Identification of skatolyl hydroperoxide and its role in the peroxidase-catalysed oxidation of indol-3-yl acetic acid

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Indol-3-yl acetic acid (IAA, auxin) is a plant hormone whose degradation is a key determinant of plant growth and development. The first evidence for skatolyl hydroperoxide formation during the plant peroxidase-catalysed degradation of IAA has been obtained by electrospray MS. Skatolyl hydroperoxide degrades predominantly non-enzymically to oxindol-3-yl carbinol but in part enzymically into indol-3-yl methanol via a peroxidase cycle in which IAA acts as an electron donor. Skatolyl hydroperoxide is degradable by catalase. Horseradish peroxidase isoenzyme C (HRP-C) and anionic tobacco peroxidase (TOP) exhibit differences in their mechanisms of reaction. The insensitivity of the HRP-C-catalysed reaction to catalase is ascribed to the formation of HRP-C Compound III at the initiation step and its subsequent role in radical propagation. This is in contrast with the TOP-catalysed process in which skatolyl hydroperoxide has a key role. Indol-3-yl aldehyde is produced not via the peroxidase cycle but by catalysis involving ferrous peroxidase. Because indol-3-yl aldehyde is one of the main IAA-derived products identified *in planta*, we conclude that ferrous peroxidases participate in IAA catalytic transformations *in vivo*. A general scheme for peroxidase-catalysed IAA oxidation is presented.

INTRODUCTION

Indol-3-yl acetic acid (IAA) is a plant hormone that regulates growth and development [1] (structures of key compounds are shown in Figure 1). In humans it has been implicated in disorders of the central nervous system such as Alzheimer's and Parkinson's diseases ([2] and references therein) because it is formed predominantly as a result of monoamine oxidase-mediated oxidative deamination of tryptamine, a putative neurotransmitter or neuromodulator. Its oxidation by molecular dioxygen catalysed by peroxidases is considered to be an important degradation pathway but there is no consensus on the mechanism.

Two schemes have been proposed for the horseradish peroxidase isoenzyme C (HRP-C)-catalysed oxidation of IAA by molecular dioxygen. One is based on the conventional peroxidase cycle that includes Compound I and II intermediate forms of the enzyme that are generated by organic hydroperoxides derived from IAA–dioxygen [reactions (1)–(3)] [3–6]:

$$E^{3+} + ROOH \rightarrow EI + ROH \quad (1)$$

$$EI + IAA \rightarrow EII + IAA^{\bullet} \quad (2)$$

$$EII + IAA + H^{+} \rightarrow E^{3+} + IAA^{\bullet} + H_2O \quad (3)$$

An alternative proposal assumes independent oxygenase and peroxidase cycles and involves only Compound II and ferric enzyme [reactions (4)–(10)] [7]:

$$E^{3+} + IAA \rightarrow E^{2+} + IAA^{\bullet+} \quad (4)$$

$$E^{2+} + O_2 \rightarrow EIII \quad (5)$$

$$EIII + IAA \rightarrow E^{2+} + P + H_2O \quad (6)$$

$$EIII + 2\ IAA \rightarrow [EI \ldots IAA] + IAA^{\bullet} + H_2O \quad (7)$$

$$[EI \ldots IAA] \rightarrow EII + IAA^{\bullet} \quad (8)$$

$$EII + IAA \rightarrow E^{3+} \ldots IAA^{\bullet} + H_2O \quad (9)$$

$$E^{3+} \ldots IAA^{\bullet} + O_2 \rightarrow EII + In\text{-}CH_2O + CO_2 \quad (10)$$

where $IAA^{\bullet+}$ is the IAA cation radical, $IAA^{\bullet}$ is the indolyl radical, P is indol-3-yl aldehyde, In-CH_2O is indol-3-yl epoxide, E^{2+} is ferroperoxidase, E^{3+}, EI, EII and EIII are native ferriperoxidase and its Compounds I, II and III respectively, and ROOH and ROH are the hypothetical organic hydroperoxide and its corresponding alcohol.

In a previous paper we used anaerobic stopped-flow kinetics [8] to demonstrate that no reaction occurred between HRP-C and IAA in the absence of oxygen. In addition, ferrous enzyme appeared only after oxygen consumption was complete. We concluded that the initiation mechanism does not involve reaction (4) and that ferrous enzyme is a reaction product. We have also shown that HRP-C behaviour in acidic medium differs significantly from that of an anionic tobacco peroxidase (TOP) under the same conditions. TOP does not form spectrophotometrically detectable amounts of Compound III in the course of the reaction [8]. In addition, the TOP-catalysed reaction is inhibited by catalase [9] but the HRP-C-catalysed reaction is not.

We have proposed [8] that plant peroxidases, like haemin and a number of metal ions [10], are able to generate IAA radicals. The reaction cycle is initiated via the formation of a ternary complex between the enzyme, IAA and oxygen-yielding IAA cation radical, [reactions (11) and (12)] [7]:

$$E^{3+} + IAA \leftrightarrow [E\text{-}IAA] + O_2 \leftrightarrow [E\text{-}IAA\text{-}O_2] \quad (11)$$

$$[E\text{-}IAA\text{-}O_2] \leftrightarrow E + IAA^{\bullet+} + O_2^{\bullet-} \quad (12)$$

where $O_2^{\bullet-}$ is superoxide anion radical.

Depending on the pH, IAA cation radical decarboxylates to

Abbreviations used: ABTS, ammonium 2,2′-azino-*bis*(3-ethyl-benzothiazoline-6-sulphonate); HRP, horseradish peroxidase; IAA, indol-3-yl acetic acid; InCH_2O, hypothetical indol-3-yl epoxide; InCH_2OH, indol-3-yl aldehyde; InCH_2OOH, skatolyl hydroperoxide; PGHSynthase, prostaglandin H synthase; SOD, superoxide dismutase; TOP, tobacco peroxidase.

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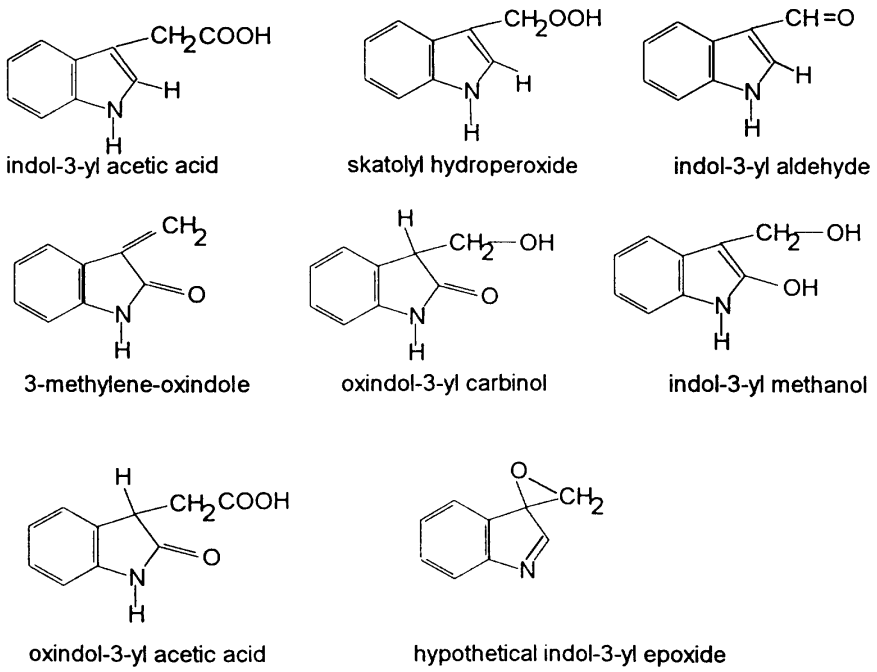
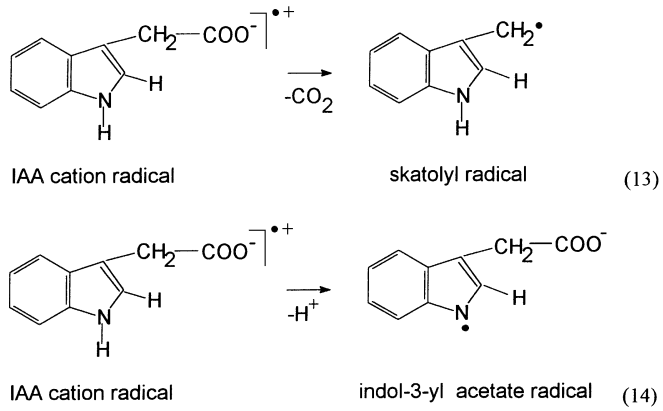


