Peroxidase-catalyzed Formation of Triplet Acetone and Chemiluminescence from Isobutyraldehyde and Molecular Oxygen*

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(Received for publication, January 29, 1985)

It has been established that the horseradish peroxidase/O2/isobutyraldehyde (IBAL) system leads to triplet acetone and formic acid formation followed by phosphorescence of the triplet acetone (see, for example, Bechara, E. J. H., Faria Oliveira, O. M. M., Durán, N., Casadei de Baptista, R., and Cilento, G. (1979) Photochem. Photobiol. 30, 101–110). In this paper many of the mechanistic details are established. The reaction is initiated by the autoxidation of IBAL to form the peracid (CH$_3$)$_2$CHC$\equiv$O(OOH). The peracid converts horseradish peroxidase into compound I which in turn is converted into compound II by abstracting the alcoholic hydrogen atom from the enol form of IBAL. This creates a free radical with two resonance forms.

\[
\begin{align*}
\text{(CH}_3\text{)}_2\text{C} & \equiv \text{C} - \overset{\text{O}}{\text{O}}\text{f18}^* \\
\text{(CH}_3\text{)}_2\text{C} & \equiv \text{C} - \overset{\text{O}}{\text{O}}
\end{align*}
\]

Addition of molecular oxygen to the latter resonance form creates a peroxy radical which abstracts a hydrogen atom near the active site of the enzyme. The newly formed α-peroxide in turn forms a dioxetane-type of intermediate which rapidly decomposes into triplet acetone and formic acid. Compound II reacts with the enol by the same pathway as compound I. Thus native horseradish peroxidase is regenerated. The hydrogen atom abstraction near the enzyme active site may occur directly from ethanol, present to solubilize IBAL or from a group on the enzyme, in which case ethanol participates in a repair mechanism. Phosphate buffer is necessary because it catalyzes the keto-enol conversion of IBAL. Thus horseradish peroxidase participates in a normal peroxidatic cycle. The only chain reaction is the uncatalyzed autoxidation of IBAL, most of which occurs prior to the mixing of IBAL with the oxygenated horseradish peroxidase solution.

The modern literature on peroxidase as an oxidase begins in 1939 (1, 2). Interest was aroused that such a comparatively simple enzyme could activate molecular oxygen for the oxidation of organic compounds. However, it has become clear that plausible mechanisms involve many elementary reactions. Complexities of peroxidase as an oxidase are illustrated with NADH (3, 4), indole-3-acetic acid (4–9), and IBAL (10–12) as substrates. The latter two substrates emit light in their reaction with O$_2$ catalyzed by horseradish peroxidase (4, 8–12). The light emission reactions (i) opened a new area of peroxidase research where the mechanism and consequences of transfer of electronic energy in biological systems have been studied (13, 14); and (ii) provide an additional probe to elucidate the mechanism(s) of action of horseradish peroxidase as an oxidase. It is the latter topic which is addressed here. Our mechanistic studies have been concerned with the IBAL/O$_2$/horseradish peroxidase system for which the overall reaction is shown in Scheme 1.

Under various experimental conditions it appeared that three different factors can control the rate of the reaction: amount of initiating peracid present in the IBAL, amount of enzyme, or rate of enolization of IBAL. Preliminary evidence has been described (15). In this paper we describe new experiments and discuss a proposed mechanism in detail.

MATERIALS AND METHODS

The analytical grade reagents were purchased from the following sources: horseradish peroxidase (type VI) and l-ascorbic acid from Sigma; K$_2$HPO$_4$, KH$_2$PO$_4$, K$_2$P$_2$O$_7$, KI from Merck; H$_2$O$_2$ from Carlo Erba. IBAL (Janssen Chimica, 98%) after drying over CaCl$_2$ was carefully distilled under N$_2$ using a Vigreux column (b.p. 64°C); stock solutions in ethanol (1:5 and 2:5) were prepared. Horseradish peroxidase (ε$_{410}$ = 1.02 × 10$^4$ M$^{-1}$ cm$^{-1}$) (16) and ascorbate (ε$_{410}$ = 1.4 × 10$^4$ M$^{-1}$ cm$^{-1}$) (17) were determined spectrophotometrically, H$_2$O$_2$ by the peroxidase assay (18). Oxygen consumption was followed with a Yellow Springs Instruments Model 53 Oxygen Monitor and a sample

\[
\text{SCHEME 1. Horseradish peroxidase-catalyzed aerobic oxidation of IBAL.}
\]

