ELSEVIER

Biophysical Chemistry 72 (1998) 231–237

Biophysical Chemistry

Anaerobic stopped-flow studies of indole-3-acetic acid oxidation by dioxygen catalysed by horseradish C and anionic tobacco peroxidase at neutral pH: catalase effect

Irina G. Gazarian [a,*], L. Mark Lagrimini [b]

[a] *Department of Chemical Enzymology, Chemical Faculty, Moscow State University, Moscow 119899 GSP, Russian Federation*
[b] *Department of Horticulture and Crop Science, Ohio State University, Columbus OH 43210–1096, USA*

Received 16 July 1997; revised 8 January 1998; accepted 8 January 1998

Abstract

The effect of order of reagent mixing in the absence and in the presence of catalase on the transient kinetics of indole-3-acetic acid (IAA) oxidation by dioxygen catalysed by horseradish peroxidase C and anionic tobacco peroxidase at neutral pH has been studied. The data suggest that haem-containing plant peroxidases are able to catalyse the reaction in the absence of exogenous hydroperoxide. The initiation proceeds via the formation of the ternary complex enzyme → IAA → oxygen responsible for IAA primary radical generation. The horseradish peroxidase-catalysed reaction is independent of catalase indicating a significant contribution of free radical processes into the overall mechanism. This is in contrast to the tobacco peroxidase-catalysed reaction where the peroxidase cycle plays an important role. The transient kinetics of IAA oxidation catalysed by tobacco peroxidase exhibits a biphasic character with the first phase affected by catalase. The first phase is therefore associated with the common peroxidase cycle while the second is ascribed to native enzyme interaction with skatole peroxy radicals yielding directly Compound II.

Keywords: Horseradish peroxidase C; Indole-3-acetic acid; Anionic tobacco peroxidase

1. Introduction

Plant peroxidases are widely spread in nature and are classified as oxidoreductases EC 1.11.1.7 catalysing the oxidation of various substrates by hydrogen peroxide. The recent refinement of the crystal structures of these enzymes [1] and progress in their protein engineering allowed the mechanism of heterolytic cleavage of hydrogen peroxide to be established [2]. However, no special binding site for peroxidase substrates has been identified. Thus, the elucidation of molecular determinants of substrate specificity of plant peroxidases remains an actual problem.

The most important physiological reaction catalysed by plant peroxidases is the oxidation of indole-3-acetic acid (IAA) by dioxygen in the absence of added hydrogen peroxide. The reaction of peroxidase-catalysed degradation of IAA, a powerful plant hormone responsible for growth and development, has been investigated since 1955 [3]. No consensus on the reaction mechanism has been achieved be-

* Corresponding author.

0301-4622/98/$19.00
PII S0301-4622(98)00098-2

cause the mechanism is significantly complicated by numerous radical reactions. The popular opinion is that the reaction mechanism is based on a common peroxidase cycle including Compound I and II species generated by the spontaneous appearance of organic hydroperoxide derived from IAA [4–7]:

$$IAA + O_2 \rightarrow IAA^{\cdot} + ? \quad (1)$$

$$IAA^{\cdot} + O_2 \rightarrow IAAO_2^{\cdot} \quad (2)$$

$$IAAO_2^{\cdot} + IAA \rightarrow IAAOOH + IAA^{\cdot} \quad (3)$$

$$E + IAAOOH \rightarrow EI + IAAOH \quad (4)$$

$$EI + IAA \rightarrow EII + IAA^{\cdot} \quad (5)$$

$$EII + IAA \rightarrow E + IAA^{\cdot} \quad (6)$$

where E, EI and EII are native enzyme, Compound I and II, respectively, IAAOOH is an exogenous hydroperoxide derived from IAA, IAAOH is the corresponding alcohol, $IAA^{\cdot}$ and $IAAO_2^{\cdot}$ are IAA radical and the corresponding peroxy radical.