Figure 1 Structures of principal compounds described in this paper



Scheme 1 Transformations of indol-3-yl acetic acid cation radical

yield a skatolyl (indole-3-yl methyl) radical [reaction (13) in Scheme 1] or releases a proton ($pK_a = 5.1$) to yield an indolyl radical [reaction (14)] as described previously [11].

The pronounced effect of pH on the rate of IAA consumption (the reaction rate at neutral pH is an order of magnitude lower than at pH < 5 [3–8]) can be ascribed to the major role of skatolyl radicals in the reaction mechanism. The inability of *N*-methylindol-3-yl acetic acid to undergo proton release [reaction (14)] explains why this is the only IAA analogue that is more reactive than IAA itself [12].

Skatolyl radicals produced in reaction (13) react with molecular dioxygen yielding the peroxy radical [reaction (15)] and subsequently skatolyl hydroperoxide in a chain propagation reaction (16) [5,6]:

$$InCH_2^{\bullet} + O_2 \rightarrow InCH_2O_2^{\bullet} \quad (15)$$

$$InCH_2O_2^{\bullet} + IAA \rightarrow InCH_2OOH + IAA^{\bullet} \quad (16)$$

where $InCH_2^{\bullet}$, $InCH_2O_2^{\bullet}$ and $InCH_2OOH$ are skatolyl radical, skatolyl peroxyl radical and skatolyl hydroperoxide respectively.

The reaction sequence, including the generation of IAA primary radicals [reactions (11) and (12)] and the subsequent appearance of skatolyl hydroperoxide [reactions (15) and (16)] switching the peroxidase cycle on [reactions (1)–(3)] can describe the mechanism at neutral pH [5,6], although it does not explain the failure to detect Compound I in transient kinetic experiments [7,8] or the lack of inhibition by catalase for HRP-C [3,4].

In spite of numerous kinetic studies on the mechanism of IAA degradation [3–8], there has been only one report on the enzyme kinetics with an attempted characterization of the stable end products and intermediates of the oxidative decarboxylation of IAA by HRP-C [12]. Although these authors postulated the formation of skatolyl hydroperoxide as the first product of the reaction, they were unable to isolate and characterize it owing to its instability.

The electrochemical and HRP-C-catalysed oxidations of IAA have been shown to yield the same products as judged by NMR and MS [2]. The electrochemical generation of the IAA radical and a subsequent decarboxylation to yield the skatolyl radical were proposed. Product identification was performed after both electrochemical and enzymic reactions were complete (10–12 h); no traces of skatolyl hydroperoxide were detected. The accumulation of oxindol-3-yl acetic acid was explained by a direct two-electron oxidation of IAA.

Modern rapid-scan spectrophotometry has now allowed us to follow the kinetics of IAA degradation at low pH catalysed by HRP-C and TOP in the presence and in the absence of catalase. We have continuously monitored the UV/visible spectra of the reaction products after separation by HPLC. The previously postulated intermediate, skatolyl hydroperoxide, has now been identified and its degradability by catalase clearly demonstrated.

MATERIALS AND METHODS

Chemicals

Indol-3-yl acetic acid (IAA), ammonium 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulphonate) (ABTS), ascorbic acid and Xylenol Orange were purchased from Sigma (Poole, Dorset, U.K.); indol-3-yl methanol and indol-3-yl aldehyde were from Aldrich (Gillingham, Dorset, U.K.); H_2O_2 and salts were from BDH Merck (Poole, Dorset, U.K.). Milli Q water was used to make up all the solutions.

Enzymes

HRP-C (RZ 3.0) was purchased from Biozyme Ltd. (Blaenavon, Gwent, U.K.) and used without further purification. The concentration of HRP-C was determined spectrophotometrically (ϵ_{403} 102 $mM^{-1} \cdot cm^{-1}$) [13]. TOP was purified from leaves of *Nicotiana sylvestris* plants overexpressing the enzyme [14]. Catalase (specific activity of 2800 units per mg of solid) and bovine superoxide dismutase (SOD) (specific activity of 3000 units per mg of protein) were purchased from Sigma (St. Louis, MO, U.S.A.) and used without further purification.

HPLC studies

The standard incubation mixture consisted of 40 nM HRP (or 80 nM TOP) and 100 μM IAA in 0.1 M sodium acetate buffer, pH 5.0. Incubation was performed at 25 °C. The reaction was initiated by enzyme addition. Catalase (0.05 mg/ml) was added before the initiation of the reaction. Aliquots (200 μl) were taken and analysed by reverse-phase HPLC on a C_{18} Columbus column, 30 nm, 5 μm, 150 mm × 4.6 mm (Phenomenex, Torrance, CA, U.S.A.) by isocratic elution in a methanol/1 % (v/v) acetic acid mixture (40:60, v/v) at a flow rate of 1.0 ml/min. A Shimadzu LC-5A instrument with absorbance monitoring at 250 nm and a Waters 626 LC system device equipped with a 996 photodiode-array detector were used throughout the experiments.

MS

Samples were analysed with a Platform I (Micromass, Manchester, U.K.) mass spectrometer equipped with an electrospray ion source. A 100 μl sample of the mixture of 100 μM IAA and 0.4 μM HRP in 0.1 M sodium acetate buffer, pH 5.0, pre-incubated for 5 min at 25 °C, was applied to the HPLC column. The eluate from the HPLC system (model 1090; Hewlett-Packard, Palo Alto, CA, U.S.A.) was split to a UV detector (monitored at 250 nm) and mass spectrometer in a ratio of approx. 35:1. Negative-ion electrospray spectra were recorded at a capillary voltage of −2.7 kV, a cone voltage of −20 V and a source temperature of 90 °C. Spectra were scanned over the mass range 50–1000 Da at a rate of 475 Da/s, with an interscan delay of 0.1 s. Data were processed with MASSLYNX software. Negative-ion electrospray spectra exhibited $[M]^-$ ions and, in some cases, additional ions 60 mass units above this ($[M + CH_3COOH]^-$) resulting from gas-phase adduct formation with acetate in the eluent buffer. Important ions were identified by mass chromatography; full-scan spectra were processed by background subtraction of solvent ions.