1 The abbreviations used are: IBAL, isobutyraldehyde; HRP, native horseradish peroxidase, EC 1.11.1.7, donor H$_2$O$_2$ oxidoreductase; enol, (CH$_3$)$_2$C$\equiv$OHOH; peracid, (CH$_3$)$_2$C(=O)O$^+$; E$_o$, total peroxidase. HEnzyme indicates source of hydrogen atoms in the active site of either the native enzyme or compound II; *Enzyme is the corresponding free radical.
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The reaction mixture was pre-incubated with ascorbate for 3 min and then horseradish peroxidase was added. The ascorbate was determined at the time of the kinetic measurements. Second, neat IBAL was shaken with aqueous solutions of KI of different concentrations to remove peracid; this IBAL was then added to the reaction mixture that already contained ethanol (0.46 M) and the remaining extent of reaction determined. Third, a biamperometric titration technique was used. In this method a known volume of a standard solution of IO: is titrated directly with iodide using horseradish peroxidase as catalyst and IO: react with the I- in acid media to form the I/I- reversible pair. The current generated by the reversible pair is measured in counts per 5 s.

To estimate the amount of peracid initiator present in the IBAL samples, four different methods were used. First, reaction mixtures containing IBAL were pre-incubated with ascorbate for 3 min and then horseradish peroxidase was added. The ascorbate was determined at the time of the kinetic measurements. Second, neat IBAL was shaken with aqueous solutions of KI of different concentrations to remove peracid; this IBAL was then added to the reaction mixture that already contained ethanol (0.46 M) and the remaining extent of reaction determined. Third, a biamperometric titration technique was used. In this method a known volume of a standard solution of IO: is added to a polarographic cell containing a dilute IBAL sample (40 mM), iodide in excess and H2SO4 (4 mM) in deaerated solution. The peracid and IO: react with the I- in acid media to form the I/I- reversible pair. The current generated by the reversible pair is measured. Subsequent additions of known volumes of IO: increase this current linearly, so that a linear plot is obtained of current (corrected for volume increase) versus [IO:]. The current of the initial mixture is taken as the zero current value and the difference of the intercept with the x axis from the value obtained for the [IO:] initially added corresponds to the I- generated by the peracid. Fourth, peracid was titrated directly with iodide using horseradish peroxidase as catalyst (15). A fifth method, which is indirect and applicable under restricted experimental conditions in which the role of initiating peracid is supplemented with added H2O2, is described below.

RESULTS

Using the IBAL/O2/horseradish peroxidase system under optimized conditions for triplet acetone production, "typical" kinetic curves for light emission were observed (10, 15). After an initial burst in light emission a steady-state phase is reached in which oxygen is consumed in an approximately zero order process. Light intensity increases moderately during oxygen depletion, since oxygen partially quenches the triplet acetone (10). The end of the reaction is normally caused by complete oxygen consumption; a sharp decrease in light emission is observed.

In contrast to the typical kinetics, we observed in the course of this work that it was also possible under "normal" conditions (42.3 mM IBAL, 1.7 μM horseradish peroxidase), to have the light emission decrease sharply before complete oxygen uptake. Concomitantly a break was observed in the oxygen consumption curve. This may be due to a lack of the peracid formed by the autoxidation of IBAL (19). By conventional procedures (see "Materials and Methods") it was not possible to remove all of the initiating peracid from the IBAL. Addition of H2O2, which also acts as an initiator, restores the light emission and the oxygen consumption by enhancing the reaction rate and prolonging the reaction time (15).

The effect of added H2O2 upon the light emission and oxygen consumption curves is shown in Fig. 1, for two values of [IBAL]. The influence of H2O2 is most striking for the lower [IBAL] with an enhancement of the initial burst and a marked prolongation of the reaction time (Fig. 1a). For higher [IBAL] (Fig. 1b) there is no clear enhancement of the initial burst with the addition of H2O2, just a prolongation of the reaction time, showing that there is sufficient initiating peracid present for the initial burst but not enough to complete the reaction. The effect of H2O2 upon the initial burst for [IBAL] = 8.46 mM is shown in Fig. 2. For this very small [IBAL] and with little or no added H2O2 the initiating peracid is rate-limiting.

In Fig. 3 the time required to reach maximum light emission
The data represent light intensity in the steady state. [IBAL] = 8.46 mM; [horseradish peroxidase] = 1.7 μM.