Computer simulations using the classical peroxidase Eqs. (4)–(6) can satisfactorily describe IAA oxidation catalysed by horseradish peroxidase (HRP) at neutral pH [6,7]. We have shown recently that IAA oxidation catalysed by tobacco anionic peroxidase (TOP) under steady-state conditions is significantly affected by catalase [8] and this supports the peroxidative mechanism. The analysis of IAA degradation products indicated the necessity of intermediate formation of skatole hydroperoxide [9]. Thus, there is no doubt that organic hydroperoxide, namely skatole hydroperoxide, should be synthesised in the reaction course. However, no data on skatole hydroperoxide or its degradability by catalase have been reported thus far. The HRP-catalysed reaction is known to be independent of catalase [4,5] and moreover, no Compound I has been detected in transient kinetic studies on both tobacco and horseradish enzymes [4,10].

The second mechanism proposed for HRP is based on the participation of Compound III [11]:

$$E + IAA \rightarrow E^{2+} + IAA^{\cdot +} \quad (7)$$

$$E^{2+} + O_2 \rightarrow EIII \quad (8)$$

$$EIII + IAA \rightarrow E^{2+} + IA + CO_2 + H_2O \quad (9)$$

$$EIII + 2IAA \rightarrow EII + 2IAA^{\cdot} \quad (10)$$

$$EII + IAA \rightarrow E\text{–}IAA^{\cdot} + H_2O \quad (11)$$

$$E\text{–}IAA^{\cdot} + O_2 \rightarrow EII + In\text{-}CH_2O + CO_2 \quad (12)$$

where EIII, E^{3+} and E^{2+} are Compound III, ferric and ferrous enzyme forms, respectively, $E\text{–}IAA^{\cdot}$ is enzyme–radical complex regenerated in the course of catalysis, IA is indole-3-aldehyde and $In\text{-}CH_2O$ is indole-3-epoxide.

In a previous paper [10] we demonstrated that no reaction Eq. (7) occurred between the enzyme and IAA in the absence of oxygen. Therefore, there is no direct evidence for the reduction of peroxidase by IAA Eq. (7) which has been proposed to explain primary radical generation [4,5,11]. Thus, the character of reaction yielding a primary radical is still unclear.

Three possible explanations could account for primary radical production, i.e. spontaneous degradation of IAA yielding a primary radical, catalytic degradation of IAA by traces of metal ions, and radical generation catalysed by the enzyme itself as we have recently proposed [10]:

$$E + O_2 + IAA \rightarrow [IAA\text{–}E\text{–}O_2] \quad (13)$$

$$[IAA\text{–}E\text{–}O_2] \rightarrow E + IAA^{\cdot +} + O_2^{\cdot -} \quad (14)$$

where $IAA^{\cdot +}$ is IAA cation radical, $O_2^{\cdot -}$ is superoxide anion radical, and $[IAA\text{–}E\text{–}O_2]$ is a hypothetical ternary complex.

The latter proposal is consistent with the established IAA oxidation by dioxygen catalysed by Fe^{3+} ions and haemin [3,12]. The existence of superoxide anion radical was clearly demonstrated by spin trapping experiments [13] and a stimulatory effect of superoxide dismutase on Compound II formation at neutral pH was shown [14]. However, to confirm the initiation mechanism proposed in Eqs. (13) and (14) one has to clarify the role of exogenous hydroperoxide.

The present study was focused on the role of exogenous hydroperoxide in the initiation mechanism. First, if organic hydroperoxide had been generated exogenously upon IAA incubation with oxygen, traces obtained with a different order of reagent mixing would be different. Second, if this process is catalytic there should be a remarkable effect of water purity on the reaction initiation. Third, if only organic hydroperoxide would be responsible for the

oxygenase cycle there should be at least some inhibition by catalase. The role of exogenous hydroperoxide has now been studied by determining the effect of order of reagent mixing on the transient kinetics of Compound II formation in the presence and in the absence of catalase and using the different quality water.