Determination of product concentrations

IAA, indol-3-yl aldehyde and indol-3-yl methanol concentrations were determined using standard solutions. The skatolyl hydroperoxide concentration was determined as follows. Skatolyl hydroperoxide was separated from other reaction products by HPLC and the fraction was added to a reaction mixture consisting of 600 nM HRP-C and 0.36 mM ABTS in sodium acetate buffer, pH 5.0. The end-point absorbance of the ABTS oxidation product was recorded at 405 nm. Skatolyl hydroperoxide concentration was estimated from a calibration graph previously obtained for H_2O_2 under the same experimental conditions [2–50 μM H_2O_2/600 nM HRP-C/ABTS/sodium acetate buffer (pH 5.0)].

Transient kinetic studies

Anaerobic stopped-flow studies were performed at 430 nm (the isosbestic point between the native enzyme and Compound I) with a Hi-Tech SF-61 stopped-flow rapid-scan spectrophotometer (High Tech Scientific, Salisbury, Wilts., U.K.) in single-wavelength mode with a xenon lamp. The stopped-flow apparatus was installed in an anaerobic glove box operating under N_2 with less than 1 p.p.m. of O_2. Temperature was controlled at 25 °C with a Techne-400 circulator [Techne (Cambridge) Ltd., Duxford, Cambs., U.K.] with an external cooler. 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. The 0.1 M Tris/HCl buffer, pH 8.7, and the 0.1 M sodium acetate buffer, pH 5.0, used in all the experiments were deoxygenated overnight in the glove box. IAA stock solution (50 mM) and the enzyme solutions were prepared anaerobically under N_2 in 0.1 M Tris/HCl buffer, pH 8.7, and 0.1 M sodium acetate buffer, pH 5.0, respectively. Oxygen-saturated and CO-saturated 0.1 M sodium acetate buffer solutions, pH 5.0, were placed into the anaerobic box in hermetically sealed serum vials.

Experiments were performed as follows. HRP-C (TOP) was mixed with IAA in O_2-containing buffer in the stopped-flow apparatus. The final concentrations were 2 μM HRP-C (TOP), 5 mM IAA and 62.5 μM O_2. SOD (0.15 or 1.5 μM) was then added to the O_2-containing syringe.

The effect of CO on the oxidation kinetics of IAA was studied by using the following final concentrations of reagents: 3 μM HRP-C (TOP), 2.5 mM IAA, 62.5 μM O_2, 0.5 mM CO. The enzyme–oxygen mixture was mixed with IAA in buffer saturated with either N_2 or CO.

RESULTS

Identification and isolation of skatolyl hydroperoxide

Figure 2(A) shows a chromatogram of the reaction mixture obtained after a 5 min incubation of 100 μM IAA with 0.4 μM

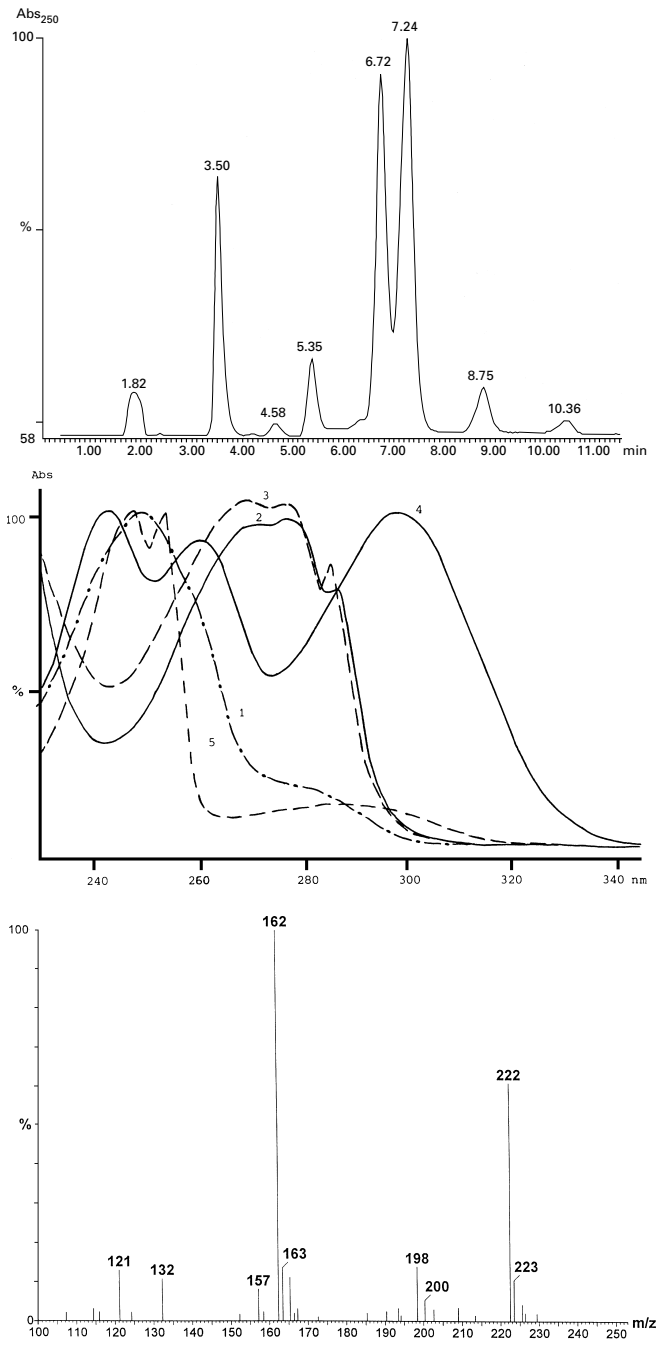


Figure 2 Separation of IAA enzymic degradation products by reverse-phase HPLC

The column used was a C_{18} Columbus column, 30 nm, 5 μm, 150 mm × 4.6 mm; elution was isocratic in methanol/1% (v/v) acetic acid (40:60, v/v) at a flow rate of 1.0 ml/min. Top panel: HPLC profile monitored at 250 nm. The peaks are: oxindol-3-yl carbinol, retention time (r.t.) 3.50 min; indol-3-yl methanol, r.t. 5.35 min; intermediate X (skatolyl hydroperoxide), r.t. 6.72 min; indol-3-yl aldehyde, r.t. 7.24 min; IAA, r.t. 8.75 min, and 3-methylene-oxindole, r.t. 10.36 min. Middle panel: the corresponding UV spectra. Trace 1, oxindol-3-yl carbinol; trace 2, indol-3-yl methanol; trace 3, intermediate X (skatolyl hydroperoxide); trace 4, indol-3-yl aldehyde; trace 5, methylene-oxindole. Bottom panel: mass spectrogram of the band corresponding to intermediate X (skatolyl hydroperoxide), (r.t. 6.72 min). A 100 μl sample of the mixture of 100 μM IAA and 0.4 μM HRP in 0.1 M sodium acetate buffer, pH 5.0, preincubated for 5 min at 25 °C, was applied to the column.

HRP at pH 5.0. Four of the five major products of IAA degradation can be easily identified from their recorded spectra (Figure 2B). These are oxindol-3-yl carbinol, indol-3-yl methanol, indol-3-yl aldehyde and 3-methylene-oxindole, with retention times of 3.50, 5.35, 7.24 and 10.36 min respectively, together with unreacted IAA, with a retention time of 8.75 min. IAA, indol-3-yl aldehyde and indol-3-yl methanol were identified by comparison with the UV absorption spectra and retention times of authentic standards. Oxindol-3-yl carbinol and methylene-oxindole were identified by comparing the absorbance spectra with those described previously [10–12]. Further confirmation was obtained by MS (results not shown).

