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Hydroxyl-radical production in physiological reactions
A novel function of peroxidase

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Peroxidases catalyze the dehydrogenation by hydrogen peroxide (H_2O_2) of various phenolic and endiolic substrates in a peroxidatic reaction cycle. In addition, these enzymes exhibit an oxidase activity mediating the reduction of O_2 to superoxide ($O_2^{\cdot-}$) and H_2O_2 by substrates such as NADH or dihydroxyfumarate. Here we show that horseradish peroxidase can also catalyze a third type of reaction that results in the production of hydroxyl radicals ($^{\cdot}OH$) from H_2O_2 in the presence of $O_2^{\cdot-}$. We provide evidence that to mediate this reaction, the ferric form of horseradish peroxidase must be converted by $O_2^{\cdot-}$ into the perferryl form (Compound III), in which the haem iron can assume the ferrous state. It is concluded that the ferric/perferryl peroxidase couple constitutes an effective biochemical catalyst for the production of $^{\cdot}OH$ from $O_2^{\cdot-}$ and H_2O_2 (iron-catalyzed Haber–Weiss reaction). This reaction can be measured either by the hydroxylation of benzoate or the degradation of deoxyribose. $O_2^{\cdot-}$ and H_2O_2 can be produced by the oxidase reaction of horseradish peroxidase in the presence of NADH. The $^{\cdot}OH$-producing activity of horseradish peroxidase can be inhibited by inactivators of haem iron or by various $O_2^{\cdot-}$ and $^{\cdot}OH$ scavengers. On an equimolar Fe basis, horseradish peroxidase is 1–2 orders of magnitude more active than Fe-EDTA, an inorganic catalyst of the Haber–Weiss reaction. Particularly high $^{\cdot}OH$-producing activity was found in the alkaline horseradish peroxidase isoforms and in a ligninase-type fungal peroxidase, whereas lactoperoxidase and soybean peroxidase were less active, and myeloperoxidase was inactive. Operating in the $^{\cdot}OH$-producing mode, peroxidases may be responsible for numerous destructive and toxic effects of activated oxygen reported previously.

Keywords: Fenton reaction; hydrogen peroxide; hydroxyl radical; peroxidase; superoxide radical.

The hydroxyl radical ($^{\cdot}OH$) constitutes the chemically most reactive species of 'activated oxygen' formed by successive monovalent reduction of dioxygen (O_2) in cell metabolism, and is primarily responsible for the cytotoxic effects of oxygen in plants, animals and micro-organisms, living in an oxygenic atmosphere [1,2]. The short-lived $^{\cdot}OH$ molecule unspecifically attacks biomolecules in a diffusion-limited reaction and is thus able to crack, for instance, polysaccharides, proteins and nucleic acids located less than a few nanometres from its site of generation [3]. Hydroxyl radicals can be produced from O_2 under a variety of stress conditions and are involved in numerous cellular disorders such as inflammations [4], embryo teratogenesis [5], herbicide effects [6,7], cell death [8,9] and killing of micro-organisms in pathogen-defence reactions [10]. There is evidence that these toxic effects can be traced back to damage by $^{\cdot}OH$ of DNA [11], proteins [12], or membrane lipids [13]. It is generally assumed [2,12,14] that $^{\cdot}OH$ is generated in biological systems from H_2O_2 by the Fenton reaction, known from inorganic chemistry:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$$

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Enymes: catalase (EC 1.11.1.6); horseradish peroxidase (EC.1.11.1.7); superoxide dismutase (EC 1.15.1.1)

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whereby Fe^{2+} can be regenerated through the oxidation by the superoxide anion ($O_2^{\cdot-}$):

$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2.$$

The combination of Eqns (1) and (2) is referred to as the iron-catalyzed Haber–Weiss reaction [15] shown below:

$$H_2O_2 + O_2^{\cdot-} \xrightarrow{Fe^{2+}/Fe^{3+}} {}^{\cdot}OH + OH^- + O_2.$$

H_2O_2 and $O_2^{\cdot-}$ are ubiquitously formed from O_2 as byproducts of electron transport process and flavin-catalyzed oxidase reactions and are thus potentially available in all aerobic cells. In contrast, the catalytic role of Fe^{2+}/Fe^{3+} in the production of $^{\cdot}OH$ by biological systems has not yet been demonstrated directly and the 'biological Haber–Weiss reaction' is so far an extrapolation from inorganic chemistry rather than an experimentally proven fact.

Peroxidases constitute a class of haem-containing enzymes ubiquitously present in prokaryotic and eukaryotic organisms [16] which catalyze the dehydrogenation of structurally diverse phenolic and endiolic substrates by H_2O_2 and are thus often regarded as antioxidant enzymes, protecting cells from the destructive influence of H_2O_2 and derived oxygen species [17]. However, in addition to this peroxidatic activity, peroxidases possess an oxidase activity by which electrons can be transferred from reducing substrates such as NADH to O_2. It has been shown that this oxidative activity involves the formation of $O_2^{\cdot-}$ and H_2O_2 as intermediates, and the conversion of the ferric form of the enzyme (Fe^{3+}-peroxidase) into the labile perferryl form (Fe^{2+}-$O_2^{\cdot-}$$Fe^{3+}$-$O_2^{\cdot-}$ peroxidase) also designated as Compound III [18]. Compound III is generally considered as enzymatically

inactive although it has been implicated in the oxidation of indole-3-acetic acid [19]. As Compound III contains Fe^{2+} in the prosthetic haem group that can be easily converted to Fe^{3+}, one can imagine that Compound III can act as a Fenton reagent in a manner similar to Fe^{2+}-chelates, such as Fe^{2+}-EDTA [20]. This idea was fostered by the fact that the unphysiological substrate dihydroxyfumarate converts horseradish peroxidase into Compound III and mediates the hydroxylation of aromatic compounds via the generation of $^{\cdot}OH$ [21–23]. To our knowledge, the obvious possibility that the Compound III/Ferr-peroxidase couple functions as a biological catalyst of the Haber–Weiss reaction has not yet been examined rigorously.

In the course of a research program aimed at the elucidation of the biochemical mechanism of cell-wall loosening during auxin-dependent elongation growth of plant organs [24] we consider the possibility that this process is mediated by a site-specific production of $^{\cdot}OH$ in the cell wall, resulting in the cleavage of load-bearing bonds within wall polymers. Peroxidase is generally present abundantly in the walls of growing plant cells [25] and has been implicated in the apoplastic generation of $O_2^{\cdot-}$ and H_2O_2 from O_2 and NADH [26]. It was therefore of interest to find out whether this enzyme could mediate the production of $^{\cdot}OH$ under these conditions.