<table>
<thead>
<tr>
<th>IBAL</th>
<th>Initiator present (μM)</th>
<th>H₂O₂ added (break in the plot) (μM)</th>
<th>% O₂ uptake at the break</th>
<th>Total initiator (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.46</td>
<td>0.40</td>
<td>11 × 10⁻⁶</td>
<td>90</td>
<td>11</td>
</tr>
<tr>
<td>16.9</td>
<td>0.25</td>
<td>5.7 × 10⁻⁶</td>
<td>90</td>
<td>6.1</td>
</tr>
<tr>
<td>33.8</td>
<td>0.54</td>
<td>5.4 × 10⁻⁶</td>
<td>97</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>0.74</td>
<td>5.5 × 10⁻⁶</td>
<td>97</td>
<td>6.2</td>
</tr>
</tbody>
</table>

(For example, arrow B in Fig. 1b) is plotted against [H₂O₂] for the reaction times. The smaller [IBAL] the shorter is the reaction time without added H₂O₂ (intercept with the y axis), because of the lower content of initiating peracid in the IBAL. The higher the value of [IBAL] the smaller is the increase in the reaction time by the addition of H₂O₂. There is indeed only a small effect for the highest [IBAL], indicating that there is already sufficient initiating peracid in the IBAL to complete the reaction. At medium [IBAL] the plot shows two straight line regions. The slope of the linear portion at higher [H₂O₂] is small indicating that further H₂O₂ is not required to complete O₂ consumption. Indeed the [H₂O₂] corresponding to the intersection of the two straight lines is sufficient for 90–100% uptake of O₂ (Table I). The increase in the reaction time with increasing [H₂O₂] may be due to compound III formation (see below).

From the dependence on [H₂O₂] the initiating peracid concentration already present in the sample can be evaluated by the extrapolation of the straight line for low [H₂O₂] to the abscissa (Fig. 4a, point x in the inset). The [H₂O₂] necessary to complete the reaction is estimated from the intersection of the two straight lines (Fig. 4a; point y in the inset). The sum of the two values (x + y) is the total initiator concentration necessary to complete the reaction (H₂O₂ + peracid). This method of adding x + y is straightforward in the theory but somewhat more difficult in practice. The problem is to determine which is the most accurate indication of a complete reaction. Three methods were used: a plot of (i) the time of the break in the O₂ consumption (or the time of 100% O₂ uptake), (ii) the time of the maximum in light emission, and (iii) the time where the light emission decreases sharply; versus [H₂O₂]. The data for the three methods correspond to arrows A, B, and C in Fig. 1b. Fig. 4 shows these plots for [IBAL] = 16.9 mM. The values obtained by the O₂ uptake fall between the values obtained by the two parameters of the light emission. For [IBAL] = 8.46 mM it was not possible to measure the time of the sharp decrease in light emission, because no end point of the reaction can be seen, presumably because O₂ diffusion to the sample can compete efficiently with the slow O₂ uptake reaction. (The reaction system in the photon counter experiments is open, whereas the sample holder for the oxygraph measurements is closed.) For [IBAL] = 67.7 mM already enough initiator is present to complete the reaction and therefore no evaluation is possible. Data obtained at the three other [IBAL] are presented in Table I.

That the reaction times are still increasing at higher [H₂O₂], when there is already enough initiator to complete the reaction, can be explained by compound III formation. The latter should slow down the reaction as it is less reactive than compounds I and II. Indeed a slower rate of O₂ uptake and a lower light intensity are seen at higher [H₂O₂] (Fig. 1b), confirming the results of Ascorbate titrations of peracid initiator present in the IBAL. The [initiator] evaluated by this method is between 3.4 × 10⁻⁴ and 67.7 mM already enough initiator is present to complete the reaction and therefore no evaluation is possible. Data obtained at the three other [IBAL] are presented in Table I.

Ascorbate titrations of peracid initiator present in the IBAL were also conducted. The ascorbate reacts with hydroperoxides or their free radicals (6). This method was used to estimate the initiator concentration in the reaction of 3-methyl-1,3-dione with horseradish peroxidase. Increasing [ascorbate] shortens the initial light emission and finally suppresses it completely. A sharp break is seen in O₂ consumption at a critical ascorbate concentration. The inhibition of the reaction by ascorbate is accompanied by the disappearance of compound II, as can be seen from the absorption spectrum in the Soret region. The critical ascorbate concentration is dependent upon [IBAL], but not upon [horseradish peroxidase], indicating that the ascorbate is destroying the initiator. The [initiator] evaluated by this method is between 3. E. J. H. Bechara and C. H. L. Soares, unpublished results.
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**FIG. 4.** Dependence of the reaction time upon [H₂O₂] at 33.8 mM IBAL: horseradish peroxidase = 1.7 μM. ●, time of the break in O₂ consumption (Fig. 1b, A) or time for total consumption; O, time of maximal light intensity (Fig. 1b, B); ×, rapid decrease of light emission (Fig. 1b, C). a, high [H₂O₂]. The inset shows schematically the method of evaluation (see text). b, low [H₂O₂] on expanded scale.

