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Evidence for a free radical chain mechanism in the reaction between peroxidase and indole-3-acetic acid at neutral pH

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Abstract

The oxidation of indole-3-acetic acid (IAA) catalyzed by horseradish peroxidase (HRP) in the absence of added H_2O_2 was studied at pH 7.4 using spectral and kinetic approaches. Upon addition of a hundred-fold excess of IAA to HRP the native enzyme was rapidly transformed to compound II (HRP-II). HRP-II was the predominant catalytic enzyme species during the steady state. No compound III was observed. HRP-II was slowly transformed to the stable inactive verdohemoprotein, P-670. A precursor of P-670, so-called P-940 was not detected. After the cessation of IAA oxidation there was neither oxygen consumption nor P-670 formation; the remaining HRP-II was spontaneously reduced to native enzyme. Single exponential kinetics were observed in the steady state for IAA oxidation, oxygen consumption and P-670 formation yielding identical first order rate constants of about $6 \cdot 10^4$ s^{-1}. A comparison of the rate of IAA oxidation by HRP-II in the steady state and in the transient state indicated that more than 1/3 of the IAA was oxidized non-enzymatically during the steady state, confirming that a free radical chain reaction is involved in the peroxidase-catalyzed oxidation of IAA. IAA oxidation stopped before IAA was completely consumed, which cannot be ascribed to enzyme inactivation because 30–50% of the enzyme was still active after the end of the reaction. Instead, incomplete IAA oxidation is explained in terms of termination of the free radical chain reaction. Bimolecular rate constants of IAA oxidation by HRP-I and HRP-II determined under transient state conditions were $(2.2 \pm 0.1) \cdot 10^3$ M^{-1} s^{-1} and $(2.3 \pm 0.2) \cdot 10^2$ M^{-1} s^{-1}.

Keywords: Horseradish peroxidase; Indole-3-acetic acid oxidation; Free radical chain reaction; Transient state kinetics; Oxygen consumption; P-670

1. Introduction

The aerobic oxidation of indole-3-acetic acid (IAA) catalyzed by peroxidase can occur in the absence of added H_2O_2 [1]. This reaction is a very complex process in which many intermediates and final products of IAA oxidation are formed [2–4]. Moreover, peroxidase is converted from the native enzyme into several catalytic forms (HRP (horseradish peroxidase)-I, HRP-II, HRP-III) and inactive forms (P-940, P-670, P-630, P-678) [5–12]. The observed behavior is highly dependent on experimental conditions.

Two main reaction mechanisms have been recog-

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nized: peroxidase and oxidase pathways. In the classical peroxidase pathway, hydroperoxide (ROOH) is the substrate of native enzyme and IAA (RH) is the substrate for the enzyme intermediates, HRP-I and HRP-II (reactions R1 to R3):

$$\mathrm{HRP} + \mathrm{ROOH} \xrightarrow{k_1} \mathrm{HRP\text{-}I} \qquad (R1)$$

$$\mathrm{HRP\text{-}I} + \mathrm{RH} \xrightarrow{k_2} \mathrm{HRP\text{-}II} + \mathrm{R}\cdot \qquad (R2)$$

$$\mathrm{HRP\text{-}II} + \mathrm{RH} \xrightarrow{k_3} \mathrm{HRP} + \mathrm{R}\cdot \qquad (R3)$$

$$\mathrm{R}\cdot + \mathrm{O_2} \xrightarrow{k_4} \mathrm{ROO}\cdot \qquad (R4)$$

$$\mathrm{ROO}\cdot + \mathrm{RH} \xrightarrow{k_5} \mathrm{ROOH} + \mathrm{R}\cdot \qquad (R5)$$

$$\mathrm{R}\cdot + \mathrm{R}\cdot \xrightarrow{k_6} \text{chain termination.} \qquad (R6)$$

The reactions R4 to R6 are the propagation and termination steps of a chain reaction which recycle ROOH, and are shown in this manuscript to be important.

In the oxidase pathway the substrate of the native enzyme is presumably either IAA itself or a free radical of IAA [7]. The substrate of Fe^{2+}-HRP is oxygen and the substrate of HRP-III is IAA.

A shift between the peroxidase and oxidase pathways can be induced by a change of the HRP/IAA ratio and pH. A high HRP/IAA ratio and high pH promote essentially the peroxidase pathway while a low ratio and low pH favor the oxidase pathway [9,12].

Extensive studies on IAA oxidation have led to an accumulation of experimental data. However, contradictions in experimental results and their interpretations abound. Some proposals are still speculative and several questions remain unanswered [12].

