Volume 220, number 1, 149-154 FEB 04976 August 1987

Oxidation of veratryl alcohol by the lignin peroxidase of *Phanerochaete chrysosporium*

Involvement of activated oxygen

Stephan D. Haemmerli, Hans E. Schoemaker*, Harald W.H. Schmidt+ and Matti S.A. Leisola

*Department of Biotechnology, Swiss Federal Institute of Technology, ETH-Hönggerberg, 8093 Zürich, Switzerland, *DSM Research, Bio-organic Chemistry Section, PO Box 18, 6160 MD Geleen, The Netherlands* and *+Givaudan Forschungsgesellschaft AG, 8600 Dübendorf, Switzerland*

Received 25 May 1987

The oxidation of veratryl alcohol by the lignin peroxidase of *Phanerochaete chrysosporium* was studied. Five products were identified: veratraldehyde, two quinones and two aromatic ring cleavage lactones. A similar product pattern was obtained with the 1-electron oxidant cerium(IV). Under anaerobic reaction conditions or in the presence of Mn(II) only traces of quinones or lactones were detected besides veratraldehyde. This indicates the involvement of activated oxygen species in the enzyme reaction. Possible mechanisms for the formation of the primary oxidation products from veratryl alcohol are discussed.

Lignin peroxidase; Veratryl alcohol; Perhydroxyl radical; Aryl cation radical; Lignin degradation

1. INTRODUCTION

Since the discovery of an extracellular enzyme (lignin peroxidase, ligninase) which plays a role in lignin degradation by *Phanerochaete chrysosporium* [1,2], white-rot fungi and their ligninolytic system have received much attention. Veratryl alcohol (3,4-dimethoxybenzyl alcohol), a metabolite from this wood-degrading fungus, is synthesized from phenylalanine and accumulates in the extracellular medium of the fungal cultures during secondary metabolism [3]. Veratryl alcohol seems to have several functions in lignin biodegradation. It induces the ligninolytic system of *P. chrysosporium* [4] increasing the H_2O_2 production rate and the levels of extracellular lignin peroxidase. Veratryl alcohol also stabilizes lignin peroxidase in the presence of excess H_2O_2 [5] and can function as a 1-electron transfer mediator between the enzyme and insoluble substrates like lignin [6,7].

Veratryl alcohol itself is oxidized by lignin peroxidase. A kinetic study of this oxidation has recently been published by Tien et al. [8]. Palmer et al. [9] measured oxygen consumption during aerobic oxidation of veratryl alcohol by lignin peroxidase and concluded that oxygen is probably a reactant and that products other than veratraldehyde must arise in the presence of oxygen. Two of these additional products have been isolated from fungal cultures and characterized [10]. The compounds are lactones, formed when the aromatic ring of veratryl alcohol is opened. Here, we have identified two further products and give evidence that active oxygen species are involved in these reactions.

Correspondence address: S.D. Haemmerli, Department of Biotechnology, Swiss Federal Institute of Technology, ETH-Hönggerberg, 8093 Zürich, Switzerland

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

2. MATERIALS AND METHODS

2.1. *Enzyme preparations*

Lignin peroxidase was isolated and purified from carbon-limited cultures of *P. chrysosporium* (ATCC 24725) as described in [7]. The enzyme with p*I* 4.15 was used in all experiments.

2.2. *Chemicals*

3,4-Dimethoxybenzyl alcohol (veratryl alcohol), 3-hydroxy-4-methoxybenzyl alcohol, 2-methoxyhydroquinone, and cerium (IV) ammonium nitrate were obtained from Fluka (Buchs, Switzerland), Fremy's salt from Aldrich (Suchema, Affoltern, Switzerland) and L-[U-^{14}C]phenylalanine from Amersham (Amersham, England).