MATERIALS AND METHODS

Chemicals

Horseradish peroxidase (grade I, mixture of basic and acidic forms in 3.2 M ammonium sulfate), catalase (from bovine liver), superoxide dismutase (from bovine erythrocytes), NADH and NADPH were from Boehringer (Mannheim, Germany); sodium benzoate, sodium formate, thiourea, Tiron, desferrioxamine mesylate, dihydroxyfumarate, Chelex 100 chelating resin and other types of peroxidase were from Sigma (Deisenhofen, Germany); diphenyleneiodonium chloride (dissolved in dimethylsulfoxide) was from Biomol (Hamburg, Germany); ascorbate was from Merck (Darmstadt, Germany), H_2O_2 was from Fluka (Buchs, Switzerland); 2-deoxy-D-ribose was from Serva (Heidelberg, Germany). All other reagents were of analytical grade. The concentration of H_2O_2 solutions was standardized photometrically using an extinction coefficient of 39.4 $M^{-1} \cdot cm^{-1}$ at 240 nm. Fe^{3+}-EDTA was prepared by mixing equal concentrations of $FeCl_3$ and Na_2-EDTA in 10 mM sodium citrate buffer (pH 6.0).

Enzyme assays

All incubations and assays were performed at 25 °C in 10 mM sodium citrate buffer (pH 6.0, except where stated otherwise). Production of $^{\cdot}OH$ by peroxidase was determined with a fluorimetric method based on the hydroxylation of benzoate [27]. The reaction mixture (1 mL in a 5 × 10-mm cell, type OS, Hellma, Germany) contained 2 mM benzoate and additions as indicated in legends. The reaction was normally started by adding enzyme and the increase in fluorescence (excitation 305 nm, emission 407 nm), mainly a result of the formation of 3-hydroxybenzoate, was recorded for up to 30 min. If, e.g. at pH < 6, the reaction demonstrated a significant background fluorescence change in the absence of benzoate, this had to be subtracted from the rate measured in the presence of benzoate. Alternatively, $^{\cdot}OH$ production was determined with a photometric method based on the degradation of deoxyribose [28]. Reaction mixtures as above, but containing 2 mM deoxyribose instead of benzoate, were incubated for 1 h. A 0.6-mL aliquot was mixed with 0.5 mL thiobarbituric-acid solution (10 $g \cdot L^{-1}$ in 50 mM NaOH) and 0.5 mL trichloroacidic acid solution (28 $g \cdot L^{-1}$). After heating in a boiling water bath for 20 min and cooling on ice, the absorbance of the pink thiobarbituric-acid adduct was measured at 532 nm in a 10-mm cell against blanks without enzyme. Turbid solutions were extracted with an equal volume of butan-1-ol and the absorbance measured in the extract. The presence of contaminating iron in the reagents was checked by passing buffer, etc. through a column of Chelex 100 chelating resin and horseradish peroxidase solutions though Sephadex G-25 (Pharmacia). As these precautions did not significantly affect the results, they were omitted in later experiments.

Preparation of different redox states of horseradish peroxidase

Compounds I, II and III were obtained following published procedures and identified by their absorption spectra [29]. As all states of the enzyme except Compound III (and ferro-peroxidase) show an isosbestic point at 452 nm [29], the change in absorbance at 452 nm (ΔA_{452}) can be used to measure Compound-III formation against the background of other forms of horseradish peroxidase. The conversion of ferri-peroxidase to Compound III and vice versa can be determined by measuring ΔA_{582} [29]. Compound I was prepared by incubating 1 μM horseradish peroxidase with 1 μM H_2O_2 for 5 min [19]. Compound II was similarly prepared by an incubation with 10 μM H_2O_2, or a mixture of 1 μM H_2O_2 and 1 μM $K_4Fe(CN)_6$ [30,31], and Compound III by an incubation with 200 μM NADH or dihydroxyfumarate [23,32]. The spectrophotometric characteristics of Compounds I, II, III generated were in agreement with published spectra [29]. Purified Compound III was prepared by incubating 1 mL of 50 μM horseradish peroxidase with 1 mM dihydroxyfumarate for 15 min and passing the solution at 5 °C through a column with 5 mL Sephadex G-25 previously equilibrated with buffer and precentrifuged (1200 ***g***, 1 min) to remove the mobile phase. The purified enzyme was recovered without dilution by a second, identical centrifugation. It was free of spectrophotometrically-detectable dihydroxyfumarate.

Statistical treatment of data

All experiments were repeated at least three times with similar results. The figures show either single representative results or means (± SE where appropriate).

RESULTS

Previous investigations have led to the assertation that NADH, in contrast with dihydroxyfumarate, is an unsuitable substrate for $^{\cdot}OH$ production by horseradish peroxidase [21,23]. This conclusion, however, was obviously based on data obtained under inappropriate experimental conditions. The $^{\cdot}OH$-producing activity of horseradish peroxidase in air-saturated buffer of pH 6.0 containing a suitable concentration of NADH could be readily demonstrated employing either the hydroxylation of benzoate [27] or the degradation of deoxyribose [28,33] to detect $^{\cdot}OH$ formation (Figs 1 and 2, Table 1). The benzoate hydroxylation assay detects primarily the highly fluorescent 3-hydroxybenzoate formed, in addition to the more weakly fluorescent 4-hydroxybenzoate [27]. Fig. 1 identifies 3-hydroxybenzoate as the major fluorescent hydroxylation product of the horseradish peroxidase-catalyzed reaction. This assay produces

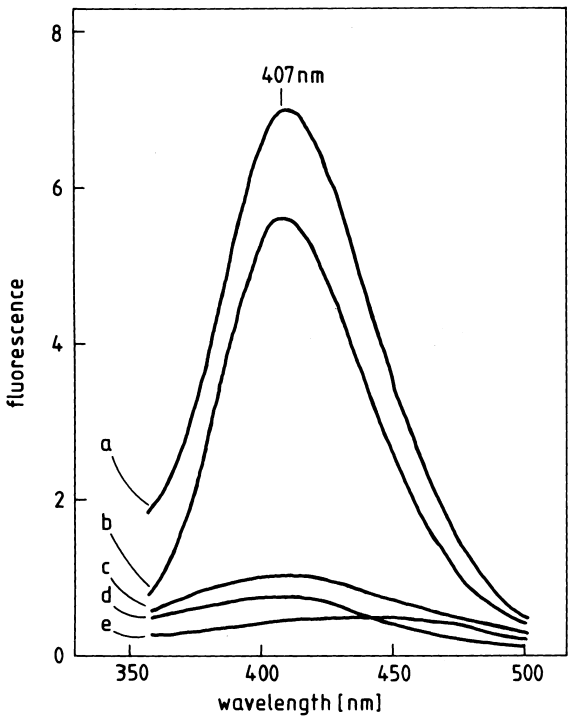

Fig. 1. Fluorescence emission spectrum of reaction product obtained in the benzoate hydroxylation assay in the presence of horseradish peroxidase and NADH. (a) Authentic 3-hydroxybenzoate (30 μM), (b) complete reaction mixture, (c) reaction mixture minus benzoate, (d) reaction mixture minus NADH, (e) reaction mixture minus horseradish peroxidase. Reaction mixtures (200 μM NADH, 30 $\mu g \cdot mL^{-1}$= 0.7 μM horseradish peroxidase, 2 mM benzoate in 10 mM citrate buffer, pH 6.0) were incubated for 60 min. Remaining NADH was eliminated by adjusting to pH 3.2 with HCl. After 60 min the pH was adjusted to 5.5 with KOH. The fluorescence spectra were not affected by pH in the range of pH 5.0–6.5.