Here we present for the first time a kinetic analysis of the conversion of enzyme compounds, of IAA oxidation and of oxygen consumption during the peroxidase-catalyzed IAA oxidation at neutral pH and high HRP/IAA ratio, on a time scale of up to two hours. Three distinct stages in the kinetics of the reaction were found. IAA oxidation and oxygen consumption took place during the first and second stages. IAA oxidation stopped even though IAA was not completely consumed and enzyme activity remained. The bimolecular rate constants for IAA oxidation by HRP-I and HRP-II were also determined. The results are explained in terms of a peroxidase free radical chain reaction.

2. Materials and methods

2.1. Materials

HRP ($RZ = 3.0$) and IAA were obtained from Sigma (St. Louis, MO), while the components of phosphate buffer were purchased from Fisher Scientific (Fair Lawn, NJ). Prepurified nitrogen (99.999%) was obtained from Praxair (Mississauga, ON).

IAA was dissolved in hot water. The HRP concentration was determined spectrophotometrically using an extinction coefficient at 403 nm of $1.02 \cdot 10^5$ M^{-1} cm^{-1} [13]. Solutions were prepared in 0.1 M pH 7.4 phosphate buffer using deionized water. The final volume of the reaction mixture was 1 ml for spectrophotometric experiments and 3 ml for oxygen consumption experiments. The reaction was initiated by the addition of enzyme. Prior to reaction initiation the solution of IAA was saturated with air; the initial oxygen concentration was 250 μM. The temperature was kept at 20.0 ± 0.5°C.

2.2. Methods

The absorption spectra and most of the kinetics were measured with a Beckman DU 650 spectrophotometer. Kinetics were corrected for lamp intensity drift during the period of measurements. Quartz cuvettes with 1 cm pathlength were used. Stopped flow experiments were made on a SX.17MV sequential stopped-flow spectrometer (Applied Photophysics, UK). Oxygen consumption was followed on a Yellow Spring Instrument Model 53 oxygen monitor.

2.3. Determination of the rate constants k_2 and k_3

The rate constant k_2 for the conversion of HRP-I to HRP-II was measured using the stopped-flow apparatus by following the absorbance at 411 nm

(isosbestic point between HRP and HRP-II). The presence of oxygen does not influence this reaction; the experiments were carried out under aerobic conditions. HRP-I was prepared by mixing equimolar amounts of HRP and H_2O_2. HRP-I was stable for the duration of the kinetic measurements. The final HRP-I concentration was 1 μM, while the final IAA concentration was varied from 100 to 500 μM.

The rate constant k_3 for the conversion of HRP-II to native enzyme was measured using the Beckman DU 650 spectrophotometer by following the absorbance at 430 nm (isosbestic point between HRP and HRP-I). This rate constant cannot be measured under aerobic conditions. The reason is that HRP-II is rapidly regenerated in the presence of oxygen because of the chain reaction which is a part of IAA oxidation. Oxygen was removed from the IAA solution by bubbling with nitrogen. We have found that the prepurified nitrogen contained an impurity which was a substrate for HRP-I and HRP-II. This impurity accumulated in the IAA solution during bubbling and prevented the peroxidase-catalyzed IAA oxidation. Therefore, we further purified the commercially available nitrogen by passing it through activated charcoal and a liquid nitrogen trap. The solution of IAA was bubbled with nitrogen for 30 min in an air-tight spectrophotometer cuvette. The solution of HRP was not deoxygenated. A small amount of concentrated HRP was then added by syringe through the rubber cover. In spite of the fact that oxygen was not removed from the HRP solution the final IAA/HRP mixture contained only traces of oxygen because of dilution. However, these traces were enough to initiate IAA oxidation. HRP was rapidly converted to HRP-II. After a short period the traces of oxygen were consumed, the chain reaction stopped and the reaction mixture contained HRP-II and an excess of IAA. HRP-II was then reduced by IAA. The single-exponential part of the kinetic trace corresponding to this reaction was used for the determination k_3. The final HRP concentration was 2 μM, and the final IAA concentrations were varied from 25 to 250 μM.

Because of the excess of IAA in the reaction mixtures the kinetics of HRP-I and HRP-II reactions were pseudo-first order. The k_{obs} values were determined by single-exponential curve-fitting with a floating endpoint using the standard program 'Enzfitter'. Bimolecular rate constants k_2 and k_3 were determined from the slopes of the linear plots of k_{obs} against IAA concentration.

Because some IAA was consumed before the exponential reduction of HRP-II was observed, the value for k_3 was corrected by an iterative procedure. In the first iteration k_3 was calculated without correction: $k_3^1 = k_{obs}/[IAA]$. In the second and third iterations corrections for IAA oxidation were introduced: $k_3^2 = k_{obs}/\{[IAA] - k_3^1[IAA][HRP\text{-}II] \cdot t\}$ and $k_3^3 = k_{obs}/\{[IAA] - k_3^2[IAA][HRP\text{-}II] \cdot t\}$. The superscripts number the iterations; t is the time of the pre-exponential phase. In the above iterations it was assumed that $[HRP\text{-}II] = [HRP]_{initial}$ and $[IAA] = [IAA]_{initial}$. Three iterations were enough to obtain acceptable convergence for the value of k_3.

