A PROPOSED MECHANISM FOR PEROXIDASE ACTION BASED ON A KINETIC STUDY OF LUMINESCENT AND NON-LUMINESCENT PEROXIDATION REACTIONS[0]

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The kinetics of three different peroxidase reactions have been examined with respect to the effects of H_2O_2 and H donor concentration on the rate of product formation.

They are (1) the balanoglossid bioluminescence reaction in which the rate of light production is measured, (2) pyrogallol peroxidation in which the rate of purpurogallin formation from pyrogallol is followed, and (3) luminol bioluminescent peroxidation in which the rate of light production is measured.

METHODS

In the balanoglossid bioluminescent reaction an unidentified luciferin is peroxidized by balanoglossid luciferase or horseradish peroxidase and H_2O_2 (1). Light is the product measured and the assay protocol has been reported in an earlier publication by Dure and Cormier (1963).

In the pyrogallol reaction the peroxidation of pyrogallol to purpurogallin by luciferase or horseradish peroxidase and H_2O_2 is followed on a Cary Model 14 recording spectro-

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photometer. The reaction protocol routinely consisted of mixing enzyme, H_2O_2, and pyrogallol with 200 μmoles of phosphate buffer, pH 7.0, in a 3 ml. volume and following the change in absorbancy at 380 millimicrons. A line drawn tangential to the portion of the curve having the greatest slope was used to ascertain the initial velocity of the reaction. The pyrogallol reaction was used to demonstrate that the kinetic data derived from the light reactions were not unique to the light reactions, but a true measure of product formation in peroxidase reactions of the classical peroxidase type.

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) as well as a number of chemiluminescent compounds, was found susceptible to enzymatic peroxidation at pH 7.0 with light as one of the products. The protocol for this reaction was identical with that reported previously for the balanoglossid bioluminescent reaction, but with luminol replacing balanoglossid luciferin and phosphate buffer, pH 7.0, replacing Tris buffer, pH 8.2.

The kinetic relationships observed using horseradish peroxidase were identical to those observed using balanoglossid luciferase, thus the bulk of the experiments were performed with horseradish peroxidase serving as the enzyme. The kinetic relationships observed in the light emitting assay when luminol served as the H donor were the same as those observed when balanoglossid luciferin was the H donor, and since the balanoglossid organism is available only in scanty quantities, the biologically artifical luminol-horseradish peroxidase system replaced the balanoglossid system in the study of light production from peroxidation reactions.

In addition to determining the effect of H_2O_2 and H donor concentration on the rate of product formation (light or purpurogallin), the effect of H donor concentration on the apparent Km and Vmax derived for H_2O_2 was determined. Likewise, the effect of H_2O_2 concentration on the apparent Km and Vmax for H donor was determined.

RESULTS AND DISCUSSION

H_2O_2 and H donor concentration effect the rate of product formation in the same ways in all three peroxidation reactions mentioned above. From this it is concluded that the mode of peroxidase action is the same in the light reactions as in the pyrogallol reaction, and that the kinetic relationships first noticed in the balanoglossid light reaction (1) are characteristic of peroxidase reactions of the classical type.

In all three peroxidase systems H_2O_2 concentration effects the rate in the typical hyperbolic fashion and the data yields the standard double reciprocal plot. However, in all three peroxidase systems H donor concentration effected the rate in a peculiar fashion that necessitated that $1/\sqrt{v}$ be plotted against 1/H donor concentration in order to achieve a linear double reciprocal plot. Some of these relationships are summarized in Table 1.

Furthermore, it was discovered that increasing the concentration of H donor caused an increase in the apparent Km and Vmax derived for H_2O_2, but in a saturating rather than linear fashion. Increasing concentrations of H_2O_2 caused a nonlinear increase in the apparent Km and Vmax derived for H donor, and this increase also approached a saturation value. These relationships were observed in both the bioluminescent light assay and the purpurogallin assay.

Any mechanism proposed for peroxidase action when H_2O_2 is the oxidant must lead to a rate equation that predicts (1) that the double reciprocal plot of rate vs. H_2O_2 concentration be linear, (3) that H donor concentration effect the apparent Km and Vmax derived for H_2O_2 in a nonlinear manner, and (4) that H_2O_2 concentration effect the apparent Km and Vmax derived for H donor in a nonlinear fashion.

Therefore, the rate equation must have the form of

$$v = \frac{Vmax S_1}{Km + S_1} \qquad (1)$$

for S_1 (H_2O_2) and have the form of

$$v = \frac{Vmax\ S^2}{(Km + S_2)^2} \qquad (2)$$

for S_2 (H donor). This second equation when reciprocated will give

$$1/\sqrt{v} = \frac{Km}{\sqrt{Vmax}} \cdot \frac{1}{S_2} + \frac{1}{\sqrt{Vmax}} \qquad (3)$$

which predicts the observed linear relationship between $1/\sqrt{v}$ and $1/S_2$.

