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ROLE OF OXYGEN DURING HORSERADISH PEROXIDASE TURNOVER AND INACTIVATION[1]

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SUMMARY: Horseradish peroxidase catalyzed oxidation of phenol has been reinvestigated to determine the requirements of facile enzyme autoinactivation. Turnover of this peroxidase was monitored spectrophotometrically at 400 nm and found dependent on the concentration of phenol and hydrogen peroxide. The inactivation of the peroxidase required both substrates, phenol and H_2O_2, but surprisingly was also potentiated by molecular oxygen. Exclusion of diffusible superoxide or hydroxyl radicals had slight effect on product formation or loss of catalytic activity. A mechanism is proposed to explain the unanticipated role of oxygen during enzyme inactivation.

Peroxidases catalyze the oxidation of a wide variety of substrates in the presence of hydrogen peroxide. Enzyme intermediates produced during catalytic turnover (eq 1 - 3) were first described spectroscopically and subsequently their reactivity was characterized with a number of electron donors (for review, see 1). Mechanistic studies using phenolic substrates are of particular interest since compounds of this class are physiological substrates for many peroxidases including thyroid peroxidase (2), ligninase (3), ovoperoxidase (4) and enzymes that are responsible for lignin (5) and yeast ascospore wall (6) formation.

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Abbreviations used: CPO, chloroperoxidase; HRP, horseradish peroxidase; LPO, lactoperoxidase; KP_i, potassium phosphate; SOD, superoxide dismutase.

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native peroxidase + H_2O_2 ⟶ compound I 1

compound I + ROH ⟶ compound II + RO· 2

compound II + ROH ⟶ native peroxidase + RO· 3

(R = phenyl)

Inhibition or irreversible inactivation of these enzymes has taken many forms. Studies on thiocarbamide antithyroid drugs have described a number of possible mechanisms for peroxidase inhibition (7 - 10). Additionally, lactoperoxidase (LPO) was shown to be inactivated in excess H_2O_2 (11, 12) and chloroperoxidase (CPO) was likewise inactivated when no halogen acceptor was included in an incubation mixture containing chloride and H_2O_2 (13, 14). Turnover inactivation of horseradish (HRP) and thyroid peroxidases has also been noted to occur in the presence of phenols (15 - 17). The origin of this is not yet understood and certainly oxygen was never implicated in the inactivation process. Data presented here, however, suggest that a phenolic intermediate may combine with molecular oxygen to cause enzyme oxidation and inactivation.

Materials and Methods: HRP (peroxidase, type VI 300 U/mg) and bovine kidney superoxide dismutase (SOD) were purchased from Sigma; analytical grade phenol was obtained from Mallinckrodt and 30% H_2O_2, from Fisher. All other reagents were of the highest grade commercially available. Hydrogen peroxide solutions were made fresh daily by dilution of the 30% commercial solution. HRP was stored (4°C) as a concentrated stock of 1.2 mg/mL in 100 mM potassium phosphate (KP_i) pH 7 and diluted before use. Concentration determination of HRP was based on $\varepsilon_{402} = 95{,}000\ M^{-1}cm^{-1}$ (18).
Kinetics: Initial rates of phenol oxidation were measured at 25°C by the formation of an absorption at 400 nm (19) and initiated with the addition of H_2O_2 to a final concentration of 5.0 mM in the presence of ca. 2.0 nM HRP, 100 mM KP_i pH 7 and phenol at various concentrations. Inactivation of HRP was also initiated by the addition of 5.0 mM H_2O_2. For these studies, incubations contained 24 nM HRP, 0.10 mM phenol and 100 mM KP_i pH 7. Time dependent loss of enzyme activity was then monitored periodically by diluting aliquots of these inactivation mixtures (25°C) into a standard V_{max} assay (5.0 mM H_2O_2, 4.5 mM guaiacol, 100 mM KP_i pH 7, 25°C) based on guaiacol oxidation (21). Oxygen was excluded during anaerobic studies by cycling samples under vacuum and argon six times (30 min). Accumulation of hydroxyl and superoxide radicals was inhibited by the addition of 0.1 M mannitol and 10 U/mL of SOD, respectively.