3. Results

3.1. Optical spectra

Upon addition of HRP to IAA at pH 7.4, the enzyme was rapidly oxidized to HRP-II, as shown by the shift of the absorption maximum in the Soret region from 403 to 419 nm and the appearance of peaks in the visible region at 527 and 555 nm (Fig. 1, spectrum 2). No HRP-III formation was observed. During the reaction the concentration of HRP-II decreased and an inactive form of peroxidase, P-670, accumulated as shown by the increase of the absorbance at 670 nm (Fig. 1, spectrum 3) [8]. A

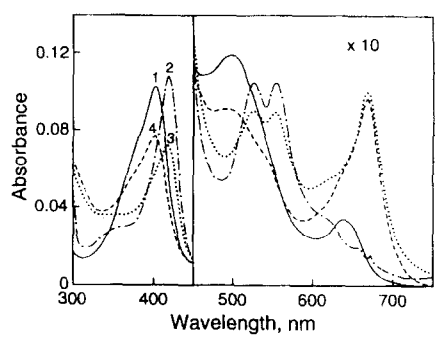

Fig. 1. Absorption spectra of 1 μM HRP in the presence of 50 μM IAA in an aerated system at different times after reaction initiation: (1) 0 min, (2) 3 min, (3) 62 min, (4) 80 min. All experiments at 20°C, pH 7.4 in 0.1 M phosphate buffer.

precursor of P-670, the species absorbing at 940 nm, was not detected. After the cessation of IAA oxidation the remaining HRP-II was reduced to native enzyme. This was accompanied by a shift of the maximum at 419 nm to 403 nm and the disappearance of the HRP-II maxima at 527 and 555 nm (Fig. 1, spectrum 4). Spectrum 3 exhibited a slight absorbance increase in the 580–640 nm region which cannot be ascribed either to HRP-II or native enzyme. Neither can the absorbance increase be ascribed to P-670 because it disappeared after the end of IAA oxidation whereas the P-670 absorbance remained. Most likely, this absorption can be attributed to HRP-I. The concentration of HRP-I contributing to spectrum 3 was estimated to be about 0.1 μM.

3.2. Kinetic studies

The following kinetics were studied as a function of IAA concentration (Fig. 2): (A) HRP-II formation and decay measured at 430 nm; (B) consumption of IAA monitored at 290 nm; (C) formation of P-670 followed at 670 nm; (D) oxygen uptake. There are clearly defined times where the rate of formation or decay of HRP-II changes (Fig. 2A). The time of