2. Materials and methods

HRP-C (RZ 3.0) was purchased from Biozyme and used without further purification. The concentration of HRP-C was determined spectrophotometrically ($\varepsilon_{403\ nm} = 102\ mM^{-1}\ cm^{-1}$) [15]. Anionic tobacco peroxidase (TOP) was purified from leaves of *Nicotiana sylvestris* plants overexpressing the enzyme, and its concentration was determined spectrophotometrically ($\varepsilon_{403\ nm} = 108\ mM^{-1}\ cm^{-1}$) [16]. Catalase with the specific activity of 2800 U per mg solid was purchased from Sigma and used without further purification.

Anaerobic stopped-flow studies were performed at 395 nm (the isosbestic point between Compound I and II), 412 nm (the isosbestic point between the native enzyme and Compound II) and 430 nm (the isosbestic point between the native enzyme and Compound I) using a Hi-Tech SF-61 stopped-flow rapid-scan spectrophotometer in a single wavelength mode with a tungsten lamp. The stopped-flow apparatus was installed in an anaerobic glove box operating under N_2 with less than 1 ppm of O_2. Temperature was controlled at 25°C using a Techne-400 circulating bath with a heater–cooler also installed in the anaerobic box. Both enzymes and the substrate powders were placed into the hermetically sealed serum vials and deoxygenated for 1 h before being placed into the glove box. 0.1 M Tris–HCl buffer, pH 8.7, and 0.1 M Na-phosphate buffer, pH 7.0, used through all experiments were deoxygenated overnight in the glove box. IAA (Sigma) and 2-methyl-indole-3-acetic acid (2-CH_3-IAA) (Aldrich, USA) stock solutions (50 mM) and the enzymes solutions were prepared anaerobically under N_2 in 0.1 M Tris–HCl buffer, pH 8.7, and 0.1 M Na-phosphate buffer, pH 7.0, respectively. The substrates purity was tested by reverse-phase HPLC on a Columbus 5 μM C18 100 A column (4.6 × 150 mm) purchased from Phenomenex (USA) using isocratic elution in methanol–1% acetic acid mixture (40:60 v/v) at a flow rate 1.5 ml/min and absorbance monitoring at 250 nm. Oxygen-saturated 0.1 M Na-phosphate buffer, pH 7.0, was placed into the anaerobic box in a hermetically sealed serum vial. Oxygen concentration was varied by premixing oxygen-saturated and deoxygenated buffer in syringes with no gas head space to avoid loss of O_2 from the solutions.

Order of mixing experiments were performed as follows: (a) HRP-C (TOP) was shot against IAA in O_2-containing buffer; (b) HRP-C (TOP) in O_2-containing buffer was shot against IAA; (c) HRP-C (TOP) premixed with IAA was shot against O_2-containing buffer. The final concentrations of the enzyme, IAA and oxygen were the same through one set of experiments. Thus, in the set with the buffers prepared on distilled water the final concentrations were 3 μM HRP-C, 2.5 mM IAA and 0.31 mM O_2. Catalase (0.05 mg/ml) was added to O_2-containing syringe. In the set with the buffers on MilliQ water the final concentrations were 3 μM HRP-C or TOP, 2.5 mM IAA, 62.5 μM O_2. Catalase (0.03 mg/ml) was added to O_2-containing syringe.

The effect of catalase on the oxidation of 2-CH_3-IAA catalysed by HRP-C was studied using the following final concentrations of reagents: 6 μM HRP-C, 5 mM 2-CH_3-IAA, 0.5 mM O_2, 0.15 mg/ml catalase. The enzyme was shot against a 2-CH_3-IAA mixture with oxygen-containing buffer in the absence and presence of catalase.

