The Behavior of Horseradish Peroxidase at High Hydrogen Peroxide Concentrations*

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ABSTRACT: The kinetics of the inactivation of horseradish peroxidase at high H_2O_2 concentrations have been studied, both by assaying for active enzyme remaining, and by following the decay of the Soret peak spectrophotometrically. Different dependences of the inactivation rate upon the enzyme and peroxide concentrations were found for the two cases, and a kinetic scheme consistent with these differences has been proposed. The results are interpreted in terms of peroxidase attack at a nonheme as well as a heme site, and the implications of such a proposal for peroxidatic activity are discussed.

Peroxidase action has not been investigated at high (>1 M) peroxide concentrations. Such experiments are hampered by the inactivation of the enzyme upon exposure to excess peroxide (Chance, 1949). Since the inactivating agent is also the primary substrate of peroxidase, a study of the inactivation process may disclose information about the binding of peroxide to enzyme.

This report compares the kinetics of horseradish peroxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) inactivation by excess hydrogen peroxide derived in one case from assay for active enzyme remaining, and in the other from following the decay of the Soret peak spectrophotometrically. The results indicate different dependences of the inactivation rate on the concentrations of enzyme and peroxide for the two cases. Implications of such a result are discussed below.

Experimental Section

Materials

Horseradish peroxidase (HRP)[1] was an electrophoretically purified preparation (HPOFF) from Worthington Biochemical Corp. (Freehold, N. J.). The samples were characterized spectrally by an R. Z. ($A_{403\ m\mu}/A_{275\ m\mu}$) of 2.9–3.0. A Worthington assay with *o*-dianisidine as hydrogen donor indicated an activity of 2800–2900 units/mg of protein (see Worthington Biochemical Corp. Handbook for assay details). The HRP was dialyzed *vs.* sodium acetate buffer, pH 4.9, for the inactivation experiments. A millimolar extinction coefficient of 91 $mM^{-1}\ cm^{-1}$ at 403 mμ (Keilin and Hartree, 1951) was used to determine HRP concentrations spectrophotometrically. The Molisch sugar test was positive for the HRP hydrolysate (1 M H_2SO_4, 110°, 4 hr, sealed tubes).

Mesidine (2,4,6-trimethylaniline) was purchased from Aldrich Chemical Co. (Milwaukee, Wis.). It was converted to the hydrochloride by addition of excess concentrated hydrochloric acid, and recrystallized from 95% ethanol several times, until the resulting crystals were white. The crystals are stable for months stored in a refrigerator freezer.

Fisher Certified Reagents 30 and 50% (stabilized) hydrogen peroxide (Fisher Scientific Co, Fair Lawn, N. J.) and Mallinckrodt Analytical Reagent 30% hydrogen peroxide (Mallinckrodt Chemical Works, New York, N. Y.) were used, suitably diluted when necessary with distilled water. Titration checks with potassium permanganate revealed that the nominal molarities calculated from the assays on the bottles were accurate and constant over periods of time long compared to that needed to complete the experiments. The H_2O_2 millimolar extinction coefficient at 230 mμ of 0.0724 (George, 1953) was also consistent with the assays.

Apparatus

A Cary Model 14 recording spectrophotometer was used. In all HRP concentration determinations and Soret peak decay experiments a base line was first obtained over the wavelength region of interest by recording with acetate buffer in both the sample and reference cuvets.

Methods

Enzyme Assay Monitor. The assay method is a modified version of that of Paul and Avi-Dor (1954), with mesidine as the hydrogen donor. In the control experiment (no inactivation), 0.01 ml of HRP is blown into a solution of 0.018 M mesidine–HCl and 0.005 M H_2O_2 in acetate buffer, pH 4.9. The dilution factor is chosen to ensure the validity of the assay (see below). The solution is poured quickly into a cuvet, and the formation of the "purple compound" [2,6-dimethyl-

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[1] Abbreviation used: HRP, horseradish peroxidase.