Results

Phenol reacts very quickly with HRP in the presence of H_2O_2 to form various light absorbing intermediate products that can ultimately lead to formation of a brownish precipitate (17, 21). Yet, steady state kinetics can

Table 1. Kinetic Constants for HRP Dependent Oxidation of Phenol[1]

		HRP	HRP - O_2	HRP + mannitol	HRP + SOD
V_{max}	$(\frac{\triangle A_{400}}{min\text{-}pM})$	84.5 ± 7.7	107 ± 28	62.0 ± 12	71.5 ± 11
K_m	(mM)	1.24 ± .15	1.61 ± .26	0.92 ± .01	0.95 ± .15

[1]Constants are based on a minimum of three sets of double reciprocal plots of initial velocity (over one min) vs phenol concentration (0.20 mM - 1.0 mM) using least squares fitting; uncertainties are given as the standard deviation. V_{max} is used for comparative analysis only and given in $\triangle A_{400}$ per min per pM HRP. Kinetic constants for standard HRP turnover were equivalent when measured in the usual 1 mL air saturated assay or after an anaerobic preparation was resaturated with air. See Methods Section for details.

be measured by absorbance change at 400 nm when the concentration of enzyme and phenol remain low. Under these conditions initial rates were found to be linear and satisfied Michaelis-Menten kinetic analysis (Table 1). As required for our comparative study, this assay is sufficient for identifying the components necessary for enzyme catalysis and inactivation. Product formation is solely dependent on the presence of H_2O_2, phenol and HRP; no change in A_{400} was noted without these three reagents. Neither freely diffusing hydroxyl radical nor superoxide appear to play a significant role in this reaction since the respective trapping agents, mannitol and SOD, had only a very small effect on the kinetic constants. Additionally, the turnover measured in this study is neither the result of or greatly influenced by the presence of molecular oxygen.

Time dependent inactivation of HRP was studied under a similar set of conditions (Table 2) using a concentration of phenol, 0.10 mM, that produced an easily measurable rate of inactivation. The primary mode of enzyme inactivation evident here was dependent on the simultaneous presence of H_2O_2 and phenol. In the absence of phenol, the k_{inact} decreased approximately an order of magnitude and conversely, when H_2O_2 was absence instead of phenol, the k_{inact} dropped even more. Turnover conditions then appear to be required for enzyme inactivation, yet molecular oxygen also affects inactivation without participating as a standard substrate of HRP. When oxygen concentration was reduced during inhibition studies, the rate of enzyme inactivation decreased noticeably from 0.337 to 0.211 min^{-1} (Figure 1) in marked contrast to the effect on turnover. This oxygen dependence can be ascribed to a requirement for molecular oxygen and not its radical derivatives since the trapping agents, mannitol and SOD, did not significantly prevent the loss of peroxidase activity (Table 2). A crucial involvement of these radicals prior to release from enzyme cannot yet be excluded.

Table 2. Time Dependent Inactivation of HRP in the Presence of Phenol and Oxygen Radical Traps[1]

peroxidase +	observed k_{inact} (min^{-1})
H_2O_2/phenol	0.337 ± .017
H_2O_2/phenol + mannitol	0.283 ± .020
H_2O_2/phenol + SOD	0.303 ± .050
H_2O_2/phenol - O_2	0.211 ± .022
H_2O_2 alone	0.031 ± .004
phenol alone	0.016 ± .006

[1]Inactivation was initiated by the addition of 5.0 mM H_2O_2. Rate constants are averaged from a minimum of four sets of semi-logarithmic plots of % remaining enzyme activity vs time; uncertainties are the standard deviations calculated by least squares analysis. Aerobic data were also generated from incubations alternatively mixed just prior to use or after deoxygenated samples were resaturated with air. See Method Section for details.

Discussion

Once phenoxy radicals are generated by HRP, a number of products may form on and off the enzyme surface. The initial formation of product(s) absorbing at 400 nm, however, still conforms to simple kinetic analysis. Enzyme turnover appears saturable and dependent on the oxidant, H_2O_2, and the reductant, phenol. Inhibition of hydroxyl and superoxide radical formation or exclusion