3. Results and discussion

3.1. Traces of HRP Compound II formation in the course of IAA oxidation in distilled water

Compound II was the only enzyme form detected at neutral pH [6,7,10]. This allowed us to follow the kinetics of its formation in a single wavelength monitoring regime. The traces corresponding to HRP Compound II formation at 430 nm, the isosbestic point between Compound I and native enzyme, show that IAA and oxygen preincubation in distilled water yields a substance that interacts with the enzyme (compare traces a with b, c in Fig. 1). The lag-peri-

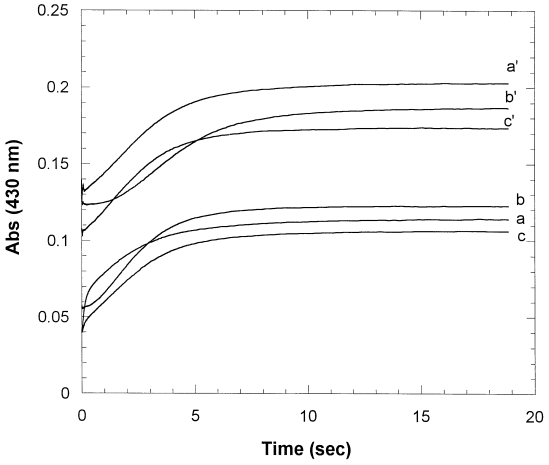


Fig. 1. Order of mixing experiments in the presence and absence of catalase using reagent solutions prepared with distilled water. (a) HRP-C was shot against IAA in O_2-containing buffer; (b) HRP-C in O_2-containing buffer was shot against IAA; (c) HRP-C premixed with IAA was shot against O_2-containing buffer. The final concentrations were 3 μM HRP-C, 2.5 mM IAA and 0.31 mM O_2. (a′), (b′), (c′) correspond to the experiments (a), (b), (c), respectively, in the presence of catalase (0.05 mg/ml) added to O_2-containing syringe. The absorbance changes were followed at 430 nm.

ods shown in traces b and c compared to the burst in trace a, indicate the existence of two processes yielding Compound II. We suggest that the first phase corresponds to the enzyme oxidation by exogenous hydroperoxide generated upon preincubation of IAA–oxygen mixture. The hydroperoxide leads to the burst in Compound II formation and removal of the lag-period (traces b and c).

The appearance of IAA radical in IAA–oxygen mixture could lead to the production of many molecules of IAA hydroperoxide since the propagation steps Eqs. (2) and (3) are rapid with rate constants in the range 10^8–10^9 M^{-1} s^{-1} [7]. We therefore believe that the substance interacting with the enzyme and yielding Compound II is more likely to be an organic hydroperoxide derived from IAA than hydrogen peroxide originated from superoxide radical dismutation.

The hydroperoxide concentration in the experiments with IAA and oxygen separated from each other before mixing is very low because of the extremely short (millisecond) contact time of the reactive species. However, the rate of exogenous hydroperoxide generation seems to be extremely high because small burst phases are still detectable on traces b and c.

In the presence of catalase (Fig. 1, traces a′, b′, c′), no initial bursts were recorded independent of the order of reagent mixing. Thus, the first phase must correspond to the initiation of the peroxidase cycle by exogenous hydroperoxide. However, the second phase of the process was catalase-independent which is consistent with the previous observations on the overall process [4,5,11].

3.2. Traces of HRP Compound II formation in the course of IAA oxidation in MilliQ (deionised) water

No initial bursts like those observed in distilled water and ascribed to exogenous hydroperoxide were recorded in the experiments with the buffer and substrate solutions prepared on MilliQ (deionised) water (compare traces a, b, c, in Fig. 1 and Fig. 2).

In MilliQ water every trace showed one and the same lag-period (Fig. 2) independent of the order of reagent mixing. The time at which oxygen consumption is complete (not shown) was also the same in all three experiments. Thus, preincubation of IAA and oxygen did not yield exogenous hydroperoxide in this case. The results obtained clearly demonstrate that there is no need for exogenous hydroperoxide to initiate the reaction, and the system IAA–oxygen–enzyme is self-sufficient to maintain the process. However, the pronounced lag-periods observed (Fig. 2) indicate the major role played by radical reactions in the initiation mechanism.