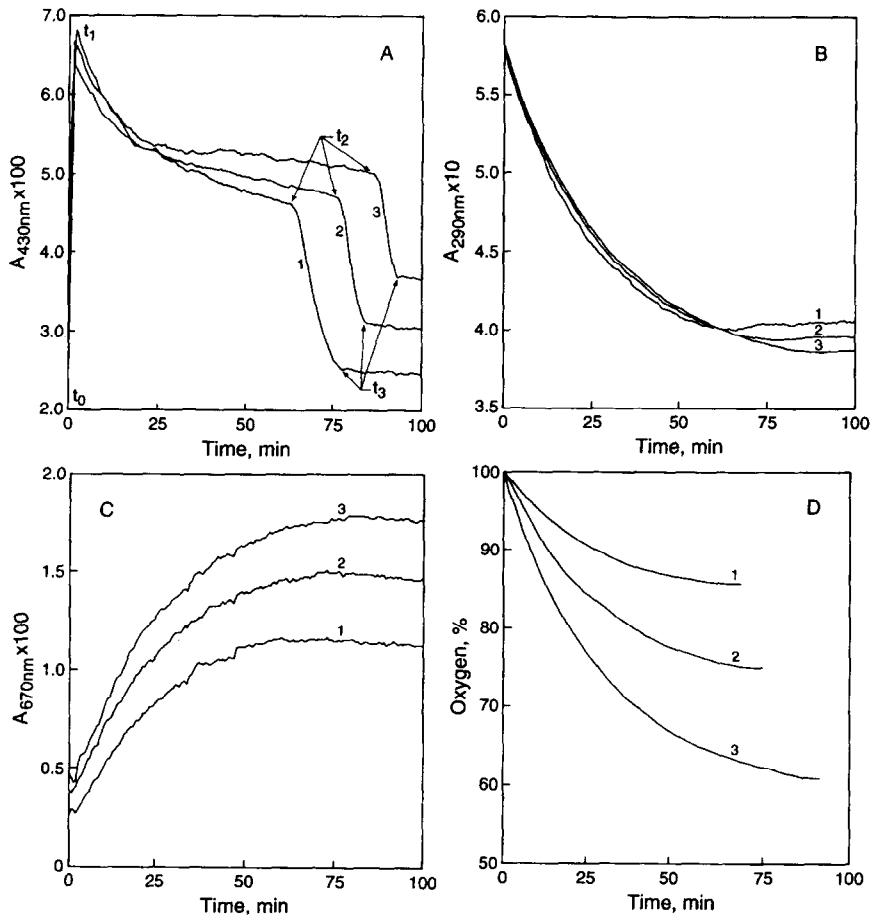

Fig. 2. Changes of concentrations with time. (A) HRP-II following at 430 nm; (B) IAA following at 290 nm; (C) P-670 following at 670 nm; (D) oxygen consumption measured electrochemically. HRP concentration 1 μM with three different IAA concentrations: (1) 50 μM, (2) 100 μM, (3) 150 μM. Traces 1 and 2 in part B were multiplied by 3 and 3/2 respectively in order to normalize them to trace 3. Thus, IAA depletion is linearly dependent upon IAA concentration. Each of oxygen consumption traces is the average of four experiments. Notations t_0, t_1, t_2 and t_3 are explained in the text.

Table 1
Rate constants of IAA oxidation, P-670 formation and oxygen consumption in the IAA/1 μM HRP/O_2 system as a function of initial IAA concentrations. The period of time $t_1 - t_2$ was used as a range of fitting

[IAA], μM	$k_{obs} \cdot 10^4$, s^{-1}		
	IAA oxidation	P-670 formation	O_2 consumption
50	6.8 ± 0.4	6.0 ± 0.4	6.5 ± 0.2
100	6.3 ± 0.3	6.3 ± 0.5	5.9 ± 0.4
150	5.5 ± 0.3	6.2 ± 0.4	6.0 ± 0.3

reaction initiation is t_0, t_1 is the time at which the maximal HRP-II concentration is reached, t_2 is the time of the cessation of IAA oxidation and t_3 marks the complete reduction of HRP-II to native enzyme.

The HRP-II concentration increased rapidly during the short period between t_0 and t_1. This period also corresponded to the detectable lag in P-670 formation (Fig. 2C). Between t_1 and t_2 the concentration of HRP-II decreased and that of P-670 increased slowly. After t_2 no more P-670 was formed while HRP-II was reduced to native enzyme.

IAA oxidation (decrease of absorbance at 290 nm) and oxygen consumption took place from t_0 until t_2 with no lag period. No decrease of IAA and O_2 concentrations was observed after t_2. (Fig. 2B,D).

For the period between t_1 and t_2 the kinetics of IAA and O_2 consumption as well the kinetics of P-670 formation yielded single-exponential traces. The pseudo-first-order rate constants for these reactions are presented in Table 1. For fixed IAA concentrations the rate constants of IAA oxidation, O_2 consumption and P-670 formation were identical within experimental error. There is a small trend of decreasing rate constants with increasing IAA concentration.

Before t_2 the decay of IAA obeyed pseudo-first order kinetics whereas after t_2 the concentration of IAA was constant (see Fig. 2B). Therefore at t_2 the differential d[IAA]/dt changes abruptly. Therefore IAA oxidation stopped before IAA was completely consumed. The concentration of unoxidized IAA in the reaction mixture at t_2 was determined using the absorbance at 290 nm:

$$[\mathrm{IAA}]^{t_2} = \frac{(A^{\infty} - A^{t_2})}{(A^{\infty} - A^{t_0})}[\mathrm{IAA}]^{t_0}$$

where A^{t_0}, A^{t_2} and A^{∞} are absorbances at t_0, t_2 and the endpoint of the single-exponential curve which fits the experimental trace from t_0 to t_2. The concentrations of unoxidized IAA at t_2 were calculated to be 4, 7 and 9 μM for initial IAA concentrations of 50, 100 and 150 μM, respectively.

