# Single-electron transfer processes and the reaction mechanism of enzymic degradation of lignin

P.J. Harvey, H.E. Schoemaker+, R.M. Bowen and J.M. Palmer

*Department of Pure and Applied Biology, Imperial College, Prince Consort Road, London SW7 2BB, England* and *+Central Laboratory DSM, PO Box 18, NL-6160 MD Geleen, The Netherlands*

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The lignin-degrading, '$H_2O_2$-dependent oxygenase' from *Phanerochaete chrysosporium* oxidised veratryl alcohol without incorporating oxygen into the substrate. It also catalysed $\alpha$-$\beta$ cleavage of a non-phenolic diarylpropane substrate in the absence of oxygen. Studies with tris(phenanthroline)iron(III) established the importance of single-electron transfer in bringing about $\alpha$-$\beta$ cleavage. We propose that the lignin-degrading enzyme functions not as an oxygenase but as a peroxidase and that the oxidation reactions are brought about by initial single-electron transfer between the aromatic ring and a high redox oxy-ferryl active site in the enzyme.

*Lignin degradation* *Single-electron transfer* *Cα-Cβ bond cleavage* Phanerochaete chrysosporium *Peroxidase compound I*

## 1. INTRODUCTION

An enzyme has been isolated from *Phanerochaete chrysosporium* which will oxidize a wide variety of lignin-related aromatic substrates, including lignin and veratryl alcohol [1–5]. It is reported to require both $H_2O_2$ and oxygen to bring about the reaction.

In the accompanying Discussion Letter [6] we postulate that most of the reactions associated with the biodegradation of lignin can be explained on the basis of a single-electron transfer from the aromatic ring to a high redox potential centre. In this paper we show that single-electron transfer reagents such as $Fe(phen)_3^{3+}$ can bring about reactions similar to those of the enzyme without the involvement of $H_2O_2$ or oxygen. We suggest that the lignin-degrading enzyme does not work as an oxygenase but as a peroxidase, and that the oxidation reactions are mediated via single electron transfer.

*Abbreviation:* $Fe(phen)_3^{3+}$, tris(phenanthroline)iron(III)

## 2. MATERIALS AND METHODS

1,2-Di(3,4-dimethoxyphenyl)-1,3-propanediol was a gift from Dr R. Pryce, Shell, UK. $Fe(phen)_3^{3+}$ was prepared according to [7]. Far-UV grade acetonitrile was obtained from BDH.

Stationary cultures of *P. chrysosporium* ATCC 24725 (10 ml, 100 ml Erlenmeyer) were grown as in [1]; oxygen was supplied on days 3 and 7. Cultures (day 10) were filtered through muslin and centrifuged at 10000 × *g* for 15 min to remove mycelial and spore contaminants. The protein was precipitated using acetone [5], resuspended in 5 mM sodium tartrate pH 4.5, dialysed against water, lyophilized, redissolved in water and the insoluble material removed by centrifugation.

The assay for veratryl alcohol oxidase activity was as [3]; one unit of activity is defined as that amount of enzyme required to oxidise 1 $\mu$mol veratryl alcohol/min. Oxygen consumption and levels were determined using a Clark-type oxygen electrode.

Reaction products were analysed by HPLC (Kontron) on a 15 cm reverse-phase Spherosorb S5

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octyl column and guard using 25% acetonitrile/water and monitoring at 230 nm. Separated peaks were integrated, characterised on the basis of retention time (RT), and identity confirmed using appropriate standards.

Absorption spectra were obtained from samples in a 1 cm light path microcuvette in a Perkin Elmer 555 spectrophotometer.

## 3. RESULTS AND DISCUSSION

The lignin-degrading enzyme from *P. chrysosporium* has been reported to need both $H_2O_2$ and oxygen to oxidize aromatic substrates. The enzyme can catalyze the oxidation of veratryl alcohol to veratraldehyde [3]; this reaction does not necessitate the incorporation of oxygen into the products and could be achieved by the transfer of two hydrogen atoms from veratryl alcohol to the $H_2O_2$. In an attempt to determine the stoichiometry of the reaction, we supplied the enzyme with excess veratryl alcohol and limiting amounts of $H_2O_2$. Simultaneous measurements of veratraldehyde production and oxygen consumption showed that for every mole of $H_2O_2$ supplied, the reaction produced 1 mol of veratraldehyde and consumed only 0.1 mol of oxygen. Furthermore, the rate of veratryl alcohol oxidation was the same in a fully aerated medium (240 nmol oxygen/ml) as that in a medium gassed with argon, in which the oxygen level was determined to be lower than 1 nmol/ml; after 5 min of reaction time the cuvette contained over 100 nmol of veratraldehyde per ml. These results both indicated that oxygen was not consumed in substrate amounts, but do not exclude a catalytic role for oxygen in the reaction. It is therefore apparent that when oxidising veratryl alcohol the enzyme reacts as a peroxidase rather than an oxygenase.