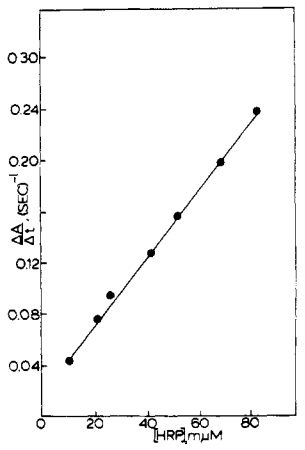

FIGURE 1: Proportionality of HRP concentration to the rate of increase of absorbance at 490 mμ determined from the mesidine assay (enzyme assay details given in "Methods" section). Temperature: 25 ± 0.5°.

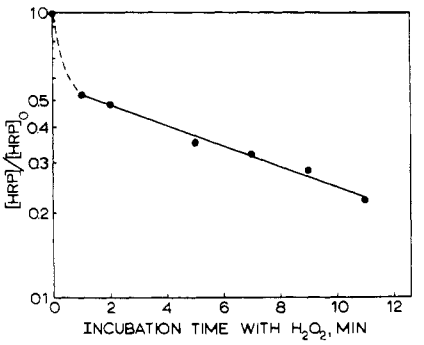

FIGURE 2: Typical plot of HRP inactivation with increasing incubation time in H_2O_2; $[HRP]_0$ = 13.0 μM, $[H_2O_2]_0$ = 1.00 M.

benzoquinone-4-(2′,4′,6′-trimethyl)anil] is followed at 490 mμ with the Cary spectrophotometer, with the cuvet compartment thermostated at 25°. When the zero-order phase of the reaction is reached ($A_{490m\mu}$ linear with time), the slope, $\Delta A/\Delta t$, is determined from the rise in absorbance and the chart speed. This slope is proportional to the HRP concentration determined spectrophotometrically over a range of 10–80 mμM at 25 ± 0.5° (Figure 1) for "fresh" (*i.e.*, undenatured) HRP samples, and hence is taken as a measure of the HRP concentration present capable of catalyzing the reaction of H_2O_2 and mesidine to form "purple compound."

In the inactivation studies, 0.01 ml of HRP is pipetted into the tip of a tapered test tube or centrifuge tube, followed, at time zero, by 0.01 ml of a suitably high concentration of H_2O_2. After the desired incubation time has elapsed, an amount of 0.018 M mesidine–HCl solution in acetate buffer is added sufficient to lower, simultaneously, the H_2O_2 concentration to about 0.005 M and the active HRP concentration to 10–80 mμM. An aliquot of the assay mixture is quickly transferred to a cuvet, and the slope determined as above. In this way the enzyme inactivation can be obtained as a function of exposure time to hydrogen peroxide.

Soret Peak Decay. A sample of HRP is diluted with acetate buffer to the proper concentration in a cuvet, and the Soret peak of the free enzyme is recorded. At incubation time zero, a known volume of concentrated H_2O_2 is added from a pipet with stirring. The Soret peak region is scanned repeatedly at a rate of 10 A/sec as the decay proceeds, the exact incubation time being noted as the Soret peak is traversed. The Soret peak absorbance values thus obtained are altered as follows. (1) The zero incubation time absorbance value (free enzyme) must be multiplied by the dilution factor arising from the addition of the peroxide. (2) Since the enzyme is converted into compound III before decay proceeds, the free enzyme absorbance value is multiplied by the ratio of the Soret peak extinction coefficients of compound III to free enzyme. That of compound III is taken to be 93 mM^{-1} cm^{-1} (Chance, 1952a). In all cases no further shift of the Soret band is noted, once compound III is formed. (3) A residual absorbance value at the Soret peak wavelengths which persists at very long incubation times is subtracted from all absorbance values. This residual absorbance was generally <5% of the initial (compound III) absorbance. The "true" Soret peak absorbance values are thus obtained as a function of the exposure time to the peroxide.

Results

The measurements taken show that a sharp initial drop of 30–50% in the enzyme activity occurs during the first minute of exposure to high H_2O_2 concentrations (Figure 2). This drop in activity is seen over the entire range of peroxide concentrations studied (0.35–2.50 M) and has no counterpart in the decay of the Soret peak, which appears uniform from incubation time zero over the peroxide concentration range studied. Thus, an early inactivation at a nonheme site is strongly suggested. The absence of an early lag phase in either series of inactivation studies reinforces the conclusion (from independent experiments) that the time for compound III to begin decay from its maximum value is small compared to the total incubation times in these studies.