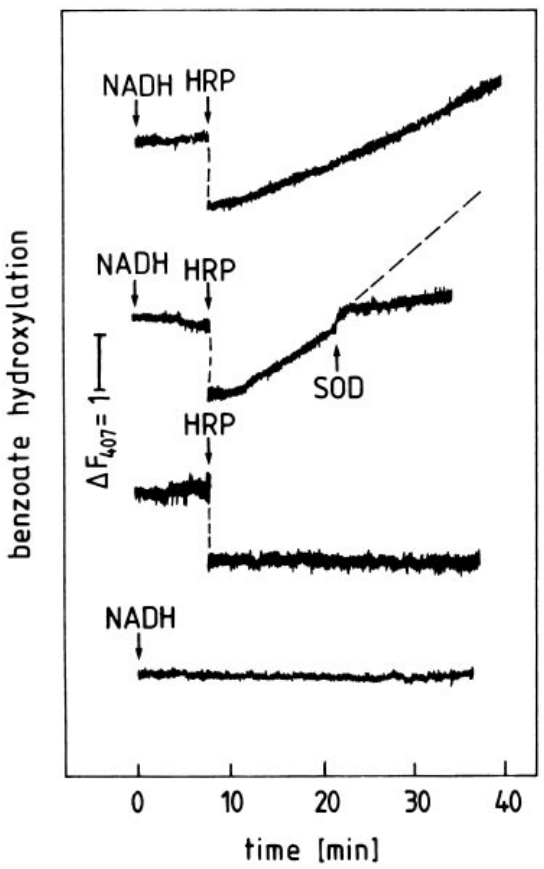

Fig. 2. Reaction kinetics of ˙OH production by horseradish peroxidase in the presence of NADH determined by benzoate hydroxylation. NADH (200 μM), horseradish peroxidase (60 $\mu g \cdot mL^{-1}$ = 1.4 μM) and superoxide dismutase (50 $\mu g \cdot mL^{-1}$) were added as indicated. ΔF_{407} = 1 corresponds to a 3-hydroxybenzoate concentration of 3.8 μM in buffer (9.5 μM in the presence of 200 μM NADH). HRP, horseradish peroxidase; SOD, superoxide dismutase.

approximately linear reaction kinetics for at least 30 min and can be used for kinetically testing the effects of inhibitors such as superoxide dismutase (Fig. 2); it was therefore used in most of the subsequent experiments. Both assay reactions were absolutely dependent on the presence of native peroxidase and a reductant such as NADH. Measurements with different horseradish peroxidase preparations revealed significant differences in activity in the standard benzoate hydroxylation assay, based on equal amounts of enzyme protein. The data reported in this paper were obtained with a single batch of Boehringer horseradish peroxidase and are therefore directly comparable.

As shown previously [18], horseradish peroxidase can be converted to Compound III in a reaction started by NADH in the presence of O_2. The conversion of the haem group from the ferric form into the perferryl form must involve $O_2^{\cdot -}$ as a reductant, as it can be prevented by superoxide dismutase (Fig. 3). Other $O_2^{\cdot -}$-scavenging reagents (100 μM Tiron or 10 μM $CuCl_2$) were as effective as superoxide dismutase, while catalase (10 $\mu g \cdot mL^{-1}$) only slightly impaired Compound-III formation (data not shown). The interconversion of ferriperoxidase into Compound III can be photometrically determined without interference from other forms of the enzyme, by measuring the increase in absorbance at 452 nm [29]. Employing this method, Fig. 4A shows that Compound-III formation demonstrates a broad plateau in the range of pH 4.5–6.0. Benzoate hydroxylation and deoxyribose degradation used for determining ˙OH production under these conditions showed optimal reaction rates in the same pH range, although the curves differed significantly, indicating different pH requirements of these reactions (Fig. 4B). The background fluorescence change of the reaction mixture in the absence of benzoate increased markedly because of NADH degradation at pH < 6.0. To minimize this effect and to approximate physiological conditions, a pH of 6.0 was used for all further experiments.

In order to scrutinize the involvement of ˙OH and other components of Eqn (3) in the peroxidase-catalyzed hydroxylation of benzoate and degradation of deoxyribose, the effects of various diagnostic inhibitors were investigated. Table 1 shows that both assay reactions can be inhibited with established ˙OH scavengers such as mannitol, formate and thiourea, in a concentration-dependent manner. In addition, these reactions can be inhibited by removing $O_2^{\cdot -}$ with Cu^{2+} [34] or superoxide dismutase, or by removing H_2O_2 with catalase. Inactivation of the haem iron of peroxidase by desferrioxamine [35], NaN_3 or KCN likewise inhibited both reactions, although the effects of desferrioxamine and NaN_3 may also be the result of ˙OH- and $O_2^{\cdot -}$-scavenging actions that have previously been attributed to these reagents [14]. The NADPH-oxidase inhibitor diphenyleneiodonium has recently been shown to interfere with the NADH-dependent $O_2^{\cdot -}$- and H_2O_2-producing activity of horseradish peroxidase [36]. The differences in sensitivity of benzoate hydroxylation and deoxyribose degradation towards most of the inhibitors are difficult to interpret as these reactions are chemically complex and different radical

Table 1. Effects of various inhibitors on ·OH production by horseradish peroxidase assayed either by benzoate hydroxylation or deoxyribose degradation. In the benzoate assay, the test substances were added to the reaction mixture 15 min after starting the reaction with horseradish peroxidase (1.4 μM), and the change in rate determined during the following 10 min as shown in Fig. 2. In the deoxyribose assay, the test substances were added before starting the reaction with horseradish peroxidase, and the increase in reaction product determined after a period of 60 min. ND, not determined.