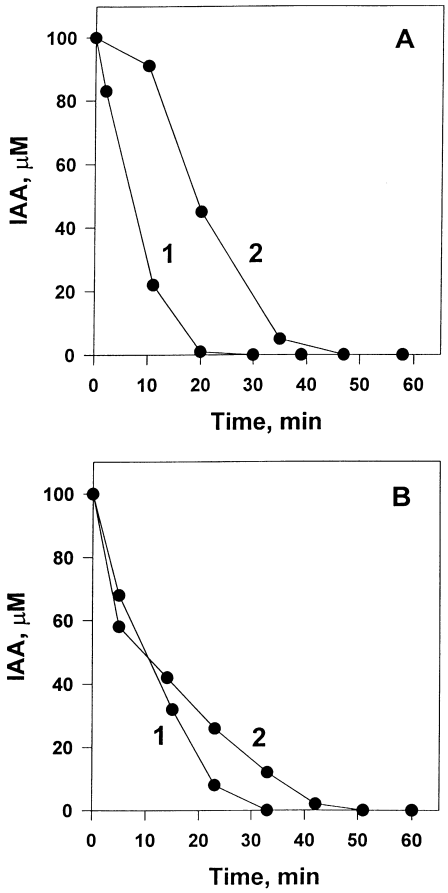


Figure 3 Effect of catalase on the kinetics of the enzymic degradation of IAA catalysed by TOP (A) and HRP-C (B)

Curve 1, in the absence of catalase; curve 2, in the presence of 50 μg/ml catalase. The incubation mixture consisted of 40 nM HRP (or 80 nM TOP) and 100 μM IAA in 0.1 M sodium acetate buffer, pH 5.0.

In addition, a new transient species with a retention time of 6.72 min was observed. Prolonged incubation of the reaction mixture (more than 1 h) caused its complete disappearance. Reinjection of the isolated fraction corresponding to the 6.72 min peak showed that oxindol-3-yl carbinol is the main degradation product of this intermediate. Reinjection of this fraction after its preincubation with ascorbic acid (a poor substrate for HRP-C) gave a HPLC peak corresponding to indol-3-yl methanol. The UV spectrum of the intermediate (Figure 2B, curve 3) showed an absorption band in the range 260–270 nm characteristic of an indolic compound. The spectrum was similar to that recorded by Nakajima and Yamazaki [3] in the course of IAA oxidation catalysed by HRP and ascribed by them to an intermediate postulated to be skatolyl hydroperoxide. They attempted no further characterization.

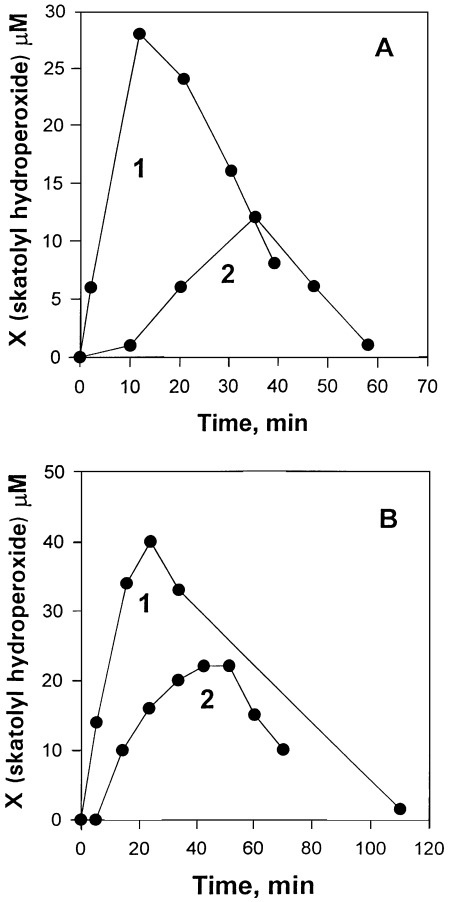


Figure 4 Effect of catalase on the kinetics of skatolyl hydroperoxide (X) accumulation in the course of the enzymic degradation of IAA catalysed by TOP (A) and HRP-C (B)

Curve 1, in the absence of catalase; curve 2, in the presence of catalase (for incubation conditions see the legend to Figure 3).

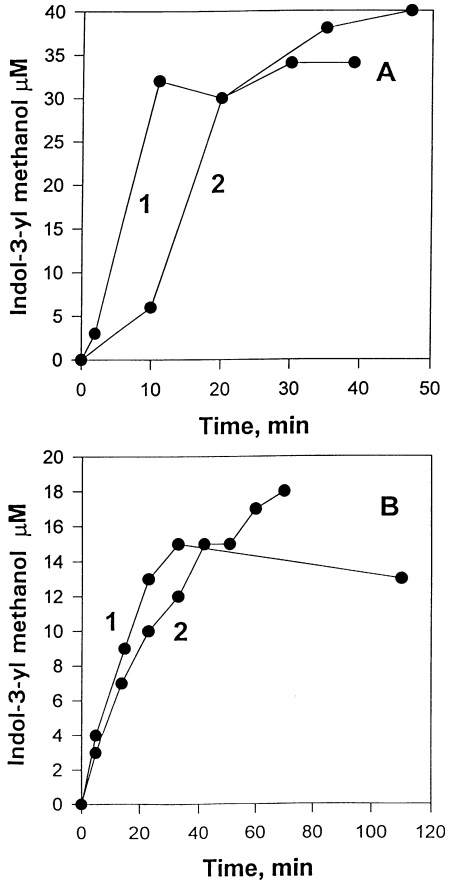


Figure 5 Effect of catalase on the kinetics of indol-3-yl methanol production in the course of the enzymic degradation of IAA catalysed by TOP (A) and HRP-C (B)

Curve 1, in the absence of catalase; curve 2, in the presence of catalase (for incubation conditions see the legend to Figure 3).

The isolated fraction was assayed with HRP-C and ABTS in the absence of H_2O_2 to determine any peroxidatic activity. Development of the green colour characteristic of ABTS oxidation strongly indicated the presence of an organic hydroperoxide derived from IAA (the HPLC separation precluded contamination by H_2O_2). In the presence of catalase no reaction was observed. All other HPLC fractions gave negative tests with HRP-C and ABTS. Fraction 6.72 was positive in a 'FOX' test, e.g. hydroperoxide determination with ferrous salt, sorbitol with the product ferric ion detected by its reaction with the indicator Xylenol Orange [15]. These results strongly suggest that the intermediate is an organic hydroperoxide derived from IAA. HPLC analysis of the products of the hydroperoxide reduction by ascorbic acid, ABTS and catalase gave further evidence for skatolyl hydroperoxide production. MS of the intermediate gave a molecular mass of 163 (negative ion 162) (Figure 2C) corresponding precisely to that of skatolyl hydroperoxide. This is the first direct evidence for skatolyl hydroperoxide production in the course of the enzymic degradation of IAA.

Effect of catalase on the enzymic oxidation of IAA

The kinetics of IAA degradation catalysed by HRP-C and tobacco peroxidase were determined by product analysis after separation by HPLC. The results are shown in Figures 3–6. Comparison of Figures 3 and 4 clearly demonstrates that skatolyl hydroperoxide concentration reaches a maximum when approx. 90% of the IAA has been consumed, independently of the type of peroxidase used. Catalase decreased the maximum concentration of skatolyl hydroperoxide to 50% (Figures 4A and 4B) and induced a lag-period of approx. 10 min for TOP and 5 min for HRP-C in skatolyl hydroperoxide formation.