At longer exposure times, the enzyme activity decay and Soret peak decay data were plotted semilogarithmically, according to a rate law of the form

$$\frac{-d[HRP]}{dt} = k'[HRP] \qquad (1)$$

where k' would depend in some way on the peroxide concentration. The variation in k' with changing $[H_2O_2]_0$ is plotted in Figure 3 and tabulated in Table I. The values of k' obtained using both techniques approach the same constant value of 0.054 at peroxide concentrations <1 M. At higher peroxide concentra-

TABLE I: Inactivation of HRP by H_2O_2.

$[HRP]_0$ (μM)	$[H_2O_2]_0$ (M)	k'^a (min^{-1})
A. Assay of Enzyme Activity		
5.5	0.35	0.055
5.7	0.35	0.053
7.8	0.60	0.054
7.8	0.85	0.068
13.0	1.00	0.084
5.0	1.32	0.109
15.9	1.60	0.135
15.9	1.80	0.164
7.5	1.99	0.179
15.9	2.50	0.215
B. Decay of Soret Peak		
6.6	0.13	0.054
8.7	0.17	0.054
8.6	0.34	0.055
6.0	0.70	0.056
5.9	1.01	0.075
5.1	1.32	0.094
5.5	1.80	0.118
5.9	2.25	0.133
6.7	2.59	0.156
6.8	3.11	0.160
6.5	3.84	0.188

[a] Determined graphically from eq 1.

tions, k' for the enzyme activity decay becomes increasingly higher than that for the Soret peak decay. In this region, then, it appears that the enzyme undergoes inactivation at a nonheme site. A reasonable kinetic scheme to fit the data of this region is given below

$$\underset{e}{\text{compound III}} + \underset{x}{H_2O_2} \xrightarrow{k_1} \underset{p_1}{E^*} \qquad (2)$$

$$\underset{p_1}{E^*} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{p_2}{E\ddagger} + \underset{y}{AH\cdot} \qquad (3)$$

$$\underset{p_1}{E^*} \xrightarrow{k_3} E_{oxp} \qquad (4)$$

$$\underset{p_2}{E\ddagger} \xrightarrow{k_4} E_{oxr} \qquad (5)$$

$$\underset{y}{AH\cdot} + \underset{y}{AH\cdot} \xrightarrow{k_5} AH_2 + A \qquad (6)$$

In this scheme, the peroxidase, in the presence of excess peroxide, is in the form of compound III. With an additional molecule of peroxide, compound III forms an inactive, transient compound, E*. E* then undergoes a rapidly reversible equilibrium step (reaction 3) in which a free radical, formally labeled AH·, appears along with another unstable form of the enzyme, E‡. Whether AH· represents a hydroxyl radical, a perhydroxyl radical, or a free radical of protein origin is not clear. However, free radicals have been detected in HRP–H_2O_2 systems (Morita and Mason, 1965). E* also may undergo a slow, irreversible oxidation of a protein residue, resulting in E_{oxp}. The rate of this reaction, $[dE_{oxp}]/dt = k_3p_1$, contributes to the over-all inactivation studied by the enzyme assay method. E‡ may also undergo a slow, irreversible oxidation of its porphyrin ring to form E_{oxr}, which does not show a Soret transition. The rate of this reaction, $d[E_{oxr}]/dt = k_4p_2$, is the one monitored by the Soret peak decay studies, and represents the remaining contribution to the over-all inactivation rate. Finally, a necessary recombination of radicals is included.

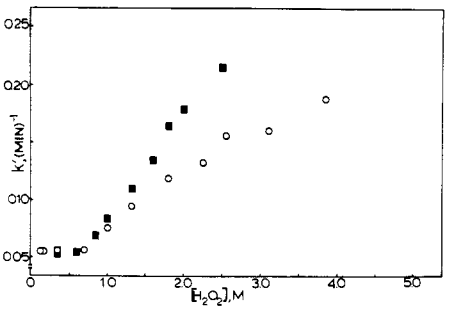

FIGURE 3: Variation of k' with initial hydrogen peroxide concentration in HRP inactivation (k' calculated from eq 1). O, Soret peak decay; ■, enzyme activity decay.