Reagent added	Benzoate hydroxylation (ΔF_{407}·10 min^{-1})	Inhibition (%)	Deoxyribose degradation (ΔA_{532}·60 min^{-1}·10^3)	Inhibition (%)
None	1.10 ± 0.04	0	23.8 ± 0.8	0
Sodium benzoate				
10 mM	ND	–	15.1 ± 0.6	37
20 mM	ND	–	11.4 ± 1.2	52
Mannitol				
20 mM	0.90 ± 0.04	22	20.7 ± 0.7	13
100 mM	0.65 ± 0.05	41	16.7 ± 1.0	30
250 mM	0.38 ± 0.05	65	12.9 ± 0.8	46
Na-formate				
10 mM	0.93 ± 0.07	15	16.9 ± 0.4	29
50 mM	0.50 ± 0.04	55	11.2 ± 0.8	53
200 mM	0.22 ± 0.02	80	ND	–
Thiourea				
5 μM	0.84 ± 0.02	24	21.7 ± 0.9	9
100 μM	0.47 ± 0.02	57	16.7 ± 0.6	30
1 mM	0.22 ± 0.03	80	13.0 ± 0.5	45
$CuCl_2$				
100 nM	1.02 ± 0.07	7	18.3 ± 0.5	23
500 nM	0.51 ± 0.07	54	15.4 ± 0.7	35
10 μM	0.10 ± 0.01	91	1.8 ± 0.7	93
Superoxide dismutase				
0.2 μg·mL^{-1}	0.73 ± 0.02	34	23.2 ± 0.3	3
2 μg·mL^{-1}	0.50 ± 0.04	54	22.7 ± 0.2	5
20 μg·mL^{-1}	0.23 ± 0.03	79	11.3 ± 0.4	53
100 μg·mL^{-1}	0.13 ± 0.03	88	ND	–
Catalase				
0.4 μg·mL^{-1}	0.71 ± 0.05	35	23.2 ± 0.5	3
2 μg·mL^{-1}	0.46 ± 0.04	58	20.1 ± 1.1	16
10 μg·mL^{-1}	ND	–	13.5 ± 0.2	43
50 μg·mL^{-1}	0.04 ± 0.02	96	ND	–
Desferrioxamine				
10 μM	0.82 ± 0.05	25	20.6 ± 0.3	13
100 μM	0.53 ± 0.08	52	17.4 ± 0.6	27
1 mM	0.38 ± 0.04	66	13.0 ± 0.3	46
NaN_3				
1 μM	0.96 ± 0.03	13	23.0 ± 0.8	4
100 μM	0.46 ± 0.03	58	16.8 ± 0.5	29
500 μM	0.14 ± 0.03	88	11.6 ± 0.1	51
KCN				
10 μM	0.95 ± 0.02	14	19.6 ± 0.3	18
1 mM	0.48 ± 0.04	56	14.1 ± 0.6	41
10 mM	0.28 ± 0.03	75	11.5 ± 1.4	52
Diphenyleneiodonium[a]				
20 nM	0.99 ± 0.05	10	ND	–
100 nM	0.50 ± 0.07	55	17.5 ± 0.6	26
1 μM	0.07 ± 0.02	94	11.8 ± 0.2	50

[a] The concentration of DMSO in diphenyleneiodonium solutions had no effect on ·OH production.

mechanisms are involved. However, these discrepancies may be partly explained by different degrees of competition between rate-limiting reactants, e.g. by different abilities of benzoate and deoxyribose to react with the extremely short-lived ·OH molecule, and thus to compete with particular scavengers, at the site of ·OH generation. Similar inconsistencies have been described by Gutteridge [27,37]. Irrespective of the precise explanation of these differences, the results of Table 1 provide qualitative evidence for the involvement of ·OH, $O_2^{\cdot -}$, H_2O_2 and reactive iron in the reactions mediated by horseradish peroxidase, which thus show the typical features of the Haber–Weiss reaction catalyzed by Fe-EDTA [15,20].

Fe^{2+}-EDTA, or Fe^{3+}-EDTA in the presence of a Fe^{2+}-forming reductant such as $O_2^{\cdot -}$, has often been used as an effective inorganic reagent for mediating ·OH production from H_2O_2 according to Eqn (1) [14]. Rowley and Halliwell [38] have shown that Fe^{2+}-EDTA and Fe^{3+}-EDTA are equally effective in catalyzing ·OH production in the presence of NADH. Comparative experiments with Fe-EDTA and horseradish peroxidase have shown, however, that at equimolar Fe concentrations up to 1 μM, horseradish peroxidase was 1–2 orders of magnitude more effective than Fe-EDTA in mediating ·OH production in the presence of 200 μM NADH and 100 μM H_2O_2 (Fig. 5). The ·OH production catalyzed by horseradish peroxidase can be

Table 2. Activity of ferri-peroxidase, Compound I, Compound II and Compound III in ·OH production. Horseradish peroxidase (1 μM) was preincubated for 5 min with various reagents. Then benzoate (2 mM) was added for determination of the ·OH production rate during the following 10 min. The presence of the indicated enzyme forms after the pretreatment was checked by measuring the absorption spectra.

Pretreatment	Enzyme form produced	·OH production rate ($\Delta F_{407.}$ 10 min^{-1})
None	Ferri-peroxidase	0
1 μM H_2O_2	Compound I	0
10 μM H_2O_2	Compound II	0
1 μM H_2O_2 + 1 μM K_4 $Fe(CN)_6$	Compound II	0
200 μM dihydroxyfumarate	Compound III	2.3 ± 0.1
200 μM NADH	Compound III	0.99 ± 0.02
200 μM dihydroxyfumarate + 200 μM ascorbate	Ferri-peroxidase	0.11 ± 0.01
200 μM NADH + 200 μM ascorbate	Ferri-peroxidase	0.10 ± 0.01

promoted by the addition of H_2O_2 indicating that H_2O_2 is a rate-limiting substrate of this reaction. Even in the absence of added H_2O_2, i.e. supported solely by the endogenously produced H_2O_2, horseradish peroxidase was more than 10 times as effective as Fe-EDTA acting at a 10-fold excess of H_2O_2. Similar to the reaction in the absence of H_2O_2 (Fig. 2), the H_2O_2-promoted ·OH production by horseradish peroxidase can be inhibited by superoxide dismutase (Fig. 6).

Various substrates that can be oxidized by horseradish peroxidase were tested for their ability to support ·OH production by the enzyme. Fig. 7 shows that NADH can be replaced by NADPH and cysteine, although these reagents are less effective than NADH. Ascorbate is totally ineffective. With the nonphysiological substrate dihydroxyfumarate, activity is substantially higher than with NADH. Because of their absorption at 305 nm, both NAD(P)H and dihydroxyfumarate interfere with the excitation of benzoate fluorescence in a concentration-dependent manner. This effect can not be disregarded in measurements utilizing different concentrations of these substrates. The measurements in Fig. 7 were corrected for this effect using calibration curves for the attenuation of 3-hydroxybenzoate fluorescence by NAD(P)H and dihydroxyfumarate under the conditions of the assay.

We tested the ability of various commercially available peroxidases to catalyze ·OH production in the presence of NADH (Fig. 8). On the basis of equal activity units of the standard peroxidase assay with pyrogallol, the highest specific activity of ·OH production was found in a preparation of alkaline horseradish peroxidase isoforms and in peroxidase prepared from the fungus *Arthromyces ramosus*. Standard horseradish peroxidase, the acidic horseradish peroxidase fraction, lactoperoxidase from bovine milk and a soybean peroxidase preparation were less active, while myeloperoxidase from human leukocytes was virtually inactive. Thus this reaction appears to be a common property of peroxidases from different sources, with the exception of myeloperoxidase; this enzyme is also exceptional because of its ability to oxidize halides to reactive products [25].