2-Methoxy-2,5-cyclohexadiene-1,4-dione (quinone II) was prepared from 2-methoxyhydroquinone by oxidation with silver oxide (in ether). Silver oxide was added portionwise to the stirred etheral solution at room temperature, until the starting material could no longer be detected in the solution [monitored by gas-chromatography: OV-17 (3%), isothermal 160°C]. The quinone was isolated from the etheral solution and from acetone extracts of the residue by treating the combined solutions with charcoal and evaporation of the solvents in vacuo at 30°C yielding yellow crystals (90% yield), m.p. 140–142°C (uncorrected). UV: λ_{max}, 252 and 354 nm. MS, *m/e* (%): 138 (M^+; 73), 123 (12), 110 (64), 108 (100), 95 (75), 82 (67), 69 (98), 55 (48), 54 (69). ^{1}H-NMR ($CDCl_3$), δ (ppm): 3.85 (s,3H)-OCH_3, 5.97 (d,1H,J = 1 Hz), 6.72 (d,1H,J = 1 Hz), 6.73 (s,1H) ring protons.

2-Hydroxymethyl-5-methoxy-2,5-cyclohexadiene-1,4-dione (quinone I) was prepared from 3-hydroxy-4-methoxybenzyl alcohol by oxidation with Fremy's salt [11] in about 80% yield, m.p. 144–145°C (uncorrected). UV: λ_{max}, 262 and 352 nm. MS, *m/e* (%): 168 (M^+;2) 150(10), 139 (49), 122 (12), 111 (36), 83 (14), 69 (100), 55 (45), 53 (37). ^{1}H-NMR ($CDCl_3$), δ (ppm): 3.84 (s,3H)-OCH_3; 4.56 (d,2H,J = 1 Hz)-CH_2OH; 5.92 (s,1H), 6.74 (t,1H,J = 1 Hz) ring protons. Quinone I could not be analyzed by gas-chromatography (OV-17, isothermal 160°C, or OV-101, isothermal 200°C) without decomposition.

2.3. *Veratryl alcohol oxidations*

The reactions were carried out at room temperature. In aerobic oxidations the reaction mixture and all solutions were flushed with oxygen 15 min prior to and during the reactions. The anaerobic reaction mixtures were flushed with argon respectively. Oxidations were followed by the formation of veratraldehyde as judged by the increase in absorbance at 310 nm. After reactions were completed, the products were immediately extracted with methylene chloride at pH 2. Extracts were evaporated to dryness under nitrogen and analysed by high-performance liquid chromatography (HPLC). In some experiments [^{14}C]veratryl alcohol was used. It was prepared by feeding uniformly labelled [^{14}C]phenylalanine to 30 h old non-agitated cultures of *P. chrysosporium*. Accumulating [^{14}C]veratryl alcohol was extracted by methylene chloride from the culture filtrate after 60 h of growth and purified by thin-layer chromatography (TLC).

Oxidation by lignin peroxidase: reaction mixtures contained 400 μM veratryl alcohol, 10 mM sodium tartrate (pH 3.0–5.5) or 100 mM sodium lactate (pH 3.5–4.5), 50–400 μM H_2O_2 and 30–50 U/l lignin peroxidase.

Oxidation by Ce(IV): reaction mixtures contained 400 μM veratryl alcohol and 800 μM cerium(IV) ammonium nitrate in water. The reaction pH was <2.

Oxidations in the presence of Mn(II): reaction mixtures contained 400 μM veratryl alcohol, 0.2–5.0 mM Mn(II) sulphate, 100 mM sodium lactate (pH 3.5–4.5), 400 μM H_2O_2 and 30–50 U/l lignin peroxidase.

2.4. *Analytical methods*

Lignin peroxidase activity was measured according to Tien and Kirk [12]. One unit of enzyme produced 1 μmol veratraldehyde from veratryl alcohol in 1 min at room temperature. Silica gel 60 F_{254} TLC plates (Merck) were developed in benzene/ethyl acetate (3:1). R_f values for compounds I–VI (for structures, see fig.1) were: I, 0.24; II, 0.67; III and IV, 0.47; V, 0.31; VI, 0.75. Reversed-phase HPLC was performed on a Merck-Hitachi 655A-12 system. A 0.46 × 12.5 cm Hyperchrome column filled with Shandon ODS Hypersil (5 μM, RP 18) was used. Samples were injected as methanol solutions and the system was run at a

flow rate of 1.0 ml/min with a gradient of methanol/water (20–100% methanol in 25 min). The absorbance detector was operated at 254 nm. UV spectra were taken in 95% ethanol on a Perkin-Elmer 557 spectrometer. ^{1}H-NMR spectra were obtained on a 400 MHz NMR spectrometer (Bruker WH 400). MS spectra were recorded on a Varian MAT CH5B spectrometer.