Catalase had no effect on traces obtained in MilliQ water solutions (not shown). Kinetic traces recorded at 412 nm, corresponding to the isosbestic point between the native enzyme and Compound II, showed no Compound I formation in the course of IAA oxidation (Fig. 2B). And this again rises a question on the actual mechanism of the main process. If the overall process is driven by hydroperoxide why is Compound I not detected?

3.3. Traces of HRP Compound I and II formation in the course of 2-CH_3-IAA oxidation in MilliQ water

2-CH_3-IAA which is known to be a poor substrate in the oxygenase reaction compared to IAA

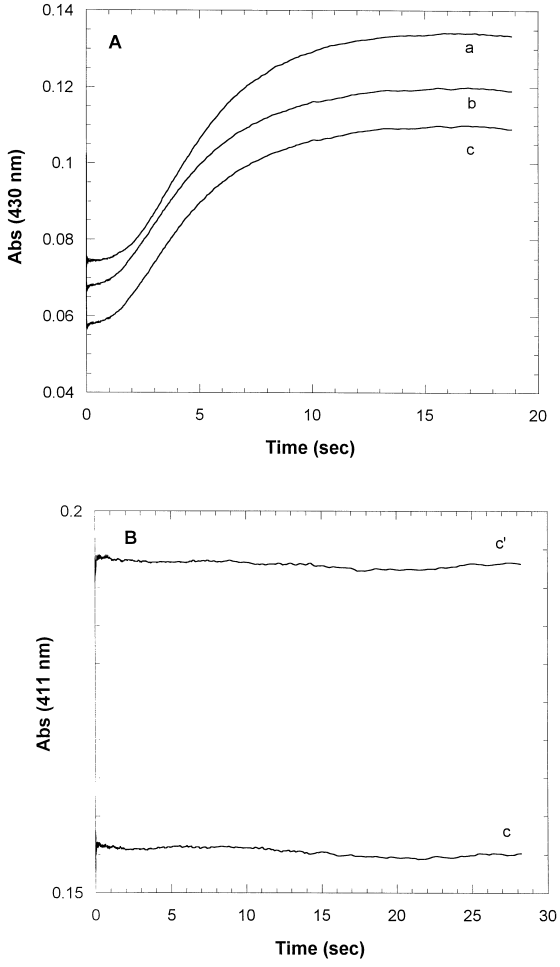


Fig. 2. Order of mixing experiments using reagent solutions prepared with MilliQ water. (a) HRP-C was shot against IAA in O_2-containing buffer; (b) HRP-C in O_2-containing buffer was shot against IAA; (c) HRP-C premixed with IAA was shot against O_2-containing buffer. The final concentrations were 3 μM HRP-C, 2.5 mM IAA and 62.5 μM O_2. The absorbance changes were followed at 430 nm. (B) Absorbance changes followed at 412 nm and corresponding to the experiment (c) performed in the absence and in the presence of catalase (c′) (0.03 mg/ml).

[9,10] was slowly oxidised through the conventional peroxidase cycle with the simultaneous appearance of Compound I and II (Fig. 3) in the absence of added hydrogen peroxide. Catalase increased the duration of a lag-period but did not suppress the process completely (not shown). Therefore, we conclude that 2-CH_3-IAA yields the corresponding hydroperoxide at the initiation step, that it is degradable by