The separation of Compound III from dihydroxyfumarate or NADH by chromatography on Sephadex G-25 [23] made it possible to investigate the substrate requirements for ·OH production by Compound III without interference of these putative substrates. Fig. 9A shows that Compound III alone does not hydroxylate benzoate but responds to the addition of H_2O_2 with a transient hydroxylation reaction. At the same time H_2O_2 induces a similarly rapid decay of Compound III (Fig. 9B). In the absence of H_2O_2, Compound III decays spontaneously with a much slower rate that is not affected by benzoate. However, the decay of Compound III can be strongly enhanced by agents reacting with $O_2^{\cdot-}$ such as superoxide dismutase (40 μg·mL^{-1}), ascorbate (200 μM), $CuCl_2$ (10 μM), Tiron (100 μM), but not by catalase (10 μg·mL^{-1}) and diphenyleneiodonium (1 μM) (data not shown). These data demonstrate that although $O_2^{\cdot-}$ can be easily removed from the enzyme by scavengers, Compound III is sufficiently stable in their absence to react with H_2O_2.

In the presence of 200 μM NADH, which allows the continuous reformation of Compound III from ferri-peroxidase by $O_2^{\cdot-}$, the addition of H_2O_2 induces a strong and long-lasting increase in benzoate hydroxylation (Fig. 9C), indicating that ·OH production utilizing H_2O_2 can be maintained in a steady-state under these conditions.

As a negative control, experiments were conducted to check whether other oxidation states of horseradish peroxidase, besides Compound III, can contribute to the ·OH-producing activity of the enzyme. Horseradish peroxidase was converted into Compound I, II, or III by established methods reported in literature and their activity tested with the benzoate hydroxylation assay. Table 2 shows that horseradish peroxidase can produce ·OH only in the state of Compound III and that this reaction can be inhibited by ascorbate, functioning as a $O_2^{\cdot-}$ scavenger rather than a substrate for ·OH production by horseradish peroxidase (see Fig. 7).

The phenol-hydroxylating reaction of horseradish peroxidase with dihydroxyfumarate + O_2 resembles the reaction of horseradish peroxidase with NADH + O_2 with respect to the production of $O_2^{\cdot-}$ and H_2O_2 and the superoxide dismutase-sensitive formation of Compound III [21,22]. However, according to another report, the dihydroxyfumarate-dependent reaction differs from the NADH-dependent reaction, in so far as the former could not be inhibited by catalase. Moreover, it apparently requires dihydroxyfumarate instead of H_2O_2 as a reaction partner in the hydroxylating activity of Compound III. Therefore, it has been concluded that dihydroxyfumarate-dependent hydroxylation by horseradish peroxidase does not involve H_2O_2 and is mechanistically different from a Haber–Weiss-type reaction [23]. However, these results were obtained at a relatively high concentration of dihydroxyfumarate (4 mM), saturated O_2, and at a low temperature (4 °C), conditions that presumably favour high concentrations of H_2O_2. Fig. 10 shows that under reaction conditions more closely resembling the experiments with NADH shown in Figs 2 and 6, the production of ·OH by dihydroxyfumarate + horseradish peroxidase can indeed be inhibited by catalase and enhanced by addition of exogenous H_2O_2. Moreover, purified Compound III has been shown to be capable of producing ·OH by utilizing H_2O_2 in the absence of dihydroxyfumarate (Fig. 9A). Thus, both dihydroxyfumarate and NADH support ·OH production by horseradish peroxidase by a reaction mechanism involving H_2O_2 as a substrate.

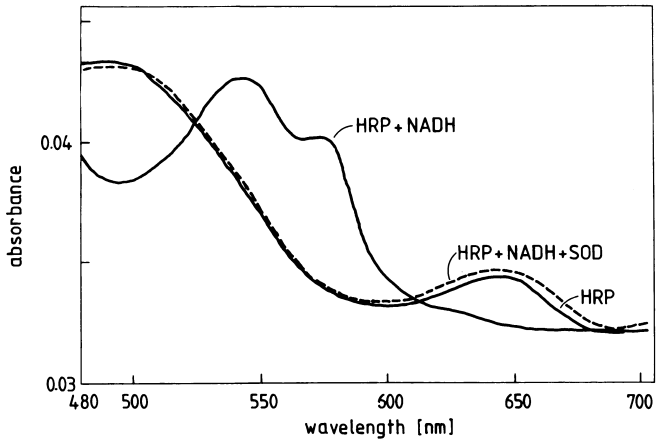

Fig. 3. Effect of NADH and NADH + superoxide dismutase on the absorption spectrum of horseradish peroxidase. Enzyme (1 μM) was incubated for 5 min with 200 μM NADH without or with 20 $\mu g \cdot mL^{-1}$ superoxide dismutase before recording the spectra. HRP, horseradish peroxidase; SOD, superoxide dismutase.

DISCUSSION

Peroxidases are very versatile enzymes that can react with numerous different substrates and catalyze different types of reactions *in vivo* [25]. The current knowledge of the basic peroxidase-dependent reactions in the peroxidatic and oxidative cycles is summarized in Fig. 11. The classic peroxidatic cycle is initiated by the transformation of the ferri-peroxidase into Compound I by H_2O_2, followed by the successive generation of two phenoxy radicals (Phe·) by hydrogen abstraction from

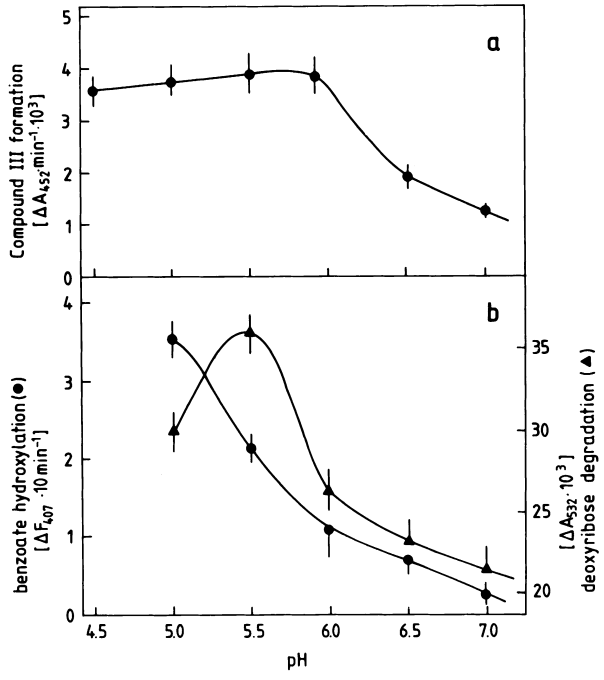

Fig. 4. pH Dependence of the transformation of ferri-horseradish peroxidase into Compound III and ·OH production in the presence of NADH. (A) Compound-III formation from horseradish peroxidase (1.4 μM) was measured as the rate of A_{452} increase in the presence of 200 μM NADH. (B) Production of ·OH was measured either with the benzoate hydroxylation assay or with the deoxyribose degradation assay in the presence of 200 μM NADH.