3. RESULTS

3.1. *Oxidation of veratryl alcohol*

When veratryl alcohol (V) was oxidised by lignin peroxidase in the presence of oxygen four other reaction products were obtained besides veratraldehyde (VI) (fig.1). When the reaction was carried out under an argon atmosphere veratraldehyde (VI) was virtually the only product. Only traces of other compounds were formed. The one-electron oxidant Ce(IV) gave essentially the same products as lignin peroxidase under both conditions. The one additional product (not identified) obtained with Ce(IV) was unaffected by the presence or absence of oxygen. Experiments with [^{14}C]veratryl alcohol showed that practically all the reaction products were extractable by methylene chloride and that all the identified products were formed from veratryl alcohol.

3.2. *Product identification*

To obtain enough material for identification of compounds I and II the enzymatic oxidation was carried out using 4 l of 0.3 mM veratryl alcohol at pH 3.0. 30 U/l lignin peroxidase were used for the oxidation and H_2O_2 (0.25 mM) was added stepwise until the reaction was completed. After extraction the reaction products were first purified by preparative TLC and then twice by analytical TLC. The yellowish compounds I and II thus isolated were analyzed by UV, ^{1}H-NMR and MS. Their spectra and retention times in TLC and HPLC allowed their identification as the known quinones I [11] and II [13], respectively (cf. fig.1). The compounds were also independently synthesized for comparison (see section 2). Compounds III and IV have been identified previously as the *E* and *Z* isomers of a lactone which results when the aromatic ring of veratryl alcohol is oxidatively cleaved [10].

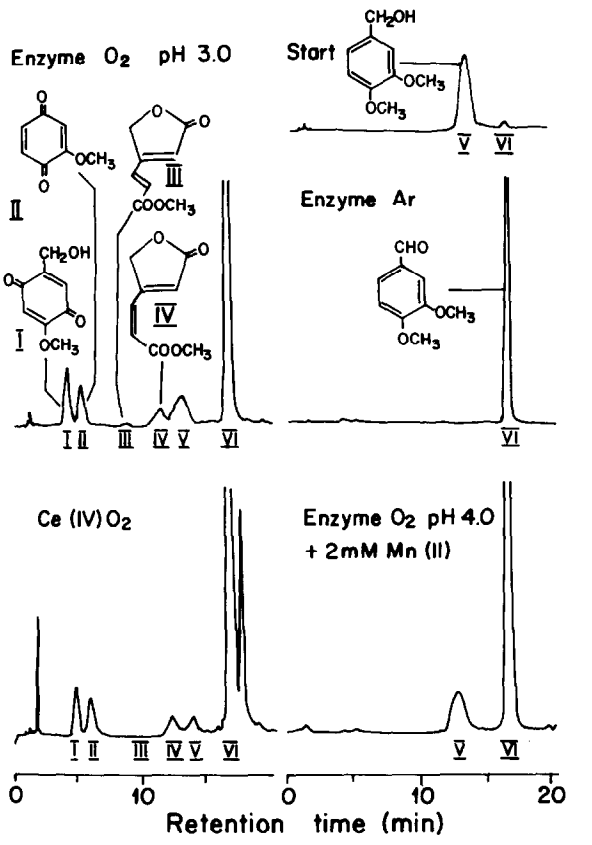

Fig.1. Oxidation of veratryl alcohol by lignin peroxidase and Ce(IV). Oxidations were carried out as described in section 2. HPLC chromatograms for oxygen and argon atmospheres are shown. Start: the substrate before oxidation.

3.3. *Effect of pH and Mn(II) on veratryl alcohol oxidation by lignin peroxidase*

The relative amounts of the various oxidation products in the enzyme reaction were dependent on the reaction pH. The highest amounts of quinones were formed at pH 3.0 while formation of the ring cleavage lactones was more favoured between pH 4.0 and 4.5. Above pH 5.0 veratraldehyde was the only product. When Mn(II) was added to the reaction mixtures, the amount of ring cleavage lactones and quinone I formed decreased considerably (table 1). The effect was more pronounced at higher pH values. However, oxidation of veratryl alcohol to the aldehyde and formation of quinone II were not significantly affected by Mn(II). Only Mn(II) concentrations of 5 mM and more had an inhibitory effect on the enzyme reaction.