**FIG. 5.** Absorption spectra in the 500–600-nm region of the reaction system with different [H₂O₂]: a, [horseradish peroxidase] = 3.4 μM; [IBAL] = 8.46 mM; [H₂O₂] = 7.4 × 10⁻³ M; Spectra recorded within 3 min after mixing the components. b, [IBAL] = 16.9 mM; [horseradish peroxidase] = 3.4 μM; [H₂O₂]; curve 1, 0.0 M; 2, 5.0 × 10⁻⁴ M; 3, 2.0 × 10⁻⁴ M; 4, 4.0 × 10⁻⁴ M; 5, 7.4 × 10⁻⁵ M.

2 and 3 × 10⁻⁵ M for the reaction with an [IBAL] of 42.3 mM. Under these conditions the initiator present is just sufficient to exhaust the oxygen.

KI also reacts with the initiating peracid and was used to estimate its concentration. The effect of KI was detected in the O₂ uptake kinetics. For a reaction that just completed the O₂ uptake the KI concentration necessary for the complete inhibition was 1.07 ± 0.09 × 10⁻⁵ M, which corresponds to 5 × 10⁻⁵ M of initiating peracid.

The determination of the peracid content by the biamperometric titration with the addition of standard, gave a value of 4.9 × 10⁻⁵ M for a reaction that just exhausted the oxygen. From the iodide titration using horseradish peroxidase as catalyst (18) the initiator concentration was estimated to be 1.0 × 10⁻⁵ M for [IBAL] equal to 42.3 mM.

Of course, the spontaneous oxidation of IBAL to the peracid should also proceed during the much faster horseradish peroxidase catalyzed reaction. This was confirmed by O₂ uptake measurements at different IBAL concentrations. The contribution of the initiator formed by uncatalyzed autoxidation during the course of the reaction is at most 10% of the initiator necessary to complete the reaction. For lower [IBAL] this contribution is even smaller (1% when [IBAL] is 10 mM).

The different methods for the determination of the initiator concentration necessary for a complete O₂ uptake gave an averaged value of 5 ± 2 × 10⁻⁵ M. The evaluation from the H₂O₂ dependence (Table I) and the biamperometric titration seem to be the most reliable methods. Clearly H₂O₂ reacts in the same way as the peracid converting native horseradish peroxidase into compound I. The value from the series of low [IBAL] is substantially higher than the values from other
determinations. This may be due to more compound III formation at low substrate concentration and therefore more consumption of H₂O₂. It is known that compound II is converted to compound III by H₂O₂.

By inhibition of the reaction with ascorbate and KI, inhibitor concentrations of 2–5 × 10⁻⁶ M were evaluated. There are good indications that the inhibition effect is due to destruction of the initiating peracid. But as there are additional effects on the reaction kinetics, which were not investigated in detail, these inhibition methods are regarded as less reliable.

In the titration with iodide using horseradish peroxidase as catalyst (18) the IBAL may interact with the horseradish peroxidase even at the low pH (pH 3.8) and low [horseradish peroxidase], thereby leading to erroneous results. The value of 4.9 × 10⁻⁵ M peracid obtained by biamperometric titration for the inhibitor concentration in a reaction sample containing 42.3 mM IBAL and just capable of completing oxygen consumption appears to be the most reliable determination.

Influence of the Aldehyde—Another rate-limiting factor in the reaction can be the rate of enol formation which is, of course, proportional to [IBAL]. Both the intensity of acetone phosphorescence and of O₂ uptake are proportional to [IBAL] (after a lag phase at low [IBAL]) (Fig. 7 and Ref. 15). This lag time can be shortened by the addition of 5.0 × 10⁻⁶ M of H₂O₂ (Fig. 7, compare with inset).

The influence of IBAL on the reaction kinetics was also tested by following the absorption at 420 nm where the maximum absorption of compound II occurs. At low IBAL this absorption decreases after a short time, indicating that compound II is converted back to the native form. Consequently, the marked slowing down of the reaction, observed in light emission and O₂ uptake is due to a decrease in [compound II] and therefore also of [compound I]. The addition of H₂O₂ prolongs the presence of compound II.

Influence of the Enzyme—The influence of the enzyme had been investigated earlier (15). We report now the effect when the reaction is catalyzed by horseradish peroxidase [H₂O₂]. The intercept for the oxygen uptake at [H₂O₂] is 6.0 × 10⁻⁸ M s⁻¹. This is due to the participation of the uncatalyzed autoxidation. The value of independent experiments for the autoxidation at the same [IBAL] is 5.8 × 10⁻⁹ M s⁻¹. Clearly there is no drastic effect of H₂O₂ upon the horseradish peroxidase dependence of the horseradish peroxidase/IBAL/O₂ system under these conditions.