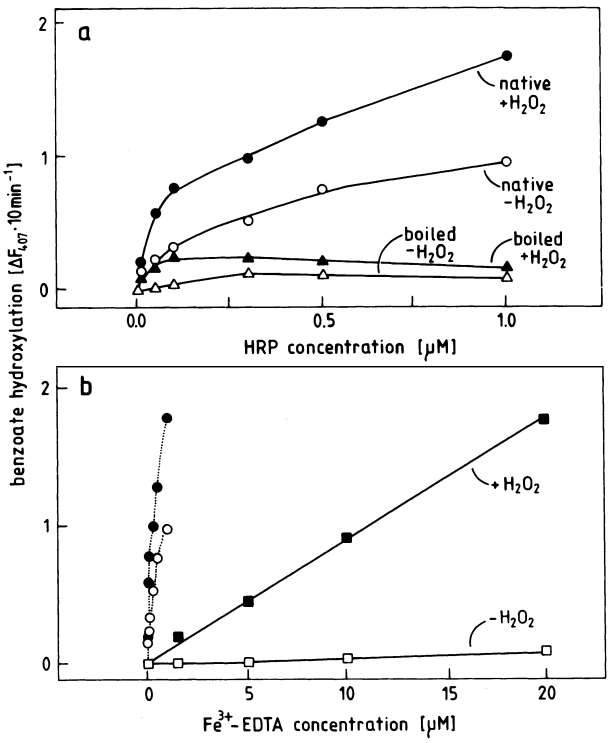

Fig. 5. Comparison of ·OH production by horseradish peroxidase and Fe^{3+}-EDTA in the presence of NADH. (A) Reaction rates produced by 0–1 μM native or boiled horseradish peroxidase in the absence and presence of H_2O_2. (B) Reaction rates produced by 0–20 μM Fe^{3+}-EDTA in the absence and presence of H_2O_2. The corresponding curves for horseradish peroxidase from panel A are included for comparison (dashed lines). Reaction mixtures contained 200 μM NADH and ± 100 μM H_2O_2.

substrates such as coniferyl alcohol. The spontaneous polymerization of these radicals leads to complex phenolic polymers such as lignins. If, however, the phenolic substrate is replaced by NADH or related reduced compounds, the resulting radicals (NAD·) initiate a nonenzymatic oxidative cycle in which O_2 can be reduced to O_2. As $O_2^{\cdot -}$ can react with another NADH molecule to produce H_2O_2 and NAD·, a chain reaction starts that provides the basis for the H_2O_2-producing NADH-oxidase activity of peroxidases. It is under these conditions that the enzyme can be partly converted to Compound III by binding $O_2^{\cdot -}$ to the haem iron. As Compound III does not support NADH oxidation, it is generally considered as an inactive form of peroxidase, at least as far as physiologically-relevant reactions are concerned [25].

The results presented in this paper add a new facet to this picture. We have shown that horseradish peroxidase, in a manner similar to chelated Fe ions, is capable of reducing H_2O_2 to ·OH in the presence of a suitable reductant. Various lines of evidence indicate that this reaction is catalyzed by horseradish peroxidase in the form of Compound III. Firstly, it has been shown that reagents such as superoxide dismutase or Cu^{2+} which promote the conversion of $O_2^{\cdot -}$ to H_2O_2, thereby preventing the formation of Compound III, strongly inhibit ·OH production in the presence of NADH (Fig. 2, Table 1). As superoxide dismutase has little inhibitory effect on NADH oxidation by Compounds I and II [39], it can be concluded that sufficient H_2O_2 is available under these conditions to maintain high levels of Compound I and II which are, however, unable to catalyze reactions leading to ·OH production. Moreover, the finding that superoxide dismutase inhibits ·OH production even in the

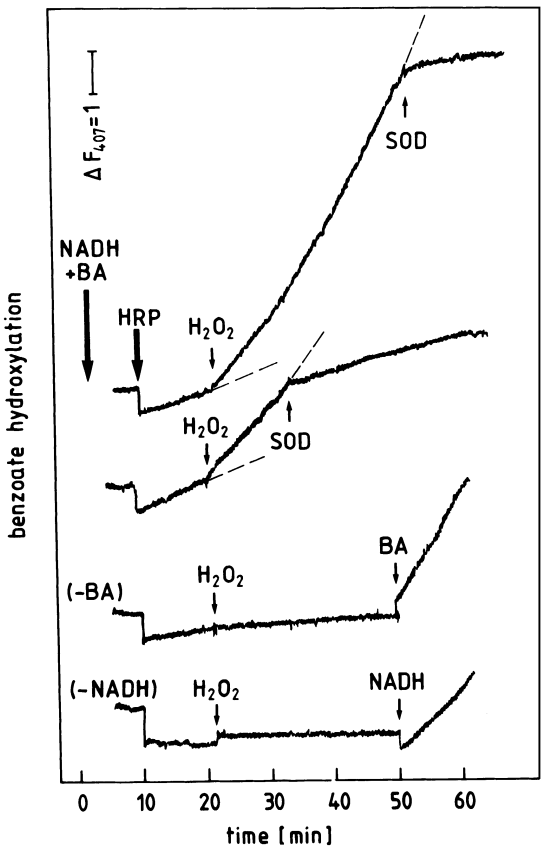

Fig. 6. Effect of superoxide dismutase on ˙OH production by horseradish peroxidase in the presence of NADH and H_2O_2, determined by benzoate hydroxylation. Benzoate (2 mM), NADH (200 μM), horseradish peroxidase (1.4 μM), H_2O_2 (100 μM) and superoxide dismutase (50 μg·mL^{-1}) were added as indicated. The two lower curves show controls omitting either BA or NADH from the initial reaction mixture. BA, benzoate, HRP, horseradish peroxidase; SOD, superoxide dismutase.

