor is complicated by the interaction of azide with the product of the mesidine oxidation to form an addition compound (Hughes *et al.*, 1954). Additional experiments have indicated that no such interaction between azide and OPD (or the product of OPD oxidation) occurs, and that the side reaction between azide and the product of mesidine oxidation does not seriously affect the kinetic conclusions drawn from the mesidine data (Weinryb, 1967a).

Discussion

Probable Chemistry of Methionine–Azide Reaction. It will be useful in discussing the results to refer to the chemistry of the reaction of azide with methionine which is somewhat obscure but probably follows the sequence outlined in eq 12 (Whitehead and Bentley, 1952). Methionine (I, symbolized simply as the thio-

TABLE III: RMS Differences between Computed and Experimental Slopes of the Inhibition Function $(v_0/v_i) - 1$ at Low Azide Concentrations.

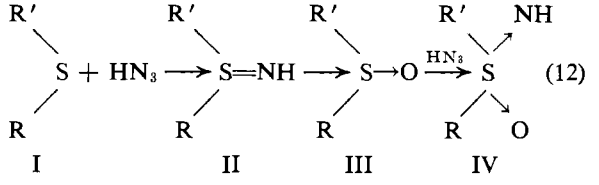
A. *o*-Phenylenediamine (K_M = 3 mM)[a]

[OPD] (mM)	Init Slope (exptl)	Rel Init Slope	Rel $[(K + [OPD])(K_M + [OPD])]^{-1}$, K (mM) = 3	7	10	13
2.1	0.094	1.00	1.00	1.00	1.00	1.00
3.4	0.071	0.76	0.63	0.70	0.72	0.73
5.3	0.050	0.53	0.38	0.46	0.49	0.51
6.6	0.028	0.30	0.28	0.35	0.39	0.41
RMS differences in relative slopes			0.12	0.06 (5)	0.06	0.07

B. Mesidine (K_M = 13 mM)

[Mesidine] (mM)	Init Slope (exptl)	Rel Init Slope	Rel $[(K + [mesidine])(K_M + [mesidine])]^{-1}$, K (mM) = 6.5	8	10	13	19.5
5.0 (5)	0.85	1.00	1.00	1.00	1.00	1.00	1.00
8.8	0.50	0.59	0.63	0.65	0.66	0.69	0.72
15.2	0.35	0.41	0.34	0.36	0.38	0.41	0.45
19.6	0.25	0.29	0.25	0.26	0.28	0.31	0.35
RMS differences in relative slopes			0.05 (2)	0.04 (8)	0.04 (5)	0.06	0.09

[a] Data from Figure 3.



(12)

ether) reacts with a molecule of hydrazoic acid to form the rather unstable sulfidimine (II), which rearranges to the sulfoxide (III). Reaction with a second molecule of hydrazoic acid would result in formation of methionine sulfoximine (IV). The above scheme predicts that lowering the pH would increase the rate of reaction of azide with methionine, since hydrazoic acid is assumed to be the reacting species. The presence of peroxide (or other oxidizing agent) would be expected to facilitate the formation of the sulfoxide (III) and hence might also increase the reaction rate. These predictions are borne out by the results of the inactivation experiments (Figure 1).

Although the results of the inactivation experiments are consistent with the view that azide inactivates HRP through reaction with a methionine residue, the effect of added peroxide deserves further comment. The marked increase in the HRP inactivation rate by such low concentrations of peroxide makes it likely that peroxide which is bound to the heme exerts its effect either by facilitating the oxidation of the essential methionine residue (presumably located very near the heme locus) or by causing (by virtue of being bound by the heme) a change in the relationship of the heme to the rest of the protein, whereby the methionine residue becomes more accessible to azide attack, or both.

The chemistry of azide–methionine reaction provides a simple explanation for the *apparent* lack of reaction of methionine, as indicated by full recovery of methionine in the amino acid analyses of azide-treated HRP (Table II). Sulfoximines are decomposed by heat, or by refluxing with acid or alkali, to form the corresponding sulfoxide (Misani *et al.*, 1951). Methionine sulfoxide appears (in variable amounts) in the hydrolysates of untreated (as well as azide treated) HRP. The lability of sulfoximines to acid hydrolysis must be considered the main drawback to the use of azide as a methionine reagent.