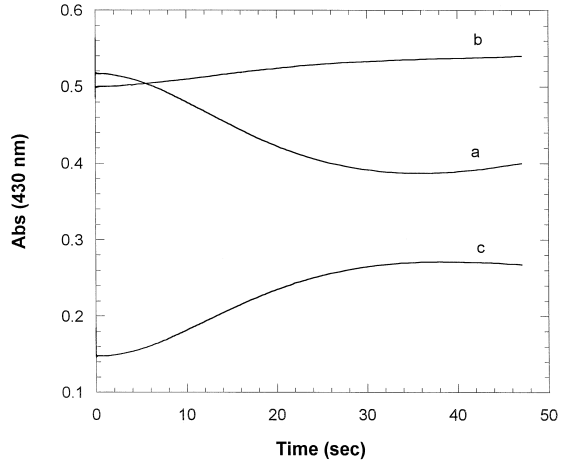


Fig. 3. Oxidation of 2-CH_3-IAA in the presence of HRP-C and oxygen followed at 395 nm (a), 412 nm (b) and 430 nm (c). HRP-C was shot against 2-CH_3-IAA premixed with oxygen. The final concentrations of reagents prepared using MilliQ water were: 6 μM HRP-C, 5 mM 2-CH_3-IAA, 0.5 mM O_2.

catalase and that 2-CH_3-IAA is oxidised via the peroxidative cycle. The difference in behaviour of IAA and its 2-methyl-analogue indicates the existence of a mechanism other than classic peroxidative in the course of IAA enzymatic degradation.

3.4. Traces of TOP Compound II formation in the course of IAA oxidation in MilliQ water

The results supporting the idea of a non-peroxidative mechanism in addition to the conventional peroxidase cycle have been obtained in the experiments with a newly isolated anionic tobacco peroxidase (Fig. 4). In our previous experiments [10] we did not pay attention to the biphasic character of the traces recorded at 430 nm (although it could be clearly seen in Fig. 2 [10]). In this study (Fig. 4) all traces in the presence and in the absence of catalase clearly demonstrate two overlapping processes yielding Compound II. The first phase is affected by catalase. This allows us to ascribe it to the peroxidase cycle.

The second phase which is better seen in Fig. 4, traces b, b′, (the order of mixing enzyme, oxygen, IAA) corresponds to the direct conversion of ferric peroxidase to Compound II. What can be the nature of the reactive species yielding Compound II upon interaction with native peroxidase? The reagent mix-

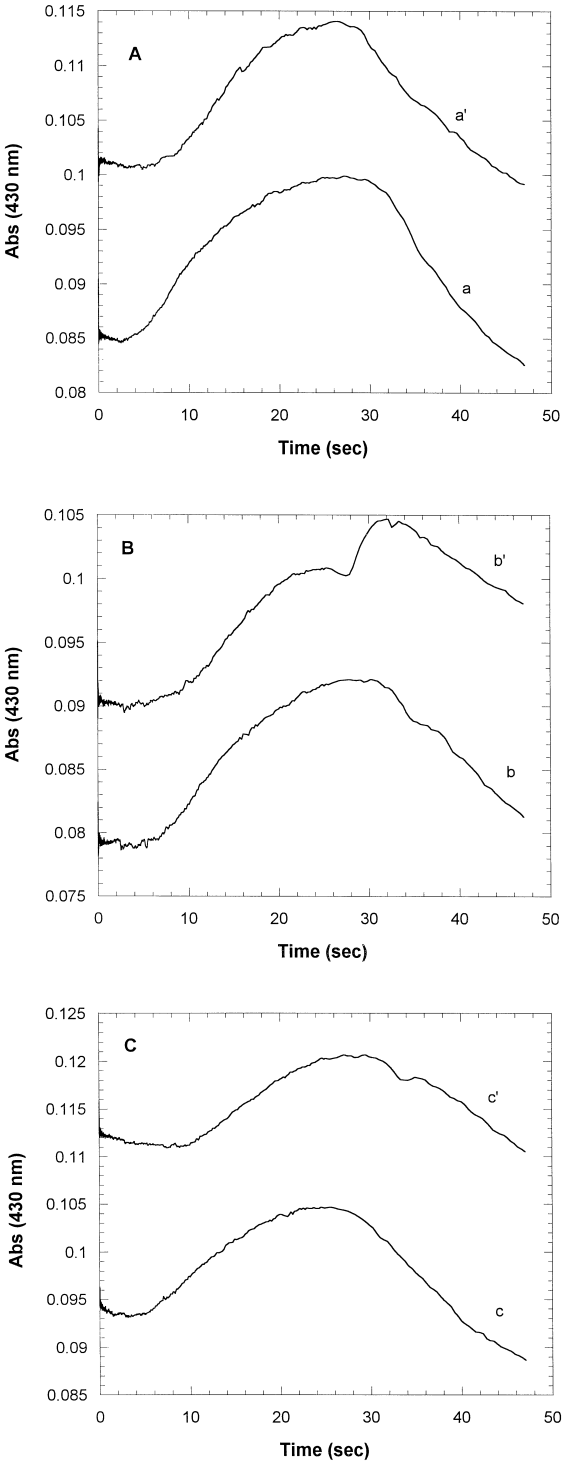
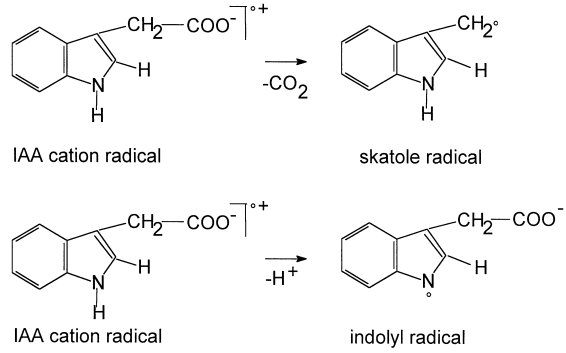