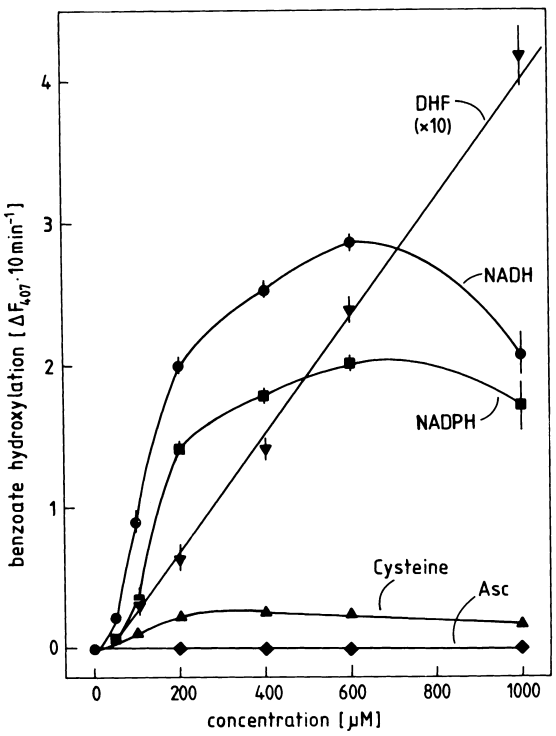

Fig. 7. Comparison of ˙OH production by horseradish peroxidase supported by various putative substrates. Reaction mixtures contained 1.4 μM horseradish peroxidase. The data were corrected for the effects of substrates (dihydroxyfumarate, NADH, NADPH) on fluorescence emisson. Note that the curve for DHF is reduced by a factor of 10. Asc, ascorbate; DHF, dihydroxyfumarate.

presence of high levels of added H_2O_2 (Fig. 6) confirms that Compound III, rather than Compounds I and II, is essential in this reaction. This conclusion is supported by the finding that neither Compound I nor Compound II can produce ˙OH from H_2O_2 (Table 2). On the other hand, purified Compound III, containing no spectroscopically detectable amounts of Compounds I and II, readily converts H_2O_2 to ˙OH and simultaneously reverts to the ferric form of the enzyme (Fig. 9A,C). We conclude therefore that Compound III catalyzes a hydroxylic cycle (Fig. 11) by which ˙OH can be continuously produced utilizing H_2O_2 and $O_2^{\cdot-}$ as formally described by the iron-catalyzed Haber–Weiss reaction (Eqn 3). According to the scheme shown in Fig. 11, peroxidase can act in two different catalytic modes. In the presence of H_2O_2 and phenolic substrates, the enzyme operates in the peroxidatic cycle and is engaged in the synthesis of lignins and other phenolic polymers. Alternatively, upon addition of $O_2^{\cdot-}$ the enzyme can be switched to Compound III and now catalyzes the reduction of H_2O_2 to ˙OH in a Haber–Weiss-type reaction cycle. This changeover in catalytic function can be brought about by NADH or a similar substrate that supports an oxidative cycle producing $O_2^{\cdot-}$. Although Compound III can also be produced by a large excess of H_2O_2 [19], this may not be of biological relevance. The operation of a NADH-dependent oxidative cycle is of course not an essential requirement for the ˙OH-producing mode of peroxidase action as the availability of $O_2^{\cdot-}$ and H_2O_2 from other sources would have a similar effect. Pyridine nucleotides can be replaced by cysteine, which is however much less effective in supporting ˙OH production by horseradish peroxidase, presumably because it also acts as an antioxidant. It has previously been shown that the oxidation of cysteine by horseradish peroxidase results in H_2O_2 production, unless the pro-oxidant function is superimposed by the antioxidant function at higher concentrations [40]. The pronounced antioxidant ($O_2^{\cdot-}$-scavenging) function of ascorbate [41] may be responsible for the inhibitory effect of this peroxidase substrate on Compound-III formation and its inactivity in the ˙OH-producing reaction of horseradish peroxidase [21,23].

In plants, peroxidases are primarily localized in the apoplastic space where they are bound ionically or covalently to cell-wall polymers [25]. No clear functions have been attributed to cell-wall peroxidases except in lignin synthesis and related oxidative cross-linking reactions [25]. In the light of the present results these enzymes may have a much wider array of physiological functions that are related to their ˙OH-producing property. This mode of peroxidase action may be fundamentally important in physiological processes involving the destructive effects of oxygen. The prediction can be made that ˙OH is likely to be produced whenever peroxidase comes into contact with suitable concentrations of $O_2^{\cdot-}$ and H_2O_2, originating either from the oxidative cycle of peroxidase or from other sources. This situation prevails, for instance, when $O_2^{\cdot-}$ and H_2O_2 levels are increased in plants in response to pathogen attack (oxidative burst) and is often followed by a hypersensitive reaction that results in host cell death [42]. However, there is accumulating evidence that plant cells can release significant amounts of $O_2^{\cdot-}$ and H_2O_2 in the absence of pathogenic stimuli [43–45]. The production of ˙OH by peroxidases bound to plant cell walls could also be relevant for the controlled breakdown of structural

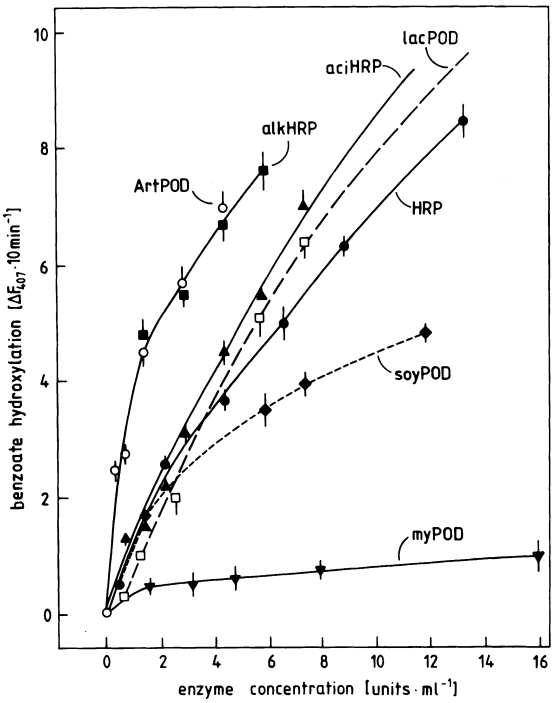

Fig. 8. Comparison of ˙OH production catalyzed by various types of peroxidase in the presence of NADH. Reaction mixtures contained 200 μM NADH and 0–20 units (standard pyrogallol assay) as indicated by the manufacturer of the following enzymes: horseradish peroxidase (unfractionated enzyme mixture), acidic horseradish peroxidase fraction, alkaline horseradish peroxidase fraction, soybean peroxidase, *Arthromyces* peroxidase, lactoperoxidase, myeloperoxidase. aciHRP, acidic horseradish peroxidase fraction; alkHRP, alkaline horseradish peroxidase fraction; ArtPOD, *Arthromyces* peroxidase; lacPOD, lactoperoxidase; myPOD, myeloperoxidase; soyPOD, soybean peroxidase .