The inhibition data are also consistent with the idea of HRP inactivation by azide reaction at a methionine residue. A comparison of eq 7–9 with eq 12 indicates a close similarity between the predicted kinetic behavior of the inhibited HRP-catalyzed reaction and the probable chemistry of azide reaction with methionine residues.

Methionine and HRP Action. The notion that a methionine residue may be required for HRP action is consistent with published reports on the peroxidatic activity of other systems. Tu (1964) synthesized heme diglutathione and found that it showed significant peroxidatic activity. Although detailed data on the purity of the HRP preparations used for comparison were not given, the results show that heme diglu-

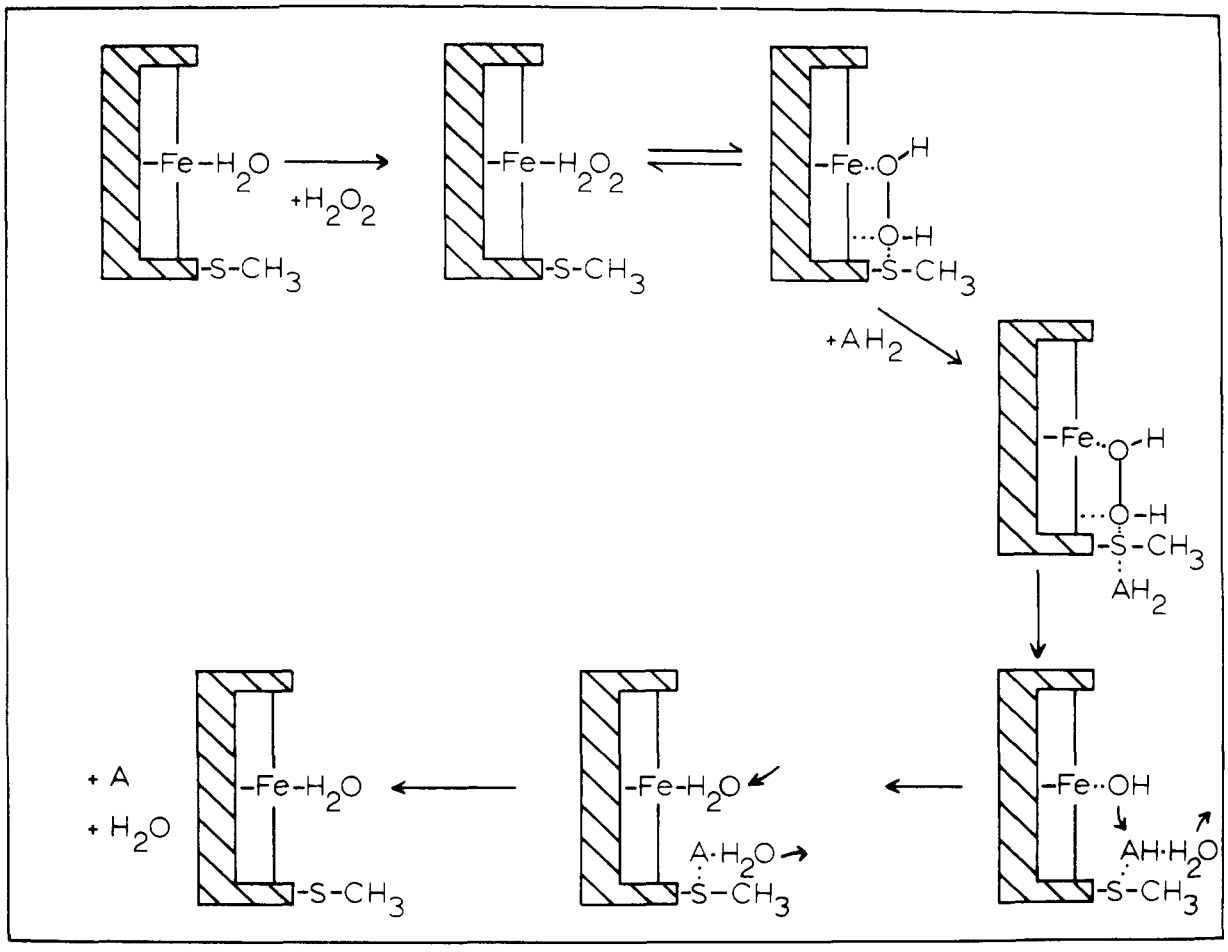


FIGURE 4: A possible model of HRP catalysis. The ribbed areas represent the remainder of the HRP apoprotein.