In terms of this kinetic scheme, the early loss of activity may be considered a "pre-steady state" manifestation of reactions 2 and 4, with E‡ not yet present in significant amounts This is an interesting region and deserves further study, but the short incubation times involved indicate that more rapid techniques may be required for detailed kinetic studies.

The steady-state analysis of this scheme yields, first, that

$$k_1ex = k_4p_2 + k_3p_1 = \frac{d[E_{oxr}]}{dt} + \frac{d[E_{oxp}]}{dt} \qquad (7)$$

i.e., that the over-all rate of inactivation is proportional to the first power of the product ex, and is, in fact, equal to k_1ex.

Manipulation also gives

$$k_1ex = 2k_5y^2\left(1 + \frac{k_3}{k_2} + \frac{k_3k_{-2}}{k_2k_4}y\right) \qquad (8)$$

It may be seen from the behavior of the experimental data that the dominant terms at large x must come from those arising from k_3p_1 (the inactivation by protein oxidation), *viz.* $(k_3/k_2)(1 + k_{-2}y/k_4)$. For $k_{-2}y/k_4 > 1$ which may hold at large x (k_4 is small)

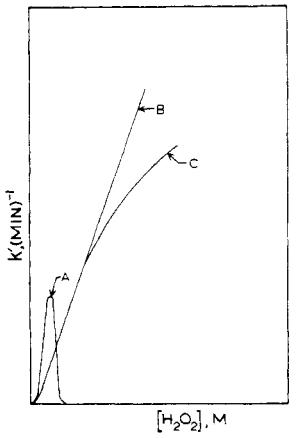

FIGURE 4: Resolution of the curves of Figure 3 into component curves (see discussion relating to reactions 14–16).

$$y = \left[\frac{k_1k_2k_4}{2k_{-2}k_3k_5}\right]^{1/3}(ex)^{1/3} \quad (9)$$

In this case

$$\frac{d[E_{oxr}]}{dt} = k_4p_2 = 2k_5y^2 = (2k_5)^{1/3}\left[\frac{k_1k_2k_4}{k_{-2}k_3}\right]^{2/3}(ex)^{2/3} \quad (10)$$

i.e., the above scheme predicts a two-thirds power dependence upon the product (*ex*) for the Soret peak decay rate.

It remains to show that the data indeed exhibit the dependences upon (ex) of eq 7 and 10. The data which were examined by eq 1 must now be examined according to the more general rate expression:

$$-\frac{d[HRP]}{dt} = K([HRP][H_2O_2])^n \quad (11)$$

This is equivalent to determining whether or not there exist values of n such that the parameter

$$K = \frac{k'[HRP]^{1-n}}{[H_2O_2]^n} \quad (12)$$

is invariant under changes in the concentrations of reactants. For the decay of activity, the kinetic scheme discussed above assigns K the value k_1. For the Soret peak decay, K is assigned a more complicated expression, $(2k_5)^{1/3}[k_1k_2k_4/k_{-2}k_3]^{2/3}$. Because of the large molar excess of peroxide employed in this study, the approximation $[H_2O_2] = [H_2O_2]_0$ in eq 12 is a very good one. While, in eq 12, the enzyme concentration, and hence K, are functions of time, the consistent use of $[HRP]_0$ for [HRP] enables one to assess directly the effect of the enzyme concentration upon K. Equation 12 is thus modified to

$$K \simeq \frac{k'[HRP]_0^{1-n}}{[H_2O_2]_0^n} \quad (13)$$

A search for the best values of n in eq 13 was undertaken. It was soon clear that integral powers of n other than one gave series of values of K with very large standard deviations. Some other values of n tried and the corresponding values of K_{mean} ($\pm\sigma$) are listed in Table II. It is apparent that the best value of K_{mean} (that with the smallest standard deviation) for the case of the enzyme activity decay data is obtained for $n = 1$, where $K_{mean} = 0.086 \pm 0.003$ M^{-1} min^{-1}. In the case of the Soret peak decay data, $n = 0.67$, 0.70, and 0.75 all give equally good values of K_{mean}. This is not unexpected, for it has been shown that the two-thirds power dependence of eq 10 arises as a limiting case; the proposed kinetic scheme allows n to vary from two-thirds to one, depending on the relative sizes of the terms in eq 8.