For the period t_1 to t_2 the decrease in absorbance at 430 nm, corresponding to decrease in the HRP-II concentration, fit a single-exponential at low IAA concentrations (50 μM) but not at higher IAA concentrations (Fig. 2A). The rate constant from the single-exponential decrease in absorbance at 430 nm was $k_{430} = (6.5 \pm 0.3) \cdot 10^{-4}$ s^{-1}. It was equal to the rate constant of P-670 formation within experimental error under the same conditions (see Table 1). Therefore the decrease of HRP-II concentration during the course of IAA oxidation was mainly the result of P-670 formation. It allowed us to determine the final concentration and the extinction coefficient of P-670 (see Appendix):

$$[\mathrm{P-670}]^{t_3} = 0.48 \pm 0.05\,\mu\mathrm{M}$$

and

$$\epsilon_{\mathrm{P\text{-}670}} = (2.4 \pm 0.2) \cdot 10^4\ \mathrm{M^{-1}\,cm^{-1}}.$$

Yamazaki et al. in their earlier work isolated P-670 by column chromatography and measured its spectrum [5]. In a later study they reported the value $(\epsilon_{670}^{\mathrm{P\text{-}670}} - \epsilon_{670}^{\mathrm{HRP\text{-}I}}) = 1.9 \cdot 10^4\ \mathrm{M^{-1}\,cm^{-1}}$ [8]. The extinction coefficient of HRP-I at 670 is $4 \cdot 10^3$ $\mathrm{M^{-1}\,cm^{-1}}$ (our measurements). Therefore using their data: $\epsilon_{670}^{\mathrm{P\text{-}670}} = 2.3 \cdot 10^4\ \mathrm{M^{-1}\,cm^{-1}}$. This value is equal to that measured in our experiments within the limits of the experimental errors.

Knowledge of $\epsilon_{670}^{\mathrm{P\text{-}670}}$ allowed us to calculate final P-670 concentrations in our IAA/HRP/O_2 reaction mixtures. For this purpose we applied Beer's law to the data at 670 nm at t_3 (Fig. 2C):

$$[\mathrm{P\text{-}670}]^{t_3} = \frac{A_{670}^{t_3}}{\epsilon_{670}^{\mathrm{P\text{-}670}}\ell} \qquad (1)$$

where A is absorbance, ϵ the extinction coefficient and ℓ the optical pathlength. It was assumed that the absorbance of P-670 at 670 nm was significantly higher than that of HRP and IAA oxidation products (see Appendix).

Table 2
Initial concentration of HRP as well as the concentration of HRP and P-670 at the time t_3 (see Fig. 2, A) in the IAA/1 μM HRP/O_2 system for different initial IAA concentrations. $[HRP]^{t_3}$ and $[P\text{-}670]^{t_3}$ were calculated using Eqs. 4 and 1 respectively

[IAA], μM	$[HRP]^{t_0}$, μM	$[HRP]^{t_3}$, μM	$[P\text{-}670]^{t_3}$, μM
50	1.0	0.52 ± 0.05	0.48 ± 0.05
100	1.0	0.40 ± 0.04	0.60 ± 0.06
150	1.0	0.33 ± 0.03	0.72 ± 0.07

Table 2 contains the final concentrations of P-670 and HRP for three different initial IAA concentrations. For these IAA concentrations the following equality is valid:

$$[HRP]^{t_3} + [P\text{-}670]^{t_3} = [HRP]^{t_0}.$$

Therefore, for three IAA concentrations, HRP-II was transformed mainly to P-670 in the time interval from t_1 to t_2.

The decrease of absorbance at 430 nm for 100 and 150 μM IAA was not single-exponential between t_1 and t_2 (Fig. 2A). Since HRP-II was transformed only to P-670 (Table 2), the deviations of the kinetic traces from single-exponential must be the result of the contribution of IAA oxidation products to the absorbance at 430 nm. In order to prove this we measured the absorbance changes at low HRP concentration so that HRP would not interfere. Fig. 3

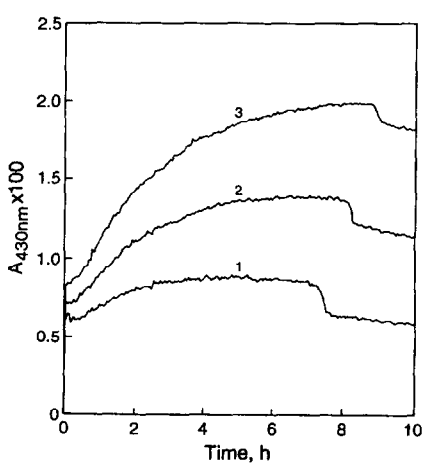

Fig. 3. Absorbance changes at 430 nm in the IAA/0.1 μM HRP/O_2 system for initial IAA concentrations of (1) 50 μM, (2) 100 μM, (3) 150 μM.