The classical mechanism for peroxidase action, which is discussed in a review by Mason (1957), is

$$E + S_1 \longrightarrow C_1$$
$$C_1 + S_2 \longrightarrow C_2 + P_1.$$
$$C_2 + S_2 \longrightarrow E + P_1.$$
$$2P_1. \longrightarrow P_2$$

and this mechanism yields a rate equation that has the form of equation 1 for both S_1 and S_2. Furthermore, it predicts that the Km_{S_1} and $Vmax_{S_1}$ increases linearly with S_2 concentration. It also predicts that the Km_{S_2} and $Vmax_{S_2}$ increases linearly with S_1 concentration.

If the classical mechanism is modified to the extent of making C_2 an enzyme substrate complex, the formation of which is reversible, the derived rate equation takes on the form of equation 2 for S_2, and so satisfies the observed relationship between rate and S_2 concentration. However, the rate equation derived from this modification alone still predicts that $Vmax_{S_1}$ and $Vmax_{S_2}$ should increase linearly with increasing S_2 and S_1 concentration respectively.

If the classical mechanism is further modified to contain a ternary enzyme substrate complex, C_3, two mechanisms

$$
\begin{aligned}
E + S_1 &\longrightarrow C_1 \\
C_1 + S_2 &\rightleftharpoons C_2 \\
C_2 + S_2 &\longrightarrow C_3 \\
C_3 &\longrightarrow E + P + h\nu
\end{aligned}
\qquad (5)
$$

$$
\begin{aligned}
E_1 + S_1 &\longrightarrow C_1 \\
C_1 + S_2 &\rightleftharpoons C_2 \\
C_2 + S_2 &\longrightarrow C_3 \\
C_3 &\longrightarrow 2P_1\cdot + E \\
2P_1\cdot &\longrightarrow P_2
\end{aligned}
\qquad (6)
$$

which yield the same rate equation,

$$
v = \frac{k_1k_2k_3k_4\ E_tS_1S_2^2}{k_2k_3k_4S_2^2 + k_1k_2k_3S_1S_2^2 + k_1k_2k_4S_1S_2 + k_1k_3k_4S_1S_2 + k_1k_{-2}k_4S_1} \qquad (7)
$$

can be obtained. This rate equation can be arranged to show the observed hyperbolic relationship between rate and S_1 concentration, and can be rearranged into the form of equation 2 for S_2. Furthermore, when its rate equation is solved for Km_{S_1}, $Vmax_{S_1}$, Km_{S_2} and $Vmax_{S_2}$, these expressions are not linearly related to S_2 and S_1 concentrations respectively. The predicted relationships between Km_{S_1}, $Vmax_{S_1}$, and S_2 concentration and Km_{S_2}, $Vmax_{S_2}$, and S_1 concentration derived from the rate equation (equation 7) are given in Table I. That these predictions are borne out experimentally is also shown in Table I.

Expressions for max $Vmax_{S_1}$ and max $Vmax_{S_2}$ can be derived from the rate equation (equation 7). Table II shows these expressions to be the same for each max Vmax, and further shows that this predicted equality is borne out experimentally in both assays systems, the results of which are given in Table II.

TABLE I

FUNCTION	PREDICTED LINEAR RELATIONSHIPS	EXPERIMENTALLY OBSERVED LINEAR RELATIONSHIPS	
		Luminol	Pyrogallol
Km_{S_1}	$\frac{1}{\sqrt{Km_{S_1}}}$ vs $\frac{1}{S_2}$	+	+
$Vmax_{S_1}$	$\frac{1}{\sqrt{Vmax_{S_1}}}$ vs $\frac{1}{S_2}$	+	+
Km_{S_2}	$\frac{1}{(Km_{S_2})^2}$ vs $\frac{1}{S_1}$	+	+
$Vmax_{S_2}$	$\frac{1}{Vmax_{S_2}}$ vs $\frac{1}{S_1}$	+	+

TABLE II

EQUATION	EXPERIMENTALLY DERIVED VALUES	
	Luminol	Pyrogallol
max $Vmax_{S_1} = k_4E_t$	1600 light units	1.40 Δ O.D. 380/minute
max $Vmax_{S_2} = k_4E_t$	1500 light units	1.37 Δ O.D. 380/minute

Since all the experimentally derived kinetic data obtained from both peroxidase assays systems are predicted by the rate equation (equation 7), one or both of the mechanisms (mechanisms 5 and 6) which yield rate equation 7 suggest themselves to be the intrinsic mode of peroxidase action, when functioning as a classical peroxidase.

REFERENCES

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