Comparison of TOP and HRP-C shows that catalase has a more pronounced effect on the TOP-catalysed reaction: lag periods of approx. 10 min were observed for the degradation of IAA (Figure 3A) and the formation of skatolyl hydroperoxide (Figure 4A), indol-3-yl methanol (Figure 5A) and indol-3-yl aldehyde (Figure 6A). The kinetics of indol-3-yl methanol accumulation during the HRP-C-catalysed reaction were not significantly affected by catalase (Figure 5B), whereas the reaction catalysed by TOP exhibited a significant lag phase (Figure 5A). The maximum concentration of skatolyl hydroperoxide for the TOP-catalysed reaction (Figure 4A) was lower than that for HRP-C (Figure 4B). However, the conversion of IAA into indol-3-yl methanol was much higher for TOP (35–40%) (Figure 5). Less than 20% of the IAA was converted into indol-3-yl methanol by HRP-C (Figure 5B). This reflects the extent of competition between the enzymic and non-enzymic trans-

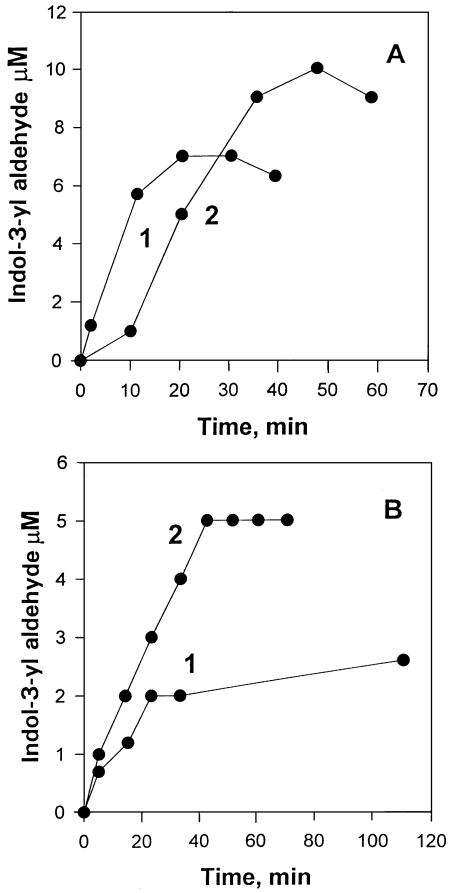


Figure 6 Effect of catalase on the kinetics of indol-3-yl aldehyde production in the course of the enzymic degradation of IAA catalysed by TOP (A) and HRP-C (B)

Curve 1, in the absence of catalase; curve 2, in the presence of catalase (for incubation conditions see the legend to Figure 3).

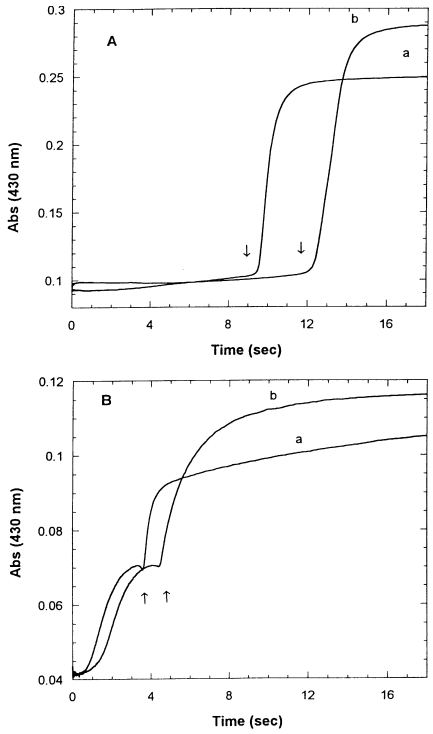


Figure 7 Effect of CO on transient kinetics of IAA oxidation by molecular dioxygen catalysed by TOP (A) and HRP-C (B) in 0.1 M sodium acetate buffer, pH 5.0, at 22 °C

Traces were recorded at 430 nm, the isosbestic point for native ferric enzyme and compound I, where increases in absorbance indicate the formation of compound II, compound III and ferrous enzyme. Trace a, no CO; trace b, 0.5 mM CO. Reagent concentrations: 3 μM HRP-C (or TOP), 2.5 mM IAA and 62.5 μM O_2. The enzyme–oxygen mixture was mixed with IAA in N_2- or CO-saturated buffer. Arrows indicate the times at which ferrous enzyme begins to appear, which corresponds to dioxygen exhaustion.

formations of skatolyl hydroperoxide. Catalase slightly increased indol-3-yl methanol production (Figure 5), consistent with the catalytic degradation of skatolyl hydroperoxide to yield indol-3-yl methanol.

The observation of significant amounts of skatolyl hydroperoxide in the presence of catalase means that it must be formed rapidly from IAA in the enzymic reaction. The main difference between TOP and HRP-C is in the reaction initiation phase. This can be explained if TOP requires skatolyl hydroperoxide to propagate chain radical reactions but HRP-C does not (see the Discussion section).

Peculiarities of indol-3-yl aldehyde synthesis

The kinetics of indol-3-yl aldehyde production is shown in Figure 6. The concentration of indol-3-yl aldehyde reaches a maximum (that for TOP is is 3-fold that for HRP-C) at the time when IAA consumption is complete. Because there were sufficient amounts of the active enzyme, skatolyl hydroperoxide and indol-3-yl methanol to maintain enzymic activity when indol-3-yl aldehyde formation had stopped, it must be concluded that indol-3-yl aldehyde is formed directly from IAA but not from indol-3-yl methanol as previously proposed [12]. This must mean that indol-3-yl aldehyde is not produced via the peroxidase cycle. For both TOP and HRP-C, catalase approximately doubled the amount of indol-3-yl aldehyde. Therefore skatolyl hydroperoxide must react with the enzyme form responsible for indol-3-yl aldehyde synthesis. Because this enzyme form cannot be part of the peroxidase cycle, it is not E, EI or EII. No Compound III form of TOP has been detected [8]. The only form left is ferrous enzyme, the appearance of which in the reaction course is usually accompanied by indol-3-yl aldehyde production [3,7,10]. We have previously shown that TOP, unlike HRP-C, generates detectable amounts of ferrous enzyme in the course of IAA oxidation by dioxygen [9]. With TOP, the level of indol-3-yl aldehyde production is much higher (Figure 6B). However, the contribution of the ferrous enzyme route under *in vitro* conditions is negligible because only low concentrations of indol-3-yl aldehyde are produced (less than 10%).