Table 1

Relative amounts of oxygenated products formed by lignin peroxidase from veratryl alcohol under various reaction conditions

pH of reaction	Relative amount of oxygenated products I, III and IV[a]				Product II[a] 0–5 mM Mn(II)
	0 mM Mn(II)	0.2 mM Mn(II)	1 mM Mn(II)	5 mM Mn(II)	
3.5	17%	12%	8%	5%	3.0%
4.0	15%	7%	4%	1%	0.7%
4.5	13%	3%	1%	<1%	0.4%

[a] The percentage of products I, III and IV relative to the total amount of products was calculated from integration of absorbance in HPLC chromatograms. The data were not corrected for the specific ϵ of the compounds

4. DISCUSSION

Tien and Kirk [12] showed that during oxidation of veratryl alcohol by lignin peroxidase one molecule of aldehyde is formed per molecule of H_2O_2 consumed. They concluded that no products other than veratraldehyde are obtained when stoichiometric amounts of H_2O_2 are used and that oxygen is probably not a reactant. According to Tien et al. [8], veratryl alcohol is oxidized by the lignin peroxidase either via a direct two-electron oxidation or via two rapid and consecutive one-electron oxidation steps. Their data did not allow the authors to distinguish between these two mechanisms. Schoemaker et al. [14] have postulated that lignin peroxidase-catalyzed veratryl alcohol oxidation proceeds via two consecutive one-electron oxidation steps with the distinct intermediacy of both the veratryl alcohol radical cation and, after proton loss, the corresponding veratryl alcohol radical. Here, we show that besides veratraldehyde at least four additional products were formed in the aerobic oxidation of veratryl alcohol by lignin peroxidase. The 1-electron oxidant Ce(IV) gave essentially the same product pattern, indicating that also in the enzymatic oxidation 1-electron transfer from the aromatic ring occurs and that a cation radical is formed.

We further provide evidence for the involvement of activated oxygen species in the enzyme reaction. Ring opening and quinone formation only occur when the reaction is carried out in an oxygen atmosphere. Palmer et al. [9] postulated the formation of activated oxygen species during the aerobic oxidation of veratryl alcohol by lignin peroxidase via the reaction of the hydroxy-substituted benzyl radical with oxygen. They further suggested that perhydroxyl radicals or superoxide anions react with radicals from veratryl alcohol and thus are responsible for the ring-opening reaction. Recently, a revised scheme was proposed by Shimada et al. [15] which also accounted for the incorporation of one oxygen atom from H_2O into the lactone. We have shown here that an activated oxygen species is indeed formed during the oxidation of veratryl alcohol by lignin peroxidase and is involved besides H_2O in the reactions leading to quinone I and the ring cleavage lactones. We believe that the perhydroxyl radical is actually the reacting species. The effects of pH and Mn(II) support this suggestion. Less quinone I or ring cleavage compounds were formed at high pH when fewer of the reactive perhydroxyl radicals are available. The same was observed when superoxide anions and thus perhydroxyl radicals were removed by Mn(II). Mn(II) chelates scavenge superoxide anions by reducing them to H_2O_2 [16]. The H_2O_2 generated in this process can be used by lignin peroxidases to produce additional veratraldehyde. It has been shown that in the presence of O_2 and superoxide dismutase or Mn(II) more than one molecule of veratraldehyde is formed per molecule of H_2O_2 supplied to the reaction mixture [17]. Our experiments confirmed these observations.