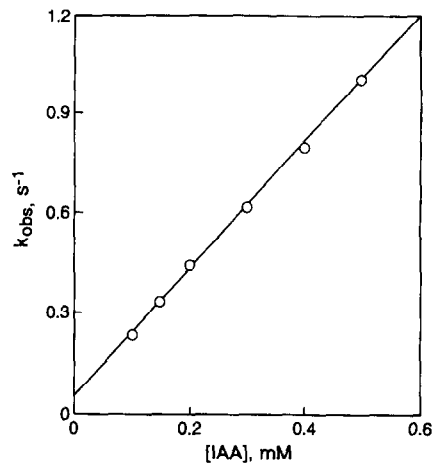

Fig. 4. Plot of the pseudo-first-order rate constant, k_{obs}, versus [IAA] for the conversion of HRP-I to HRP-II by IAA. The slope, k_2, is $(2.2 \pm 0.1) \cdot 10^3$ M^{-1} s^{-1}.

shows an increase of absorbance at 430 nm, which cannot be ascribed to peroxidase compounds. The kinetics of the absorbance at 430 nm are the sum of the single-exponential decay of HRP-II and the slower single-exponential formation of IAA oxidation products (Fig. 2A).

3.3. Determination of k_2 and k_3

The dependence of k_{obs} on IAA concentration was linear for the reaction of HRP-I with IAA. (Fig.

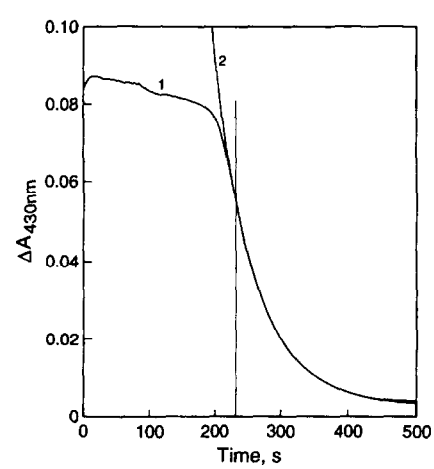

Fig. 5. The kinetics of HRP-II formation and decay in the reaction of 100 μM IAA with 2 μM HRP under anaerobic conditions (1). A single-exponential decay of HRP-II occurred after 230 s (2).

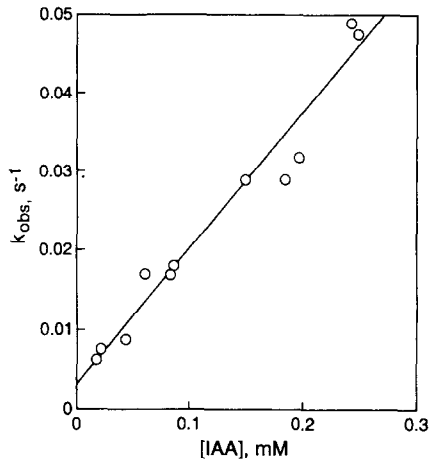

Fig. 6. Plot of the pseudo-first-order rate constant, k_{obs}, versus [IAA] for the reduction of HRP-II to HRP by IAA. The slope, k_3, is $(2.3 \pm 0.2) \cdot 10^2$ M^{-1} s^{-1}.

4). The rate constant k_2 was determined from the slope:

$$k_2 = (2.2 \pm 0.1) \cdot 10^3\ M^{-1}\ s^{-1}.$$

A typical kinetic trace of HRP-II formation and decay in the IAA/HRP system under anaerobic conditions is presented in Fig. 5. The part of the trace after 230 s, which corresponds to reaction R3, fit a single-exponential curve and was used for the determination of k_{obs}. The bimolecular rate constant k_3 was calculated from the slope of linear plot of k_{obs} against IAA concentration (Fig. 6) and yielded:

$$k_3 = (2.3 \pm 0.2) \cdot 10^2\ M^{-1}\ s^{-1}.$$

4. Discussion

During IAA oxidation HRP-II is the main catalytic form of enzyme; no HRP-III was detected. Therefore for our experimental conditions IAA oxidation goes through the peroxidase pathway (Eqs. R1–R3). The free radical R · formed in Eqs. R2 and R3 is responsible for oxygen consumption and the chain reaction (Eqs. R4–R6). The free radical R · consumed in reaction R4 is regenerated in R5; hence these two reactions are the propagation steps of the chain reaction.

In the steady state the ratio between the different peroxidase forms is:

$$[\text{HRP}]/[\text{HRP-I}]/[\text{HRP-II}] = 1 / \frac{k_1[\text{ROOH}]}{k_2[\text{RH}]} / \frac{k_1[\text{ROOH}]}{k_3[\text{RH}]}.$$

Nakajima and Yamazaki determined the following values for k_1 and [ROOH] in the system 100 μM IAA/1 μM HRP: $2 \cdot 10^6$ M^{-1} s^{-1}, [ROOH] $\approx$ 1 μM [7]. Using these values as well as $k_2 = (2.2 \pm 0.1) \cdot 10^3$ M^{-1} s^{-1}, $k_3 = (2.3 \pm 0.2) \cdot 10^2$ M^1 s^{-1} and [RH] = 100 μM we can estimate the above ratio:

$$[\text{HRP}]/[\text{HRP-I}]/[\text{HRP-II}] \approx 1/10/100.$$

Thus, HRP-II reduction, reaction R3, is the rate-determining step. This is consistent with our experimental results.