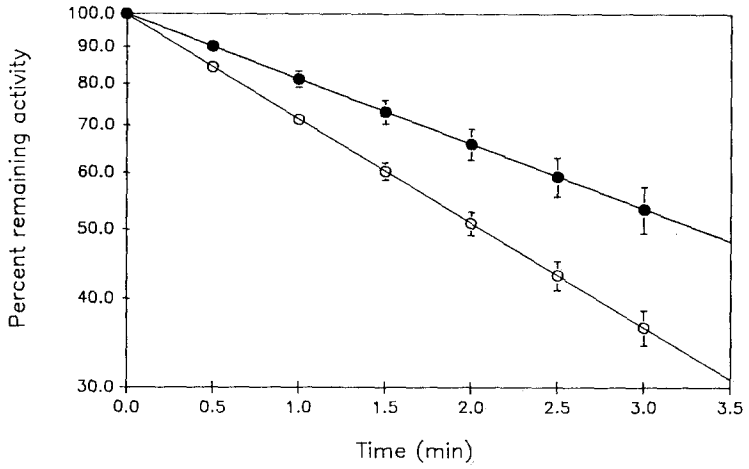
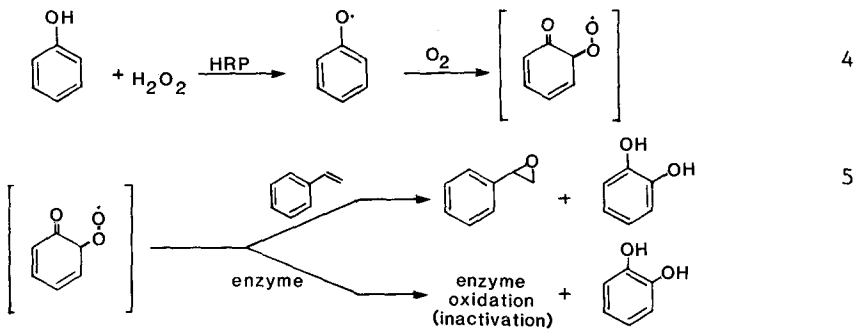


Figure 1. Time dependent loss of HRP activity in the presence of 5 mM H_2O_2 and 0.10 mM phenol in air saturated buffer (-○-○-) or after exclusion of oxygen (-●-●-). Each kinetic trace is the average of 4 sets of data. Details given in Methods Section.

of O_2 had little effect on the catalytic rate and if the V_{max}/K_m ratios of these experiments are compared, the turnover values differ by no more than 8%. Therefore, the major product absorbing at 400 nm was not likely formed by reaction with oxygen or inorganic oxygen radicals. An oxygen effect then differentiates between turnover and inactivation since at least a component of inhibition requires oxygen along with H_2O_2 and phenol. This dependence is not likely to involve diffusible species such as superoxide or hydroxyl radicals since neither SOD nor mannitol could lower the rate of inactivation to a level comparable to that measured under anaerobic conditions. The minimal effect of the trapping agents on inactivation may only be a secondary manifestation of their slightly greater influence on turnover, an integral part of autoinactivation.

Our inability to fully suppress enzyme inactivation in the absence of oxygen might be due to the presence of an underlying inhibitory process that does not require oxygen. Just as HRP may catalyze the formation of many products so may it promote a number of processes detrimental to its activity. The most likely mechanism of autoinactivation that does not require oxygen would simply involve repeated phenol radical coupling to the holoenzyme. Since enzyme-generated phenol radicals polymerize so readily (17), these same radicals might easily modify the aromatic groups on the surface of the enzyme. How oxygen could directly or indirectly facilitate this process is certainly not clear and thus, a second and distinct mechanism must be invoked to explain the surprising influence of oxygen on inactivation. Only a minimal dependence on oxygen can actually be estimated from this study since oxygen very likely remained to contaminate our anaerobic studies. The inevitable disproportionation of H_2O_2 and the low catalase activity associated with peroxidases (11) limited our attempts to exclude all oxygen.

Autoinactivation of HRP should now join the list of other oxygen consuming reactions catalyzed by this enzyme. The previously characterized reactions, glutathione oxidation (22), phenol hydroxylation (23) and styrene epoxidation (15) are not necessarily related by mechanism but do share a common requirement for oxygen. Data so far gathered for HRP inactivation is most consistent with a requisite formation of the bracketed hydroperoxy radical (eq 4), an intermediate first proposed to explain phenol/HRP/H_2O_2/O_2 dependent epoxidation of styrene (15). Neither this epoxidation as described by Ortiz de Montellano and Grab nor the inactivation documented here were quenched by SOD or mannitol. By analogy to the pathway for oxygen transfer to styrene, a mechanism of oxygen transfer to HRP can now be proposed to explain the loss of catalytic activity (eq 5). Further work towards identifying the full range of enzyme, intermediate and product species generated during inactivation is currently in progress.



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