Anaerobic stopped-flow kinetics in the presence of CO, catalase and SOD

To investigate the role of ferrous enzyme we have studied transient kinetics of IAA oxidation in the presence of CO (Figure 7). The duration of the first phase is a measure of the oxygen consumption rate [8]. When the oxygen is exhausted, an increase in absorbance occurs owing to the formation of ferrous enzyme. With HRP-C, essentially identical traces were obtained in the absence and in the presence of CO (Figure 7B). This was in contrast with TOP (Figure 7A), where CO inhibited oxygen

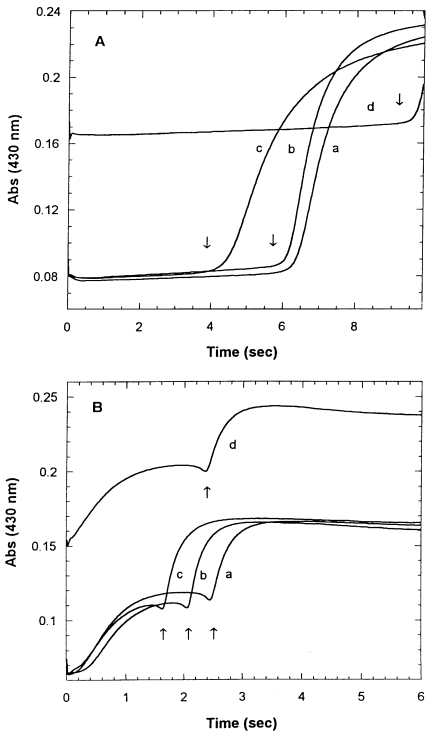


Figure 8 Effect of SOD and catalase on transient kinetics of IAA oxidation by molecular dioxygen catalysed by TOP (A) and HRP-C (B) in 0.1 M sodium acetate buffer, pH 5.0, at 22 °C

Traces were recorded at 430 nm, the isosbestic point for native ferric enzyme and compound I, where increases in absorbance indicate the formation of compound II, compound III and ferrous enzyme. Trace a, no SOD or catalase; trace b, 0.15 μM SOD; trace c, 1.5 μM SOD; trace d, 0.15 mg/ml catalase. Reagent concentrations: 2 μM HRP-C (or TOP), 5 mM IAA and 62.5 μM O_2. The enzyme was mixed with IAA–O_2 mixture; SOD (catalase) was added to the substrate mixture. Arrows indicate the times at which ferrous enzyme begins to appear, which corresponds to dioxygen exhaustion.

consumption as indicated by the prolongation of the first kinetic phase. We conclude that ferrous peroxidase has a significant role in the TOP-catalysed reaction of IAA degradation.

SOD and catalase were used to investigate further the differences in the initiation mechanism for TOP and HRP-C (Figure 8). SOD in both cases stimulated oxygen consumption (compare curves a, b and c in Figures 8A and 8B), shortening the first kinetic phase. This supports our proposal that superoxide anion radical is released from the ternary complex (enzyme-IAA-oxygen) formed at the initiation step [reactions (11) and (12)] [8]. However, although the effect of SOD on the transient kinetics for both HRP-C and TOP was similar (stimulation of oxygen consumption), the HRP-C-catalysed reaction was insensitive to catalase, whereas the TOP-catalysed reaction was significantly inhibited; for example, the duration of the oxygen consumption phase was increased from 6 to 10 s (compare curves a and d in Figures 8A and 8B). This observation was in agreement with the previous result obtained at neutral pH [9]. Thus the main difference between HRP-C and TOP seems to be related to HRP-C Compound III formation at the initiation step.

Summary of results

1. The first direct evidence for skatolyl hydroperoxide formed as an unstable product of the peroxidase-catalysed degradation of IAA has been obtained.
2. Skatolyl hydroperoxide is degraded by catalase.
3. After exhaustion of IAA, skatolyl hydroperoxide is not degraded by peroxidases but decomposes non-enzymically to oxindol-3-yl carbinol.
4. The mechanism of IAA oxidation depends on the nature of the peroxidase because: (a) the HRP-C-catalysed reaction is insensitive to catalase and CO, whereas the TOP-catalysed reaction is inhibited; (b) the rate of skatolyl hydroperoxide generation in the HRP-C-catalysed reaction is much higher than that for TOP; (c) the contribution of enzyme-catalysed transformations (via peroxidative and ferrous enzyme cycles) is higher for TOP than for HRP-C (50 % and 20 % respectively).

DISCUSSION

Role of skatolyl hydroperoxide in the reaction initiation and propagation

The results presented in Figures 2–4 show unequivocally for the first time that skatolyl hydroperoxide is the main product of the peroxidase-catalysed oxidation of IAA and that it is degradable by catalase. The formation of skatolyl hydroperoxide in this reaction was first postulated by Nakajima and Yamazaki [3]. The instability of this intermediate explains why Kobayashi et al. [12] failed to isolate the hydroperoxide. However, the precise role played by this intermediate in IAA degradation clearly depends on the peroxidase origin (i.e. HRP-C or TOP).

The anaerobic stopped-flow data obtained in this study (Figure 8) and previously [8] support a mechanism for the reaction initiation step via the formation of a ternary complex [reactions (11) and (12)] that yields the superoxide radical, the IAA cation radical and the ferric enzyme. The stimulation of dioxygen consumption with either enzyme in the presence of SOD is consistent with our proposal that reactions (11) and (12) comprise the mechanism of initiation. The IAA cation radical at low pH converts into the skatolyl radical via decarboxylation [reaction (13)] (18000 s^{-1} [11]); the latter interacts quickly with oxygen. The rate constants for the reactions between organic radicals and dioxygen are usually in the range 10^8–10^9 $M^{-1} \cdot s^{-1}$ (for the benzyl radical it is approx. 2×10^9 $M^{-1} \cdot s^{-1}$ [16]). Thus skatolyl radicals produced in the presence of an excess of dioxygen immediately yield peroxyl radicals and skatolyl hydroperoxide via reactions of chain propagation [reactions (15) and (16)]. Skatolyl hydroperoxide starts the common peroxidase cycle [reactions (1)–(3)], thereby generating IAA radicals.

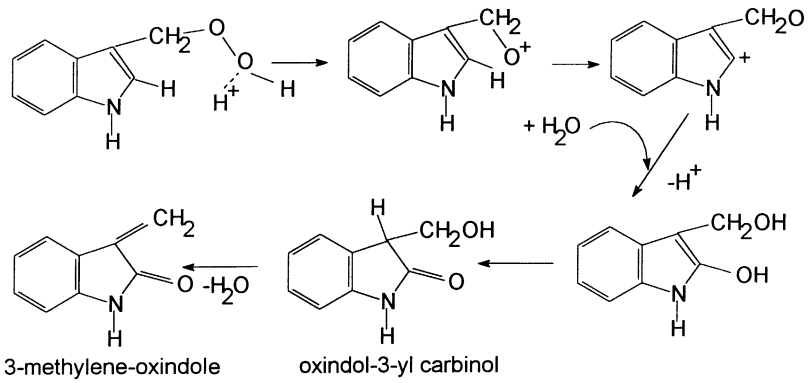
For HRP-C at low pH the ternary complex, formed according to reaction (11), yields Compound III and the IAA cation radical:

$$[\text{E-IAA-O}_2] \rightarrow \text{E-O}_2^{\bullet -} + \text{IAA}^{\bullet +} \quad (17)$$

where $\text{E-O}_2^{\bullet -}$ is Compound III.

This explains why HRP-C is insensitive to catalase, in contrast with TOP. The properties and stability of Compound III should therefore determine the reaction mechanism. HRP mutants with the altered properties of Compound III ($Arg^{38} \rightarrow$ Ser, $Arg^{38} \rightarrow$ Leu) [17] are currently being used in this laboratory to test this hypothesis. The two-electron reduction of Compound III first described by Ricard and Job [reactions (7) and (8)] provides the second route to Compound II without Compound I formation (i.e. not a conventional peroxidase cycle). If only one IAA molecule is oxidized by Compound III it might yield oxindol-3-yl acetic acid (oxiIAA):

$$\text{EIII} + \text{IAA} \rightarrow \text{EII} + \text{oxiIAA} \quad (18)$$



Scheme 2 Non-enzymic degradation of skatolyl hydroperoxide

Skatolyl hydroperoxide, generated via the non-enzymic radical reactions (15) and (16), accumulates in the system and presumably also degrades non-enzymically. The reaction sequence in Scheme 2 illustrates a plausible route to oxindol-3-yl carbinol and methylene-oxindole, which are the main products of IAA degradation *in vitro* [12].