The ratio of [ROOH] to [RH], an important factor in determining the steady state ratios of the various enzyme forms, is dependent upon [RH]. Therefore, it is possible to shift the steady state concentrations of the HRP compounds by changing [RH].

IAA oxidation occurs in reactions R2, R3, and R5. It can be calculated from:

$$k_{obs} = 2k_3[\text{HRP-II}] + k_5[\text{ROO} \cdot].$$

Table 1 shows that the experimentally determined k_{obs} for the oxidation of 50 μM IAA is $(6.8 \pm 0.4) \cdot 10^4$ s^{-1}. Since [HRP-II] $\leq$ 1 μM and k_3 is $(2.3 \pm 0.2) \cdot 10^2$ M^{-1} s^{-1}, the value of k_5[ROO ·] can be calculated from the following:

$$k_5[\text{ROO} \cdot] = k_{obs} - 2k_3[\text{HRP-II}] \geq (2.2 \pm 0.6) \cdot 10^4\ s^{-1}$$

and thus we can calculate the ratio:

$$k_5[\text{ROO} \cdot]/k_{obs} \geq 1/3.$$

Therefore more than 1/3 of the IAA oxidation occurred in reaction R5. By this result we have for the first time determined quantitatively the contribution of the nonenzymatic steps in IAA oxidation, and confirmed that the peroxidase-catalyzed IAA oxidation involves a free radical chain reaction.

We found that after the cessation of IAA oxidation HRP-II was reduced to native enzyme and HRP-I

was not reformed. Therefore, hydroperoxide was not in the reaction mixture at t_2. Otherwise, we would observe an increase in HRP-I concentration after t_2. This confirms the results of Nakajima and Yamazaki that maximal hydroperoxide concentration is in the earlier stages of IAA oxidation and it decreases with IAA consumption [7].

We observed a termination of IAA oxidation even though IAA was not completely consumed. Acosta et al. reported similar behavior for IAA oxidation at pH 6.3 [11]. The authors ascribed incomplete IAA oxidation to conversion of the enzyme to inactive P-670. However, we found that 30–50% of HRP was not inactivated (Table 2). We explain the incomplete IAA oxidation in terms of the free radical chain reaction. IAA oxidation takes place up to the time when the free radical multiplication factor (FRMF) becomes less than unity. FRMF is probably a complex function of enzyme, substrate and oxygen concentrations which are changing during IAA oxidation. We believe that enzyme inactivation is only one of the reasons of incomplete IAA consumption. It should be noted that the kinetic traces of IAA oxidation have singular-like points t_2 where the differential $d[IAA]/dt$ is changed abruptly. Such nonlinear effects can only be readily explained by the propagation of a chain reaction [15]. Thus, the kinetics shows the autocatalytic nature of the peroxidase-catalyzed oxidation of IAA at neutral pH.

Identification of the initiation step of peroxidase-catalyzed IAA oxidation is one of the many complex problems in this system which remains unsolved. Ricard and Job [14] reported that the formation of HRP-II was preceded by that of HRP-III. Thus, it was proposed that the initiation step is the oxidase pathway. Later, Nakajima and Yamazaki reported that they did not find HRP-III formation prior to that of HRP-II [7]. Discrepancies probably arose from differences in experimental conditions and also from the complexity in spectral analysis of the enzyme intermediates in the pre-steady state. The problem of the initiation step is still not solved. Nakajima and Yamazaki presented evidence that IAA oxidation is an autocatalytic reaction [7], which is confirmed by our present and previous studies [16,17]. The autocatalytic peroxidase pathway (reactions R1–R6) may be initiated by traces of organic hydroperoxide in the IAA solution. Neither sublimation nor recrystallization of IAA influences the reaction initiation [12,18], which means either that the oxidase pathway is the initiation step or that the reaction can be initiated by a very low concentration of organic hydroperoxide formed or remaining after purification of IAA.

P-670 is an inactive verdohemoprotein. It is formed in the reaction of HRP with peroxides [8,19,20] or during the peroxidase-catalyzed aerobic oxidation of IAA [6–12]. There is agreement about the requirement for peroxide to form P-670. There is evidence that P-940 is a precursor of P-670. With regards to which peroxidase compound reacts with peroxide to form P-940, there are several different points of view involving every species from native HRP to HRP-III [8,12,19,20].