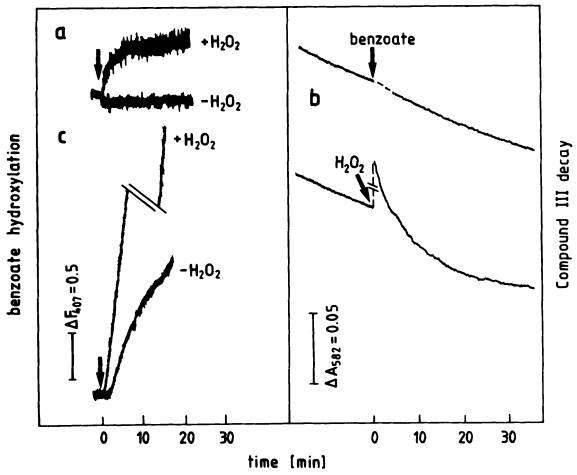

Fig. 9. Production of ˙OH from H_2O_2 by Compound III and the simultaneous conversion of Compound III to ferri-peroxidase. (A) Reaction kinetics of benzoate hydroxylation induced by adding 3 μM freshly purified Compound III (arrow) to assay mixture without NADH, in the absence and presence of 100 μM H_2O_2. (B) Reaction kinetics of Compound-III conversion induced by adding 100 μM H_2O_2 to buffer containing 50 μM freshly prepared Compound III, measured as decrease in A_{582} [29]. Control: Compound III in buffer; without and with added benzoate (2 mM). (C) Reaction kinetics of benzoate hydroxylation induced by adding 1.4 μM horseradish peroxidase (ferri-peroxidase, arrow) to assay mixture containing 200 μM NADH in the absence and presence of 100 μM H_2O_2. H_2O_2 produced no measurable fluorescence increase in the reaction mixture without NADH.

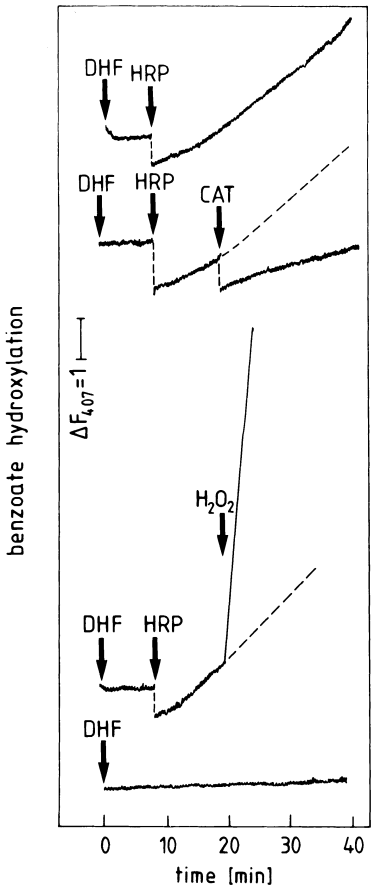

Fig. 10. Effect of catalase and H_2O_2 on the production of ˙OH by horseradish peroxidase in the presence of dihydroxyfumarate, determined by benzoate hydroxylation. Dihydroxyfumarate (50 μM), horseradish peroxidase (1.4 μM), catalase (50 μg·mL^{-1}) and H_2O_2 (100 μM) were added as indicated.

polymers. This could occur in the form of site-specific scissions in polymers to which the peroxidase molecules are attached. As ˙OH can also cause oxidative cross-linking reactions [46], the as yet unexplained covalent binding of peroxidase to cell-wall polymers could be another consequence of the ˙OH-producing activity of this enzyme. Moreover, it is well known that enzymes can be structurally modified or damaged by ˙OH [12]. Thus, taking into account the short life-time of ˙OH, which restricts the effective range of action to a few nanometers from its site of generation, it seems unavoidable that the peroxidase protein itself becomes a target of ˙OH. This suicide action provides a plausible explanation for the unique and bewildering abundance of peroxidase isoforms generally found in the cell walls of plants (e.g. 47 electrophoretically-separable forms in tobacco tissues [47]), which could be partly degraded products of a smaller number of true isoenzymes.

In this context it is also interesting to note that the depolymerization of lignins by wood-rotting fungi is mediated by secreted peroxidases, designated as ligninases, in the presence of H_2O_2 [48]. The biochemical mechanism of this ecologically extremely important peroxidase reaction is not yet resolved and it may be of interest to test the hypothesis that ligninases initiate the biodegradation of lignins via the ˙OH-producing mode of peroxidase action. It has been shown that ligninase Compound III can produce ˙OH in the presence of dihydroxyfumarate in a similar fashion as horseradish peroxidase

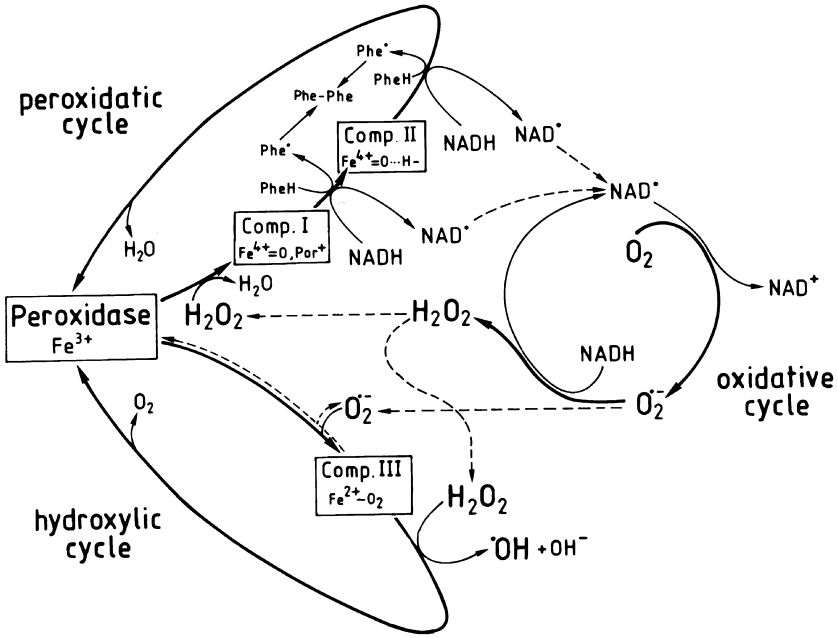

Fig. 11. Reaction scheme combining the peroxidatic, oxidative and hydroxylic cycles of peroxidase catalysis. The classic peroxidatic cycle mediates the oxydation by H_2O_2 of phenolic substrates (PheH) to phenoxy radicals (Phe·) that can polymerize to form molecules such as lignins. The corresponding reduction of substrates such as NADH (or dihydroxyfumarate) initiates the self-sustained oxidative cycle that reduces O_2 to $O_2^{\cdot-}$ and H_2O_2. $O_2^{\cdot-}$ can be utilized to produce Compound III that reduces H_2O_2 to OH in the hydroxylic cycle.

Compound III [32]. The observation that the ligninase-like peroxidase secreted by the fungus *A. ramosus* [49] displays a particularly high activity of ·OH production (Fig. 8) provides further evidence for this idea. It can also be conceived that cationic cell-wall peroxidases possessing a similarly high ·OH-producing activity (Fig. 8) are involved in the depolymerization of lignins under particular physiological conditions in the living plant. Thus, depending on the level of $O_2^{\cdot-}$ and H_2O_2 in the cell-wall space, peroxidases could mediate either wall-stiffening by lignin synthesis [50] or wall-loosening by lignin depolymerization. These and other biologically relevant implications of our results can now be explored experimentally.

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