Parallels with prostaglandin H synthase mechanism

The chemistry of IAA conversion into indol-3-yl aldehyde is still unresolved. We have shown that when IAA consumption is complete, the peroxidases are still active, and there is sufficient skatolyl hydroperoxide to oxidize added reducing substrates such as ABTS or ascorbate in a conventional peroxidase cycle. However, indol-3-yl aldehyde synthesis is stopped completely (Figure 6); moreover, for the TOP-catalysed reaction, the concentration of indol-3-yl aldehyde reaches a maximum and then decreases, possibly owing to indol-3-yl aldehyde polymerization reaction [12]. This raises two questions: (i) Why is no indol-3-yl methanol synthesized from skatolyl hydroperoxide after IAA consumption? (ii) Why is indol-3-yl methanol formed from skatolyl hydroperoxide at the enzyme active centre not oxidized further to indol-3-yl aldehyde? At this point it is interesting to compare plant peroxidases with prostaglandin H synthase (PGHSynthase) [18–20], which exhibits both oxygenase and peroxidase activities. Arachidonic acid forms a radical at the active site and then via dioxygenation it is converted into hydroperoxy endoperoxide, prostaglandin G_2 (the detailed mechanism of the initiation of the reaction and the formation of prostaglandin G_2 is still unclear). To complete the peroxidase cycle PGHSynthase needs an electron donor other than arachidonic acid [18], yielding the corresponding hydroxy endoperoxide, prostaglandin H_2. A peculiarity of the reaction is that Compound I has not been detected in the reaction course [17,18], although it has been detected spectrophotometrically during titration of the enzyme with hydroperoxy endoperoxide, prostaglandin G_2 [19]. Prostaglandin H synthase has a unique substrate-binding site located approx. 12 Å from the distal site, at which hydroperoxide cleavage occurs [20]. This could allow arachidonic acid and the corresponding hydroperoxide (prostaglandin G_2) to be bound to the enzyme simultaneously. In this case the ternary complex enzyme–hydroperoxide–arachidonic acid could convert directly into a Compound II–arachidonic acid radical complex, releasing the reaction product prostaglandin H_2.

Our identification of skatolyl hydroperoxide as a key product of IAA enzymic degradation and the absence of Compound I from the reaction suggested similarities between the mechanism of action of plant peroxidases and PGHSynthase.

If IAA has a role as a second electron donor to complete the peroxidase cycle started by skatolyl hydroperoxide, the absence of Compound I is easily rationalized. Skatolyl hydroperoxide should generate indol-3-yl methanol ($InCH_2OH$) at the active site of peroxidase via reaction (19); indol-3-yl methanol would then be quickly oxidized, yielding indol-3-yl methanol radical and Compound II via reaction (20).

$$E^{3+} + InCH_2OOH \rightarrow EI\text{-}InCH_2OH \quad (19)$$

$$EI\text{-}InCH_2OH \rightarrow EII\text{-}[InCH_2OH]^{\bullet} + H^{+} \quad (20)$$

To complete the cycle, an electron donor other than indol-3-yl methanol is needed because it is a poor substrate for HRP-C (k_3 400 $M^{-1} \cdot s^{-1}$ [21]). This then allows IAA itself to act as the second electron donor [reaction (21)]:

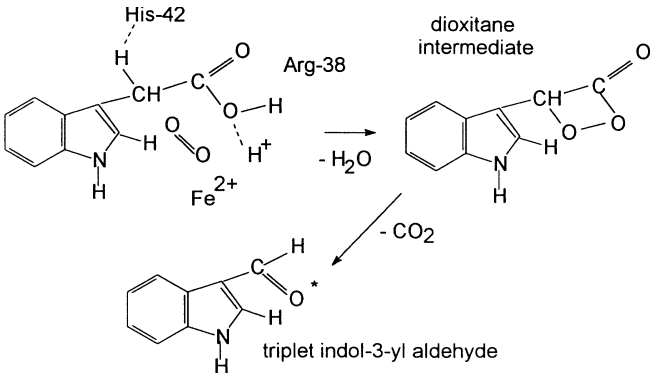
$$EII\text{-}[InCH_2OH]^{\bullet} + IAA \rightarrow EII\text{-}InCH_2OH + IAA^{\bullet} \quad (21)$$

This proposal finds a precedent in the existence of complexes between peroxidase Compound II and a substrate radical (EII-S$^{\bullet}$) responsible for the substrate–substrate activation phenomenon in the case of co-oxidations catalysed by plant and fungal peroxidases [22,23]. A special role for this type of complex has been shown for veratryl alcohol oxidation catalysed by lignin peroxidase [24,25]. The co-oxidation phenomenon is also a characteristic of the enzymic degradation of IAA: the oxidation of coniferyl alcohol by cell wall peroxidases with indol-3-yl acetic acid and O_2 has been proposed as a model for the lignification of plant cell walls in the absence of H_2O_2 [26]. It is likely that a co-oxidation phenomenon in both peroxidase and oxygenase reactions catalysed by haem-containing plant peroxidases involves a complex formed between Compound II and a substrate radical.

The reaction sequence (19)–(21) is proposed to explain the absence of Compound I, skatolyl hydroperoxide accumulation and its non-enzymic degradation. It also explains the fast inactivation of the enzymes in the course of IAA oxidation *in vitro*. However, it leaves open the question of the mechanism of indol-3-yl aldehyde appearance.

Plausible role of ferrous peroxidase

Because indol-3-yl methanol is not oxidized by skatolyl hydroperoxide to indol-3-yl aldehyde in the peroxidase cycle, Compound III and ferrous enzyme are the only forms that can be considered as catalysts for indol-3-yl aldehyde production from



Scheme 3 A plausible mechanism for indol-3-yl aldehyde formation (adapted from [7])

IAA. The direct transformation of IAA into indol-3-yl aldehyde at the active site of HRP-C Compound III has been proposed [7] (see Scheme 3). Two other groups have previously suggested that indol-3-yl aldehyde production is related to the appearance of ferrous enzyme [3,10] but no direct experimental data were presented to support this conclusion.

The stimulation of indol-3-yl aldehyde synthesis in the presence of catalase described in this study can be easily explained if ferrous peroxidase is responsible for IAA conversion into indol-3-yl aldehyde via reaction (22), which is also represented in Scheme 3:

$$Fe^{2+} + IAA + O_2 \rightarrow Fe^{2+} + CO_2 + H_2O + \text{indol-3-yl aldehyde} \quad (22)$$

Ferrous enzyme should react with skatolyl hydroperoxide to yield Compound II and indol-3-yl methanol:

$$E^{2+} + InCH_2OOH \rightarrow EII + InCH_2OH \quad (23)$$

Reaction (23) will decrease the concentration of ferrous enzyme and subsequently the concentration of indol-3-yl aldehyde formed via reaction (22). Degradation of skatolyl hydroperoxide by catalase will maintain the concentration of ferrous enzyme and thereby increase the production of indol-3-yl aldehyde. The inhibition of oxygen consumption by CO indicates a higher contribution of the ferrous enzyme route to the overall reaction with TOP than with HRP-C.