Influence of Oxygen and H₂O₂ on the Peroxidase Cycle—Additional experiments were performed to test the influence of O₂ and added H₂O₂ on the length of the peroxidase cycle monitored in absorption at 420 nm (maximum absorption by compound II). Nitrogen was bubbled through the sample for 15 min prior to IBAL and horseradish peroxidase addition to remove most of the oxygen. The length of light emission from samples treated in this way was very short, showing that almost all O₂ was removed. In contrast, the peroxidase cycle showed the normal behavior also under these almost anaerobic conditions. Under aerobic conditions the length of the peroxidase cycle is linearly dependent on the [H₂O₂]. The cycle continues also when all O₂ is consumed (indicated in parallel experiments in light emission). Therefore it can be concluded that the length of the peroxidase cycle is only dependent on the initiator (peracid or H₂O₂) and not on O₂.

**DISCUSSION**

In the previous communication (15) it was pointed out that three distinct processes need to be considered in the peroxidase-catalyzed oxidation of IBAL: (i) the uncatalyzed autoxidation of the keto form of IBAL, (ii) the horseradish peroxidase-catalyzed autoxidation of the keto form of IBAL, and (iii) the horseradish peroxidase-catalyzed autoxidation of the enol. Process ii can be eliminated. The possibility of a branching chain reaction exists which would greatly amplify the slowest of reactions. However, the keto form of IBAL appears to be totally unreactive with horseradish peroxidase (15); it would appear that horseradish peroxidase is incapable of forming an acyl radical by direct abstraction of the aldehydic hydrogen from IBAL.

We discuss processes i and iii. The generally accepted mechanism for aldehyde autoxidation (19) is shown for IBAL in Scheme 2.

The initiation step in Scheme 2, production of the acyl radical with rate constant k₁, can occur thermally or photochemically. In elegant studies of the autoxidation reactions the rate of initiation was controlled exactly by photolysis of added initiator (19). Because of their high reactivity, aldehydes were studied at 5 °C. Equations 2 and 3 in Scheme 2 with rate constants k₂ and k₃ constitute the propagation steps

**Scheme 2.** Mechanism for the uncatalyzed autoxidation of IBAL to form the corresponding peracid. Initiation, propagation, and termination rate constants are indicated by k₁, k₂, and 2k₃ respectively (19).
of a chain reaction. The acyl radical converted into an acyl peroxo radical in the first step of the chain (Equation 2) is regenerated in the second (Equation 3) with formation of the peracid product. Thus with one initiation event the reaction would go on forever except for chain termination (rate constant $k_t$, Equation 4). The steady state approximation may be applied to the acyl radical and the corresponding acyl peroxo radical. With the valid assumption that the number of acyl radicals generated in the initiation step is negligible compared to the number which came from the propagation step one obtains the following.

$$\frac{-d[O_2]}{dt} + \frac{d[peracid]}{dt} = k_p \sqrt{\frac{k_p[IBAL]}{2k_o} [IBAL]}$$

Thus in the uncatalyzed autoxidation of IBAL there is a 1:1 correspondence between $O_2$ consumption and [peracid] generation. The rate is proportional to the three-halves power in [IBAL].

The peracids are excellent oxidizing substrates for peroxidases. Therefore compound I formation is readily understood in the IBAL/O$_2$/horseradish peroxidase system. The enol form of IBAL (Equation 6) is highly reactive toward the peroxidases (Refs. 10 and 11, and see below). Scheme 2 combined with Scheme 3 indicate a major part of our proposed mechanism for triplet acetone formation from IBAL.

Equations 7-9 (Scheme 3) are a conventional peroxidase cycle. They are followed by formation of the $\alpha$-peroxy radical (Equation 10) from the resonance-stabilized alkyl radical and abstraction of a hydrogen atom from within the enzyme (Equation 11). This leads to rapid formation of triplet acetone and formic acid (Equation 12) probably via a dioxetane intermediate (10, 14). The hydrogen atom abstraction within the enzyme is indicated in Equation 11 by the conversion $\text{HEnzyme} \rightarrow *\text{Enzyme}$ with the former species representing both the compound II generated in Equation 8 and the native horseradish peroxidase formed in Equation 9. We have at this time no basis to discriminate between the relative reactivities of horseradish peroxidase and compound II towards the $\alpha$-peroxy radical.

Equation 11 replaces the tentative proposal that the $\alpha$-peroxy radical abstracts the crucial hydrogen atom from the active site of compound II thereby converting it into compound I (10).

$$\text{Comp II} + \text{H}_2\text{O}_2 \rightarrow \text{Comp I} + \text{HCOOH} + \text{Acetone}$$

The combination of Equations 8, 10, and 13 would constitute the propagation step of a chain reaction in which compound I is regenerated in each cycle through the chain. We found it impossible to fit the kinetic data with Equation 13 included in the mechanism.

For reasons discussed below it would appear that the IBAL/
O₂/horseradish peroxidase system contains a built-in repair mechanism for damage to the enzyme such as is indicated in Equation 12. Therefore we shall not concern ourselves here with the fate of the "Enzyme free radical species.