A number of possible reactions of the intermediate radical cation Va from veratryl alcohol are depicted in fig.2. Proton loss from Va would yield the hydroxy-substituted 3,4-dimethoxybenzyl radical Vb, which under anaerobic conditions can

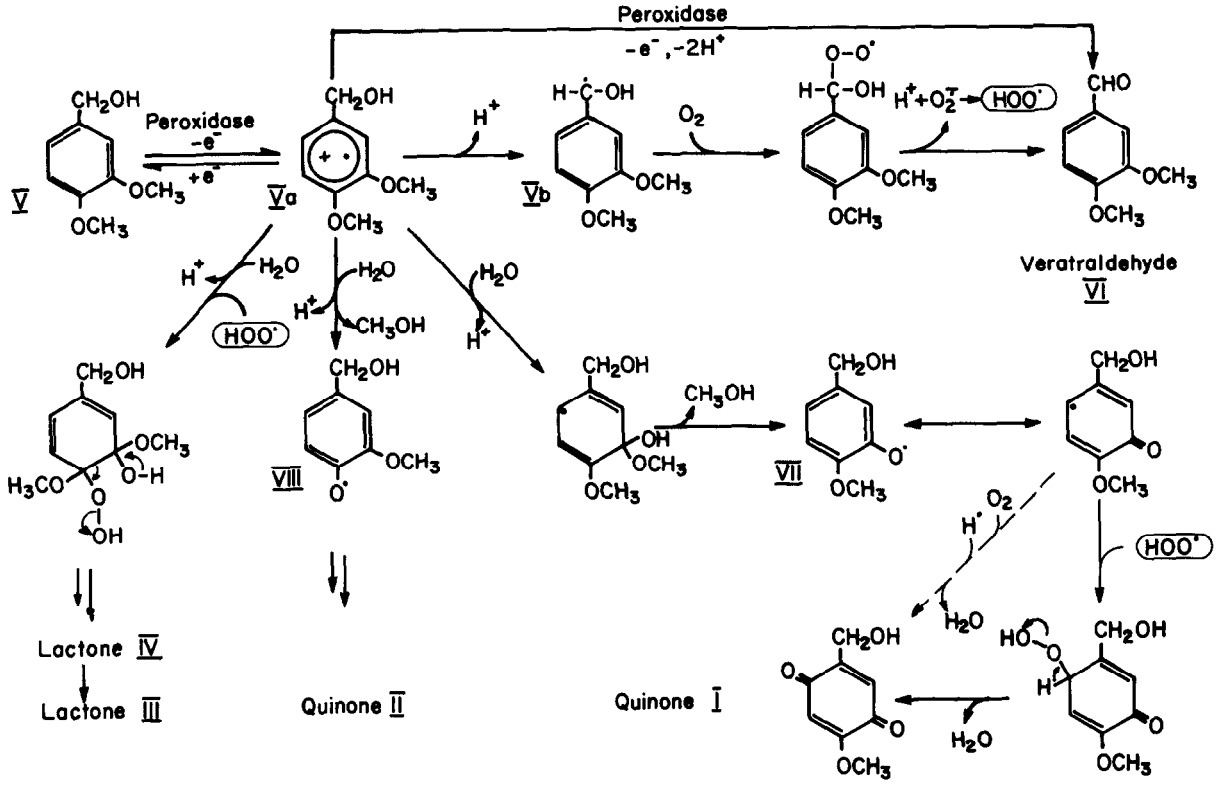

Fig.2. Possible reactions of the veratryl alcohol cation radical and a hypothetical mechanism for the formation of quinone I.

be directly oxidised to veratraldehyde (VI). Under aerobic conditions oxygen activation can occur. The superoxide anion formed will be protonated at the acidic pH of the reaction to the perhydroxyl radical. Reaction of the radical cation Va with a perhydroxyl radical and H_2O will then lead to aromatic ring opening and lactone formation. Alternatively, the radical cation Va can react with the nucleophilic solvent water, giving rise to the formation of at least two possible phenoxy radicals VII and VIII. These are then expected to react further to quinones I and II, respectively. A hypothetical scheme for the formation of quinone I is given in fig.2.

We conclude that during lignin peroxidase catalysis oxygen activation can occur. The perhydroxyl radical thus formed might be of importance in the microbial degradation of lignin itself. Aromatic ring cleavage and quinone formation from a non-phenolic substrate by a peroxidase can be explained by the involvement of activated oxygen species. The two quinones will further be of interest as intermediates in the metabolism of veratryl alcohol by *P. chrysosporium*.

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