Discussion

At peroxide concentrations <1 M, where the enzyme is apparently inactivated by attack at the heme site alone, a "plateau" inactivation rate of 0.054 min^{-1} is measured, as has already been noted above. If this value is subtracted from the values of k' determined at higher peroxide concentrations and values of K are then recomputed for the values of n in Table II, it is found that, in general, σ is a much greater percentage of K_{mean}, in each case, than if k' had not been "corrected." This is an indication that the region of constant k' does not represent a different inactivation process which acts in parallel with those operative at higher peroxide concentrations.

Recognizing that the data of Figure 3, if extrapolated to $[H_2O_2]_0 = 0$, must pass through the origin, one may view the region of constant k' as the resultant of two inactivation processes, as shown in Figure 4. Curve A might represent a process governed by kinetics of the following kind

$$E + H_2O_2 \xrightarrow{k_a} \text{compound I} \quad (14)$$

$$\text{compound I} \xrightarrow{k_b} D \quad (15)$$

$$\text{compound I} + H_2O_2 \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} \text{compound III} \quad (16)$$

$$\text{compound III} + H_2O_2 \xrightarrow{k_t} E^*, \text{ etc.} \quad (2)$$

The essential part of this scheme is that compound I (or some other intermediate proportional in concentration to compound I) undergoes a first-order reaction to an inactive species, D. It is probable that reaction 15, which may represent the "catalytic" process in

TABLE II: Values of K as a Function of n (from eq 13).[a,b]

n	$K_{mean} \pm \sigma$ (± %)	
	Soret Peak Decay	Act. Decay
0.50	0.22 ± 0.03 (±14)	...
0.60	0.17 ± 0.01 (±6)	...
0.67	0.14 (2) ± 0.006 (±4)	0.23 ± 0.05 (±22)
0.70	0.13 (1) ± 0.005 (±4)	0.21 ± 0.04 (±19)
0.75	0.11 (5) ± 0.005 (±4)	0.18 ± 0.03 (±17)
0.80	0.10 (1) ± 0.006 (±6)	0.15 (5) ± 0.023 (±15)
0.90	0.079 ± 0.008 (±10)	0.11 (5) ± 0.010 (±9)
1.00	0.060 ± 0.010 (±17)	0.086 ± 0.003 (±3.5)
1.10	...	0.065 ± 0.004 (±6)
1.20	...	0.048 ± 0.005 (±10)
1.33	...	0.033 ± 0.007 (±21)

[a] Units of K are $(\mu M\ HRP)^{1-n}$ $(M\ H_2O_2)^{-n}$ min^{-1}. [b] $\sigma = [\Sigma_p(K_{mean} - K_p)^2/(p-1)]^{1/2}$.

HRP, occurs *via* the formation of compound IV (Keilin and Hartree, 1951). It can be shown by steady-state analysis of reactions 14–16 and 2 that: (1) at low $[H_2O_2]$, where [compound III] is negligible, d[D]/dt is proportional to $[H_2O_2]$. (2) For intermediate $[H_2O_2]$ such that [compound III] is still not substantial, d[D]/dt is independent of $[H_2O_2]$. (3) At large $[H_2O_2]$, where [compound I] and [compound III] are both substantial, d[D]/dt is proportional to $[H_2O_2]^{-1}$ and hence tends to zero. Thus, the above reactions can lead to an inactivation curve similar to curve A of Figure 4. Superposition of curve A with curves B and C of the type found at higher $[H_2O_2]$ can give a region of approximately constant k'.

The proposal that HRP is inactivated by high concentrations of hydrogen peroxide through attack at a nonheme site implies a role for that site in HRP peroxidatic action at lower $[H_2O_2]$. Such an idea is not new. It has been postulated (Naylor and Saunders, 1950; Saunders and Watson, 1950) that an activated complex of peroxidase, peroxide, and hydrogen donor may be formed in the course of peroxidase action. While such a complex does not, in itself, require the participation of the apoenzyme, it is interesting to note that in the absence of exogenous hydrogen donor, the protein may furnish an "endogenous hydrogen donor," similar to that described by Chance (1952b). This interpretation implies that such a donor be in close proximity to the heme group in the native enzyme.