Consider Equations 7–12. In the steady-state phase the steady state approximation may be applied to native horseradish peroxidase, compound I, compound II, the α-peroxy radical, and the α-peroxy radical (see Appendix). This leads to the following equation:

\[
\frac{d[O₂]}{dt} = \frac{d[\text{acetone}]}{dt} = -\frac{2k₇[E₀][Eₐ]}{k₇[\text{peracid}]} + \frac{k₇[\text{peracid}]}{k₇[\text{peracid}]} + 1
\]  

(14)

Since compound I usually reacts at least an order of magnitude more rapidly than compound II, \(k₇[\text{peracid}]\) may be neglected. If \(k₇[\text{enol}] \ll k₇[\text{peracid}]\) then Equation 14 becomes

\[
\frac{d[O₂]}{dt} = \frac{d[\text{acetone}]}{dt} = 2k₇[\text{enol}][E₀]
\]  

If, on the other hand, \(k₇[\text{enol}] \gg k₇[\text{peracid}]\) then

\[
\frac{d[O₂]}{dt} = \frac{d[\text{acetone}]}{dt} = 2k₇[\text{enol}][E₀]
\]  

(15)

(16)

It should be noted that enol is being generated continuously during the course of the reaction, but peracid is formed almost entirely by autoxidation of IBAL prior to the horseradish peroxidase-catalyzed reaction. If Equation 15 is valid then \([E₀] \approx [\text{compound II}]\); and if Equation 16 is valid \([E₀] \approx [\text{horseradish peroxidase}]\) (Appendix, Equations 31 and 33).

If the rate of conversion of the enol form of IBAL back to the keto form is negligibly small compared to its reactions with compounds I and II, a steady state in [enol] is given by:

\[
\frac{d[\text{enol}]}{dt} = k₇[\text{IBAL}] - k₇[\text{compound II}][\text{enol}]
\]  

(17)

\[
= k₇[\text{IBAL}] - 2k₇[\text{compound II}][\text{enol}]
\]  

From Equations 15 and 17 and with \([E₀] = [\text{compound II}]\) the rate of oxygen consumption is given by

\[
\frac{d[O₂]}{dt} = k₇[\text{IBAL}]
\]  

(18)

Thus predictions may be made from the mechanism that under varying conditions the reaction may depend upon the concentration of enol (Equation 15), peracid (Equation 16), or IBAL (Equation 18). We discuss the corresponding evidence for each of these predictions in turn. First, dependence upon peracid.

Low [peracid] initiator can become rate-limiting in the initial burst as shown in Fig. 2. The linear part of the saturation curve at low \([H₂O₂]\) indicates that the rate is linearly dependent on the initiator concentration. For \([H₂O₂] > 5 \times 10^{-5} \text{M}\), the reaction rate no longer depends upon the initiator concentration; enough initiator is present to complete the initial burst. The dependence of the reaction rate upon the initiator concentration can indeed only be seen at low [IBAL] (see "Results"). For small [IBAL], and therefore low initiator concentrations, compound II is converted back to the native form after a short time, as can be seen from the absorption experiments at 420 nm. At low initiator concentration, the conversion of the native form of the enzyme to compound I becomes rate-limiting and therefore \(O₂\) consumption and light emission are slowed down, as predicted by the steady state derivation (Equations 31 and 33, Appendix).

According to the proposed mechanism, one molecule of peracid initiator can lead to two molecules of triplet acetone and the consumption of two oxygen molecules. As the \(O₂\) concentration in the sample is \(\approx 2 \times 10^{-3} \text{M}\) (20), and since \([O₂] \ll [\text{IBAL}]\) the initiator necessary for complete \(O₂\) consumption is \(1.0 \times 10^{-4} \text{M}\). The experimental data show that the initial peracid concentration, when not sufficient to allow complete \(O₂\) consumption, can determine the length of the reaction time. The much smaller oxygen uptake observed in the later stages of the reaction can be attributed to the uncatalyzed autoxidation of IBAL proceeding as the reaction continues. As this is producing the initiating peracid, the light emission reaction also goes on. During the fast horseradish peroxidase-catalyzed reaction with the enol form of IBAL the uncatalyzed autoxidation of the keto form contributes to a negligible extent to the total amount of initiator present and hence to the rate of \(O₂\) uptake.