In our study we did not observe measurable concentrations of P-940 which implies that the rate-limiting step in P-670 formation is not the reaction P-940 → P-670. The rate-limiting step is probably the reaction of one of the oxidized peroxidase compounds with hydroperoxide. The observed rate constant of P-670 formation is obviously the rate constant of the rate-limiting step. This rate constant does not depend on IAA concentration (Table 2), which means that either the concentrations of peroxidase compound and ROOH do not depend on [IAA] or they depend on [IAA] in opposite ways. It is obvious from the reactions R1-R6 that [ROOH] increases with increasing [IAA]. It is confirmed also by increase of critical inhibitor concentration with increasing of IAA concentration [17]. Therefore, the concentration of the peroxidase compound which reacts with ROOH has to decrease with increasing [IAA]. The concentration of HRP-II is almost independent of [IAA]. Therefore, the compound reacting with ROOH is not HRP-II, but some other minor compounds. Unfortunately, it is very difficult to measure the concentrations of HRP, HRP-I, and HRP-III during the steady state of IAA oxidation. So, the question 'What HRP compound does react with ROOH?' remains unanswered.

Acknowledgements

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Appendix A

In order to determine P-670 concentration we used the fact that HRP-II was transformed mostly to P-670 during the course of IAA oxidation in the 50 μM IAA/1 μM HRP/O_2 system.

At t_0 the enzyme is in native form. At the moment t_1, HRP is almost completely oxidized to HRP-II (see Fig. 1):

$$[\text{HRP-II}]^{t_1} = [\text{HRP}]^{t_0}.$$

Then, during the period from t_1 to t_2 HRP-II was converted to P-670. So, at t_2 (the end of IAA oxidation) the concentration of P-670 was:

$$[\text{P-670}]^{t_2} = [\text{HRP}]^{t_0} - [\text{HRP-II}]^{t_2}. \quad (2)$$

After the cessation of IAA oxidation P-670 was not formed:

$$[\text{P-670}]^{t_2} = [\text{P} - 670]^{t_3}$$

and HRP-II was completely reduced to native enzyme during the period from t_2 to t_3:

$$[\text{HRP-II}]^{t_2} = [\text{HRP}]^{t_3}.$$

Using the last two equalities we were able to rewrite (2) as:

$$[\text{P-670}]^{t_3} = [\text{HRP}]^{t_0} - [\text{HRP}]^{t_3} \quad (3)$$

and to calculate the final HRP concentration using the known expression:

$$[\text{HRP}]^{t_3} = \frac{A_{430}^{t_2} - A_{430}^{t_3}}{\left(\epsilon_{430}^{\text{Co-II}} - \epsilon_{430}^{\text{HRP}}\right)\ell} \quad (4)$$

the difference between extinction coefficients was measured: $(\epsilon_{430}^{\text{Co-II}} - \epsilon_{430}^{\text{HPP}}) = 4.05 \cdot 10^4\ \text{M}^{-1}\,\text{cm}^{-1}$. Finally, the concentration of P-670 was calculated using expression (3) and the value of $[\text{HRP}]^{t_3}$ precalculated from (4). Table 3 contains the concentrations of HRP, HRP-II and P-670 at different times in the system IAA 50 μM/HRP 1 μM/O_2.

Knowledge of $[\text{P-670}]^{t_3}$ allowed us to calculate P-670 extinction coefficient at 670 nm. Indeed, the final reaction mixture at t_3 consisted of P-670, HRP and the products of IAA oxidation. In order to measure the absorption of IAA oxidation products at 670 nm we used the system 50 μM IAA/0.1 μM HRP/O_2 instead of 50 μM IAA/1 μM HRP/O_2. It was found that absorption at 670 nm ($A_{670}^{t_3}$) for the system with [HRP] = 0.1 μM was 10 times less than that for the system with [HRP] = 1 μM. It means that IAA oxidation products did not absorb at 670 nm. Moreover, HRP also does not absorb at this wavelength. Therefore, the only species which absorbs at 670 nm is P-670, which enabled us to determine its extinction coefficient:

$$\epsilon_{670}^{\text{P-670}} = \frac{A_{670}^{t_3}}{[\text{P-670}]^{t_3}\ell} = (2.4 \pm 0.2) \cdot 10^4\ \text{M}^{-1}\,\text{cm}^{-1}.$$

Table 3
The concentrations of native enzyme HRP, HRP-II and P-670 in the 50 μM IAA/1 μM HRP/O_2 system for different moments of time highlighted in Fig. 2, A

Time	[HRP], μM	[HRP-II], μM	[P-670], μM
t_0	1.0	0	0
t_1	0	1.0	0
t_2	0	0.52 ± 0.05	0.48 ± 0.05
t_3	0.52 ± 0.05	0	0.48 ± 0.05

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