Other workers have discussed possible roles for the protein in peroxidase activity. Nicholls (1962) criticized the theory that the protein provided a crevice for the hematin group and hence exerted steric constraints upon the enzymic reaction (George and Lyster, 1958); he advanced instead a theory involving an indirect effect of the protein upon the heme group. The experiments of Kurozumi *et al.* (1961) concerning the peroxidase activities of hemoproteins indicated that the specific role of the HRP apoprotein lay in the enhancement of the rate of combination of enzyme and peroxide molecules. This is consistent with the proposal of this paper that oxidation of a certain amino acid residue (or possibly residues) near the heme group would disrupt the heme–apoenzyme "active site configuration" and render the enzyme relatively inactive.

If such a model is proposed, an explanation for the fact that HRP reacts with H_2O_2 "reversibly" (no net loss in activity) as compounds I–III at lower peroxide concentrations must be brought forth. Consider the following series of reactions, where P represents the protein site, or endogenous hydrogen donor, P* the protein site oxidized reversibly, P** the protein site oxidized irreversibly, and AH_2 exogenous hydrogen donor

$$^1/_2\,H_2O_2 + P \xrightarrow{k} P^* \qquad (17)$$

$$P^* \xrightarrow[AH_2]{k_{ii}} P \qquad (18)$$

$$P^* \xrightarrow[H_2O_2]{k_{iii}} P^{**} \qquad (19)$$

At H_2O_2 concentrations approximately equimolar with that of HRP, P is oxidized to P*, which represents the condition (reversibly reducible) of the protein site in compounds I–III (see reaction 17). This is consistent with the widely held view that the spectral differences between compounds I–III are due to differences at the heme site, for instance, in the iron oxidation state (George, 1952, 1953), and that the donor specificities are about the same for compounds I and II, since there is a protein site involved. If exogenous hydrogen donor is present, reaction 18 proceeds, with P* representing the protein in compounds I and II. If no such donor is present, or if excess peroxide is present, P* (compound III) will be formed and reaction 19 will proceed. The higher the peroxide concentration, the greater the extent of reaction 19 relative to reaction 18, and the greater the irreversible damage. At lower H_2O_2 concentrations (<1 M) the data (Figure 2) indicate that reaction 19 is not proceeding to a significant extent.

The proposed model, then, is one in which the amino acid residue(s) making up the second site can be altered oxidatively, first in a reversible way, and then drastically and irreversibly. The indole group of tryptophan, the imidazole group of histidine, and the phenolic group of

tyrosine are all possibilities for this site in the sense that one can conceive of a minor oxidative change (*e.g.*, hydroxylation) brought about by peroxide, followed by a major, irreversible change (ring cleavage in each case). The recent analyses of Klapper and Hackett (1965) indicate the presence of histidine and tyrosine in HRP. However, Maehly and Paléus (1950) have stated that tryptophan is absent. Before the assignment of the second site can be made with confidence, considerably more data on the chemistry of the HRP molecule must accrue.

It should be mentioned that inactivation at the protein part of the HRP "active site" could occur indirectly through, *e.g.*, the oxidation of a disulfide bridge in another region of the protein, resulting in a change in secondary structure which could reflect itself in disruption of the "active site." However, the fact that H_2O_2 is not an efficient reagent for disulfide bridge scission, even under very severe reaction conditions, makes this possibility less likely than a direct attack on an amino acid residue actually forming part of the catalytic site. Wholesale (generalized) oxidative degradation of the protein is ruled out by the presence of an early nonheme inactivation even at relatively low peroxide concentrations, by the modest (five- to tenfold) decrease in enzyme activity in the studies discussed, and by the kinetic analyses of the process, which indicate the presence of HRP intermediates common to both high- and low-H_2O_2-concentration inactivation processes, and hence suggest specific protein attack.

Nothing in the data obtained precludes the possibility that the carbohydrate moiety of HRP is involved in the second site. However, its role in HRP is presently obscure, and hence its possible involvement in the second site in the model proposed here will not be dealt with in this paper.

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