Our estimated average value for the total amount of initiator to complete the reaction \((5 \pm 2 \times 10^{-5} \text{M})\) and our best value determined by biamperometric titration \((4.9 \times 10^{-5} \text{M})\) are not too different from the theoretical value \((1.0 \times 10^{-4} \text{M})\). Also the qualitative observation that the reaction effectively ends without sufficient initiator present confirms our mechanistic prediction. Both facts rule out any chain mechanism such as the compound I ⇌ compound II shuttle (Equation 13). The only chain reaction of importance is the uncatalyzed autoxidation of the keto form of IBAL leading to peracid formation, occurring prior to the horseradish peroxidase-catalyzed reaction. Additional evidence against the shuttle (Equation 13) is the fact that the peroxidase cycle is independent of \(O₂\) in contrast to the prediction from this mechanism.

The rate of autoxidation of the keto form of IBAL to form peracid should be put in perspective. Compared to the autoxidation of other hydrocarbons, aldehydes react with \(O₂\) very rapidly (19). But compared to the rate of the peroxidase-catalyzed autoxidation of the enol form the uncatalyzed autoxidation of the keto form of IBAL is negligibly small. Therefore essentially all of the peracid which initiates the peroxidase-catalyzed oxidation is formed prior to incubation of the reaction system with peroxidase.

Provided there is enough initiator present in the reaction mixture, the rate of enolization and hence [IBAL] is the rate-limiting factor. That the enol form of the aldehyde is the reactive form in the horseradish peroxidase-catalyzed oxidation has been proposed earlier (10); recently evidence for that prediction was found (21). The reactive species might be the corresponding enolate. The steady-state evaluation is not substantially changed by taking this into account. Therefore we have indicated the enol form only as the reactive form in the evaluations. The reaction rate depends linearly on [IBAL] above 10 mM IBAL (15). For lower concentrations a lag phase is clearly observed, which may be due to a lack of peracid initiator. In the presence of more initiator the lag is substantially shortened and the dependence becomes linear also in the lower concentration range (Fig. 7 compared with the inset). The slope of the straight line was estimated from three separate determinations in 0.1 mM phosphate buffer to be \(2.0 \pm 0.2 \times 10^{-5} \text{s}^{-1}\). According to Equation 18 this corresponds to \(k₇\), the enolization rate constant. The rate constant for enolization in water is known. The keto-enol equilibrium constant in water is \(1.28 \times 10^{14}\) and the rate constant for ketonization is \(4.2 \times 10^{10} \text{s}^{-1}\). Therefore the rate constant for enolization is \(5.4 \times 10^{-8} \text{s}^{-1}\) (22). Thus the phosphate is catalyzing a nearly 400-fold increase in rate. The catalytic influence of phosphate on the enolization was proposed earlier.

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(21) and has been observed in another system (23). Another indication for the reactivity of the enol form with horseradish peroxidase is that the reaction rate of 1,3-dicarbonyl compounds, which are known to be present in aqueous solution predominantly in the enol form, show at best only a slight dependence upon the phosphate concentration.

The third factor that can become rate-limiting is the enzyme concentration (15). Since at low [horseradish peroxidase] the reaction rate slows down after a short time, the horseradish peroxidase dependence was repeated in the presence of H$_2$O$_2$. At low enzyme concentration the bimolecular reaction of the native form of the enzyme with the initiator to form compound I can become rate-limiting. Of course addition of more initiator speeds up the initiation reaction and by this a slowing down of the reaction. The dependence of the reaction rate on the enzyme concentration in the presence of H$_2$O$_2$ shows the same behavior as without H$_2$O$_2$ (15) (data not shown).

In an earlier communication (15), the relative scales for O$_2$ consumption and light emission were chosen so that in the saturation region (high horseradish peroxidase concentrations) the two curves match. The small difference in the linear region (low horseradish peroxidase concentrations), where the O$_2$ consumption rate is slightly faster than the light emission rate, can be accounted for as a contribution of the autoxidation to the oxygen consumption. It could be concluded that with both methods the same process is monitored. Therefore the steps after O$_2$ consumption leading to triplet acetone (and phosphorescence emission), cannot be rate-limiting. By using the same relative scales for the IBAL dependence, fairly good agreement between the O$_2$ uptake and light emission data can again be seen (Fig. 7).

H-Enzyme + H$_2$C-C-OC$^-$→H-Enzyme + H$_2$C-C-OC$^-$ (19)

2 H$_2$C-C-OC$^-$→H$_2$C-C-OC$^-$ + CH$_3$CHOH (20)

H$_2$C-C-OC$^-$ + H$_2$C-C-OC$^-$ + CH$_3$CHOH

There are two obvious possibilities for the source of hydrogen atoms for the α-peroxy radical: (i) the enzyme itself or (ii) the ethanol which is used to dissolve the IBAL. Equation 12 could be followed by the repair mechanism indicated in Equations 19 and 20. On the other hand, hydrogen atom abstraction could occur directly from the ethanol in which case Equation 11 would be replaced by Equation 21 followed by Equation 20. In support of the former possibility the sugar groups attached to horseradish peroxidase could be readily oxidized. A sugar residue may be present near the active site (24). On the other hand, ethanol is known to bind weakly in the active site of horseradish peroxidase (25). In either case a considerable amount of acetaldehyde would be produced which is in accordance with the experimental observations (11). However, the source of hydrogen atoms must be within the enzyme in order for the acetone phosphorescence to be observed at all.