Fig. 4. Order of mixing experiments in the presence and absence of catalase using reagent solutions prepared with MilliQ water. (a) TOP was shot against IAA in O_2-containing buffer; (b) TOP in O_2-containing buffer was shot against IAA; (c) TOP premixed with IAA was shot against O_2-containing buffer. The final concentrations were 3 μM TOP, 2.5 mM IAA and 62.5 μM O_2. (a′), (b′), (c′) correspond to the experiments (a), (b), (c), respectively, in the presence of catalase (0.03 mg/ml) added to O_2-containing syringe. The absorbance changes were followed at 430 nm.



Scheme 1.

ture in the course of IAA degradation contains different reactive species originating from IAA cation radical formed at the initiation step (Scheme 1).

Skatole radicals produced in reaction (15) react with molecular dioxygen yielding skatole peroxy radical Eq. (17) and finally skatole hydroperoxide Eq. (18):

$$InCH_2^{\cdot} + O_2 \rightarrow InCH_2O_2^{\cdot} \quad (17)$$

$$InCH_2O_2^{\cdot} + IAA \rightarrow InCH_2OOH + IAA^{\cdot} \quad (18)$$

where $InCH_2^{\cdot}$, $InCH_2O_2^{\cdot}$ and $InCH_2OOH$ are skatole radical, skatole peroxy radical and skatole hydroperoxide, respectively.

Skatole hydroperoxide must give Compound I upon interaction with native enzyme. Therefore Compound II should be formed from native enzyme upon interaction with oxygen-containing species other than skatole hydroperoxide. The only choice is skatole peroxy radical:

$$E + IAAO_2^{\cdot} \rightarrow EII + In\text{-}CH_2O \quad (19)$$

The product of this hypothetical reaction could be indole-3-epoxide or indole-3-aldehyde. According to computer simulations [6,7] the concentration of IAA

peroxy radicals reaches a maximum before the hydroperoxide does, and thus, under transient kinetics conditions Eq. (19) can contribute into IAA degradation mechanism.

Eq. (19) is analogous to Eq. (12) proposed by Ricard and Job [11]. These authors stressed a true enzymatic character of IAA degradation sensitive to minor changes in the substrate structure. Our data also indicate that 2-CH_3-IAA is consumed in the conventional peroxidase cycle and this substrate is unable to undergo a reaction like Eq. (19). Therefore, the intermediate In-CH_2O is more likely to have the activated second position of pyrrole ring that could explain incorporation of the labelled air oxygen into 3-methylene-oxindole observed earlier [9].