tathione possessed about 0.03 (an appreciable fraction) of the specific activity of HRP. Heme diglutathione contains two glutathione molecules (γ-L-glutamyl-L-cysteinylglycine) in which the cysteine residues are bound covalently to the heme vinyl groups, forming *thioether* linkages (in necessarily close proximity to the heme group). No other significant chemical feature appears to have been added to the heme, which alone showed no peroxidatic activity at all under the conditions of the experiments. Similarly, the cytochrome *c* heme peptide (11 residues) of Paléus *et al.* (1955) in which thioether linkages to the heme are also present, was found to possess about 1% of the specific activity of HRP. Here, however, the presence of histidine in the peptide hampers a simple interpretation; *viz.*, that the thioether linkage is the protein function necessary (along with the heme) for HRP catalytic activity.

It should be pointed out that these analogies with model compounds, and the new data presented in this paper, leave open the question as to whether methionine might be directly or passively involved. Direct participation of a methionine residue is favored by the following considerations. The extraordinarily broad donor specificity of HRP would seem to require an extraordinarily reactive protein functional group (or groups). An oxidized form of methionine such as the sulfoxide appears to fulfill these requirements more closely than oxidized forms of the other residues under consideration. Sulfoxides of the class

possess perhaps three reactive sites: a partially positive sulfur atom, a partially negative oxygen atom, and by analogy to the more familiar chemical properties of dimethyl sulfoxide, a methyl group possibly subject to electrophilic attack due to the acidic character of one of the protons (Kharasch and Thyagarajan, 1966). It becomes easy to see how compounds of many kinds would suffice as hydrogen donors. The reactivity of methionine sulfoxide should lead not only to broad donor specificity, but to inhibition by diverse groups of compounds. HRP has been reported to be inhibited by many groups of compounds which do not appear to act at the

heme locus, such as sulfonamides and related compounds (Saunders *et al.*, 1964). In particular, Lipmann (1941) has found sulfanilamide, sulfathiazole, and sulfapyridine to be good inhibitors. This observation should be considered along with the knowledge that aryl or alkyl sulfoxides are known to condense with sulfonamides to give rather unstable compounds (Tarbell and Weaver, 1941); thus HRP inhibition by sulfonamides can be interpreted in terms of reaction at an oxidized methionine residue.

A Chemical Mechanism for HRP Catalysis. It is possible to begin to formulate a chemical mechanism for HRP action in terms of an interaction with a methionine residue of the protein. A possible scheme is outlined in Figure 4. The general features of the model are the following. (1) HRP first binds peroxide at the heme site with high efficiency. (2) The binding of peroxide probably causes a change in the relation of the heme group to the apoprotein, more fully exposing a methionine residue to the bulk solution. Such a concept not only is consistent with the peroxide enhancement of the rate of azide inactivation of HRP discussed earlier, but is suggested by the observation that vigorous conditions are necessary for HRP inactivation through the alkylation of methionine residues in the free enzyme (Weinryb, 1967a,b). In free HRP, the methionine residue may be shielded from the bulk solution by the vinyl or propionic acid side chains of the prophyrin. As the methionine residue is exposed, it may receive, as has a methine bridge, virtual oxidation equivalents from the coordination sphere of the iron. The involvement of a methine bridge carbon atom has been suggested in the past (Chance, 1949a; Brill and Williams, 1961). (3) The virtual oxidation of methionine to form the sulfoxide enhances the reactivity of this residue, enabling it to bind hydrogen donor efficiently. (4) The association of oxidized methionine and hydrogen donor results in a rapid oxidation of the donor. (5) The tertiary structure of the HRP apoprotein probably contributes to the efficient binding of peroxide to heme and hydrogen donor to protein.

The above model may be interpreted as ascribing the catalytic properties of HRP to the possibility that it binds the two substrates in close proximity to one another. In this sense the role of HRP may be to increase the rate of oxidation of hydrogen donor not only by decreasing the over-all activation energy of the reaction, but by increasing the probability of interaction of peroxide and hydrogen donor relative to that in the bulk solution.

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