The mechanism of ferrous enzyme production is still unclear. Ferrous peroxidase can be formed when ferric enzyme is reduced by skatolyl radicals [reaction (24)], as proposed by Nakajima and Yamazaki [3]:

$$E^{3+} + InCH_2^{\bullet} \rightarrow E^{2+} + InCH_2^{+} \quad (24)$$

Hydrolysis of skatole cations can yield indol-3-yl methanol:

$$InCH_2^{\bullet+} + OH^- \rightarrow InCH_2OH \quad (25)$$

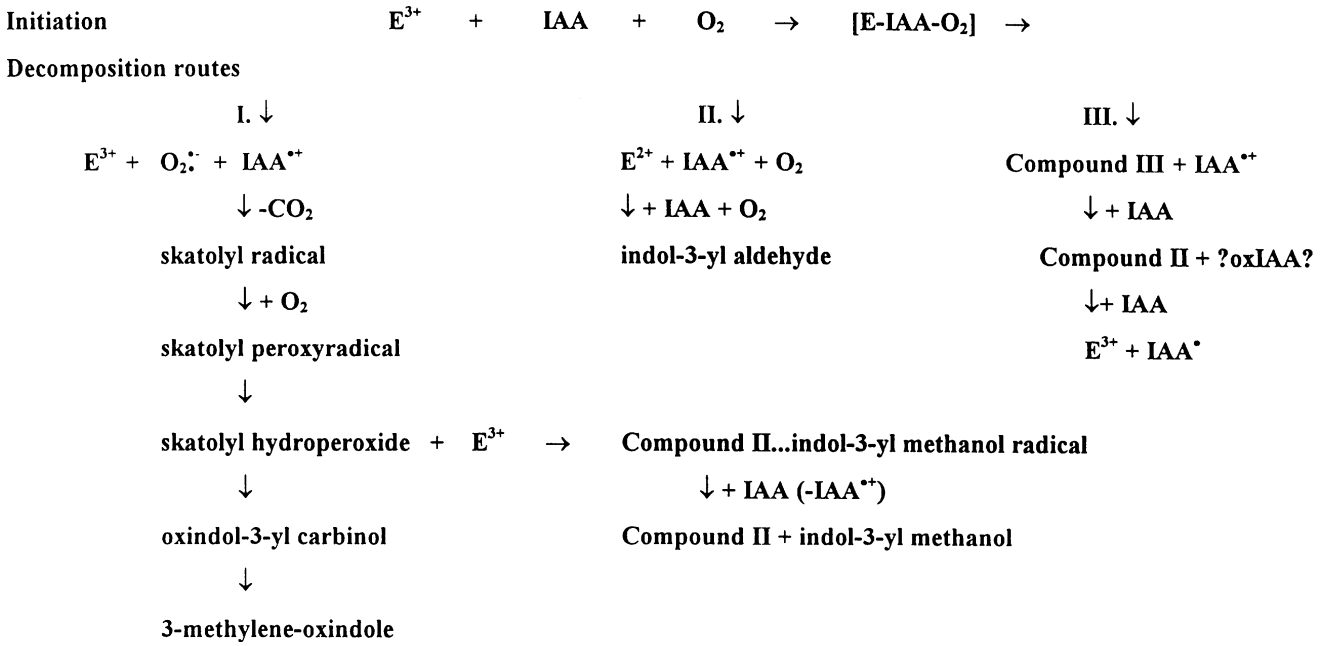
Another possibility is the release of ferrous enzyme from the ternary complex, enzyme-IAA-oxygen:

$$[\text{E-IAA-O}_2] \rightarrow E^{2+} + IAA^{\bullet+} + O_2 \quad (26)$$

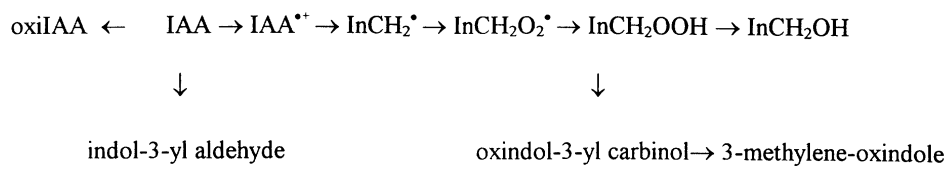
The results obtained in the present study allow us to conclude that indol-3-yl aldehyde production is catalysed by ferrous enzyme, but at this stage we prefer not to speculate on the mechanism.

Conclusions

The results of these and previous stopped-flow studies on the reaction mechanism [8] support the following mechanism of IAA degradation catalysed by plant peroxidases (Scheme 4). The system peroxidase-IAA-oxygen is self-sufficient to initiate the process. The initiation proceeds via the formation of a ternary complex responsible for primary radical generation. The IAA



Scheme 4 Mechanism of indol-3-yl acetic acid degradation catalysed by plant peroxidases



Scheme 5 Indol-3-yl acetic acid transformations occurring during peroxidase-catalysed oxidation

cation radical released from the ternary complex finally yields skatolyl hydroperoxide. The present study provides, for the first time, direct evidence for skatolyl hydroperoxide formation and accumulation in the system. Skatolyl hydroperoxide is converted enzymically into indol-3-yl methanol via the peroxidase cycle only in the presence of IAA, which acts as an electron donor to complete the cycle. This explains why the products of the non-enzymic degradation of skatolyl hydroperoxide, i.e. oxindol-3-yl carbinol and methylene-oxindole, have been identified as the main products of IAA degradation *in vitro* but not *in vivo*: plants contain sufficient quantities of peroxidase substrates to complete the cycle that finally yields indol-3-yl methanol from skatolyl hydroperoxide.

The prinicipal finding is that skatolyl hydroperoxide is degradable by catalase and that the presence of catalase significantly increases the amount of indol-3-yl aldehyde product. The inability of catalase to inhibit completely the reaction of IAA oxidation is indirect evidence for an initiation step different from the usual peroxidatic reaction postulated by others [3,5,6]. We have previously proposed that initiation requires ternary complex formation [8].

Scheme 4 summarizes our mechanism. The predominant route for ternary complex decomposition depends on the type of peroxidase. The insensitivity of the HRP-C catalysed reaction to catalase can be ascribed to Compound III formation at the initiation step and its role in radical propagation (route III). This is in contrast with the TOP-catalysed process, in which no Compound III is detected and skatolyl hydroperoxide has a key role in radical propagation (route I). In both cases the stimulating effect of SOD provides evidence for superoxide radical production (route I). Inhibition by CO indicates that ferrous enzyme participates in the reaction (route II). Thus for HRP-C we conclude that all three routes for ternary complex decomposition operate but with a minimum contribution from route II. For TOP, only routes I and II are involved in the inititation mechanism.

The results obtained in this work, combined with the recent identification of oxindol-3-yl acetic acid as a product [2], support the scheme shown in Scheme 5 for IAA transformations.

The principal difference between Scheme 4 and that proposed by Kobayashi et al. [12] is the route that yields indol-3-yl aldehyde, which is not produced via the conventional peroxidase cycle but by catalysis involving ferrous peroxidase. Because indol-3-yl aldehyde is one of the main IAA-derived products identified *in planta*, we conclude that ferrous peroxidases participate in IAA catalytic transformations *in vivo*.

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