4. Conclusions

To answer is the chemistry of IAA transformations catalysed by plant peroxidases using kinetic data only is practically impossible. However, this kinetic study allows a number of important conclusions on the reaction mechanism to be made.

First, IAA degradation in aerobic water solutions is sensitive to water purity and thus, it is a catalytic process. Thus, the problem of the reaction initiation is actual only for in vitro conditions but not in planta where IAA radicals can be easily generated due to the presence of metal ions or UV-irradiation [10]. Second, haem-containing plant peroxidases like metal ions and haemin are able to catalyse the initiation step, e.g. generation of IAA radicals necessary to start chain reactions. We suppose that a ternary complex enzyme → IAA → oxygen is responsible for the reaction initiation and release of IAA cation radical and superoxide radical. Third, 2-CH_3-IAA is oxidised via the conventional peroxidase cycle with the simultaneous appearance of Compound I and II. Organic hydroperoxide derived from 2-methyl-IAA under these conditions, e.g. 2-methyl-substituted skatole hydroperoxide, is degradable by catalase. Thus, skatole hydroperoxide itself should be degradable by catalase. Fourth, the initiation of the horseradish peroxidase-catalysed reaction is independent of catalase which contrasts to the tobacco peroxidase-catalysed reaction. This indicates the principal differences between these two enzymes in terms of the reaction mechanism.

Thus, the present physico-chemical study unequivocally indicates production of intermediate hydroperoxide in the course of IAA oxidation and this inspires us to work further on skatole hydroperoxide isolation and testing with different plant peroxidases. The work on skatole hydroperoxide identification and isolation by means of HPLC and mass-spectrometry is in progress.

Acknowledgements

The authors thank Dr. R.N.F. Thorneley (UK) for the unique anaerobic facilities provided and both Dr. R.N.F. Thorneley and Prof. A.D. Ryabov (Russia) for the critical comments on the manuscript.

References

[1] D.J. Schuller, N. Ban, R.B. van Huystee, A. McPherson, T.L. Poulos, Structure 4 (1996) 311.
[2] S.L. Newmyer, P.R. Ortiz de Montellano, J. Biol. Chem. 271 (1996) 14891.
[3] R.H. Kenten, Biochem. J. 59 (1955) 110.
[4] R. Nakajima, I. Yamazaki, J. Biol. Chem. 254 (1979) 872.
[5] A.M. Smith, W.L. Morrison, P.J. Milham, Biochemistry 21 (1982) 4414.
[6] K.K. Krylov, H.B. Dunford, Biophys. Chem. 58 (1995) 325.
[7] K.K. Krylov, H.B. Dunford, J. Phys. Chem. 100 (1996) 913.
[8] I.G. Gazaryan, L.M. Lagrimini, Phytochemistry 42 (1996) 1271.
[9] S. Kobayashi, K. Sugioka, H. Nakano, M. Nakano, S. Tero-Kubota, Biochemistry 23 (1984) 4589.
[10] I.G. Gazaryan, L.M. Lagrimini, G.A. Ashby, R.N.F. Thorneley, Biochem. J. 313 (1996) 841.
[11] J. Ricard, D. Job, Eur. J. Biochem. 44 (1974) 359.
[12] R.L. Hinman, J. Lang, Biochemistry 4 (1965) 144.
[13] C. Mottley, R.P. Mason, J. Biol. Chem. 261 (1986) 16860.
[14] D. Metodieva, M. Pires de Melo, J.A. Escobar, G. Cilento, H.B. Dunford, Arch. Biochem. Biophys. 296 (1992) 27.
[15] P.-I. Ohlsson, K.G. Paul, Acta Chem. Scand. B 30 (1976) 373.
[16] I.G. Gazarian, L.M. Lagrimini, Phytochemistry 41 (1996) 1029.