Acknowledgments—We are grateful to Dr. D. A. M. Zaia for the biamperometric titrations and to Dr. Etelvino J. H. Bechara for many helpful discussions.

APPENDIX

Application of the steady state approximation to Equations 7–12 (Scheme 3) leads to:

\[
\frac{d[HRP]}{dt} = k_1[HRP][\text{peracid}] - k_4[\text{compound II}][\text{enol}] = 0
\] (22)

\[
\frac{d[\text{compound I}]}{dt} = k_1[HRP][\text{peracid}] - k_4[\text{compound I}][\text{enol}] = 0
\] (23)

\[
\frac{d[\text{compound II}]}{dt} = k_4[\text{compound I}][\text{enol}] - k_0[\text{compound II}][\text{enol}] = 0
\] (24)

From Equations 8–11 and 24

\[
\frac{d[(\text{CH}_2)\text{C}-\text{CH}]}{dt} = 2k_0[\text{compound II}][\text{enol}]
\]

From Equation 22

\[
[\text{HRP}] = [\text{compound II}] \frac{k_0[\text{enol}]}{k_1[\text{peracid}]}
\] (27)

From Equation 23

\[
[\text{HRP}] = [\text{compound I}] \frac{k_0[\text{enol}]}{k_1[\text{peracid}]}
\] (28)

From Equation 24

\[
[\text{compound I}] = [\text{compound II}] \frac{k_0}{k_4}
\] (29)

Conservation:

\[
[\text{total enzyme}] = [E]
\]

\[
[E] = [\text{HRP}] + [\text{compound I}] + [\text{compound II}]
\] (30)

For [HRP] use Equations 27, 28, and 30

\[
[E] = [\text{HRP}] + [\text{HRP}] \frac{k_0[\text{peracid}]}{k_4[\text{enol}]} + [\text{HRP}] \frac{k_1[\text{peracid}]}{k_4[\text{enol}]}
\]

\[
[\text{HRP}] = \frac{[E]}{1 + \frac{k_0[\text{peracid}]}{k_4[\text{enol}]} + \frac{k_1[\text{peracid}]}{k_4[\text{enol}]}}
\] (31)


\[
[\text{compound I}] = \frac{[E]}{k_0[\text{enol}]} \frac{k_4[\text{enol}]}{k_4[\text{peracid}]} + 1 + \frac{k_4}{k_4[\text{peracid}]}
\] (32)

For [compound II] use Equations 27 and 31.
[compound II] = \frac{[E]_0}{k_0[enol] + k_0 + \frac{k_0[peracid]}{k_7[peracid] + k_8 + 1}}

For

\[ \text{0} \hspace{1cm} \text{O} \hspace{1cm} \text{[(CH\textsubscript{3})\textsubscript{2}CCH]} \]\n
use Equations 25 and 33

\[ \text{0} \hspace{1cm} \text{O} \hspace{1cm} \text{[(CH\textsubscript{3})\textsubscript{2}CCH]} = \frac{2k_0[enol]}{k_0[H\text{Enzyme}]} \frac{[E]_0}{k_0[enol] + \frac{k_0[peracid]}{k_7[peracid] + k_8 + 1}} \]

For

\[ \hspace{1cm} \text{O} \hspace{1cm} \text{O} \hspace{1cm} \text{[(CH\textsubscript{3})\textsubscript{2}C-CH]} \]\n
use Equations 26 and 34

\[ \hspace{1cm} \text{O} \hspace{1cm} \text{O} \hspace{1cm} \text{[(CH\textsubscript{3})\textsubscript{2}C-CH]} = \frac{2k_0[enol]}{k_0[H\text{Enzyme}]} \frac{[E]_0}{k_0[enol] + \frac{k_0[peracid]}{k_7[peracid] + k_8 + 1}} \]

Rate: From Equations 11 and 35

\[ \frac{d[acetone]}{dt} = k_{11[H\text{Enzyme}][(CH\textsubscript{3})\textsubscript{2}C-CH]} \]

\[ = \frac{2k_0[enol][E]_0}{k_0[enol] + \frac{k_0[peracid]}{k_7[peracid] + k_8 + 1}} \]

From Equations 10 and 34

\[ \frac{d[O_2]}{dt} = k_{12}[O_2][(CH\textsubscript{3})\textsubscript{2}CCH] \]

leads to Equation 14.

See Equations 14–18 of text for special conditions.

REFERENCES