Peroxidases mediate their reactions by oxidizing the haem with $H_2O_2$ to create a high redox potential centre, the oxo-iron(IV) porphyrin cation radical [8] (oxy-ferryl complex), which can then extract two electrons from the substrate via two single-electron transfer steps [9]. The absorption spectra presented in fig.1 show the changes in absorption bands when the enzyme is reacted with $H_2O_2$. The native enzyme has detectable absorption bands at 407, 500, 538, 574 and 626 nm. The addition of $H_2O_2$ causes bleaching at 407, 500 and

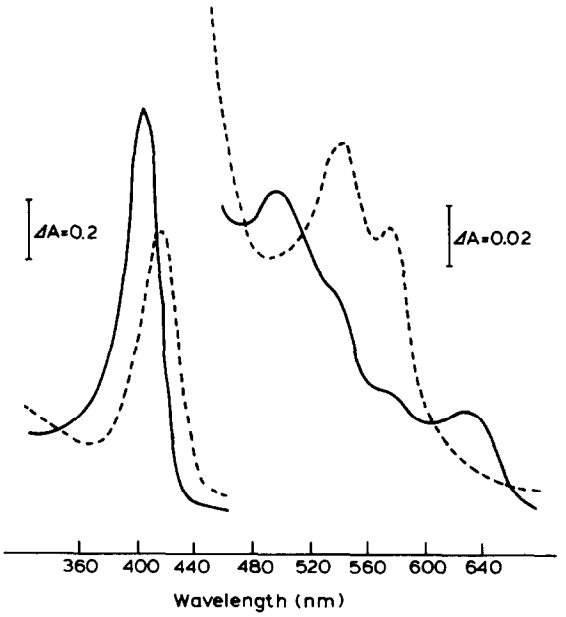


Fig.1. Absorbance spectra of the lignin-degrading enzyme (0.6 units/ml in 20 mM acetate pH 3.0) before (——) and after (– – –) the addition of 0.875 mM $H_2O_2$.

626 nm which is very similar to spectral changes associated with the formation of the oxy-ferryl complex in catalase [10] and is indicative of a similar process in the lignin degrading haem. The nature of the absorption peaks at 538 and 574 nm which are strongly enhanced by $H_2O_2$ is not known. They seem likely to be associated with a second haem since the protein used was not extensively purified. However, similar spectral features have been observed by Tien and Kirk [3] and Gold et al. [5], therefore the 538 and 574 nm bands could be additional features of the lignin-degrading enzyme.

There have been many reports [3,4,11] that when the enzyme degrades dimeric lignin model compounds, $^{18}O$ from labelled dioxygen is inserted onto the $\beta$-carbon of the substrate. Such observations have led to the view that the enzyme acts as an oxygenase. In an attempt to reconcile the latter observation with our own view that the enzyme acts as a peroxidase, we turned our attention to recent investigations using metal-centered oxidants such as ceric ammonium nitrate [12,13] and $Fe(phen)_3^{3+}$ complexes [7] which have established the importance of single-electron transfer as the

primary step in the oxidation of side chains in aromatic compounds. The data presented in fig.2A show the HPLC analysis of the products formed when $Fe(phen)_3^{3+}$ reacts with the 1,2-di(3,4-dimethoxyphenyl)-1,3-propanediol substrate. In the presence of oxygen (trace b), the substrate (RT 15 min) is completely degraded with the production of large quantities of veratraldehyde (RT 25 min). Trace c shows that similar products are formed when argon is used as the gas phase, although the amount of aldehyde produced is slightly less. Ceric ammonium nitrate in 75% acetonitrile gave the same results. Since the aldehyde is produced as a consequence of $\alpha$-$\beta$ cleavage, it is clear that one-electron oxidation will bring about $\alpha$-$\beta$ cleavage and that neither oxygen, $H_2O_2$ nor water are required for the cleavage reaction. Similar observations have been made by other workers, using diarylethanols [12] and diarylethanes [13]. Fig.2B shows data obtained using the isolated enzyme; trace e shows that in the presence of $H_2O_2$ and oxygen, the products formed are identical to those produced by $Fe(phen)_3^{3+}$ whilst trace f shows that in the argon-treated assay, although the rate of substrate oxidation was slightly reduced, the products were the same as those observed in the corresponding $Fe(phen)_3^{3+}$ system and indicated a substantial production of veratraldehyde. No products were observed in the absence of $H_2O_2$.

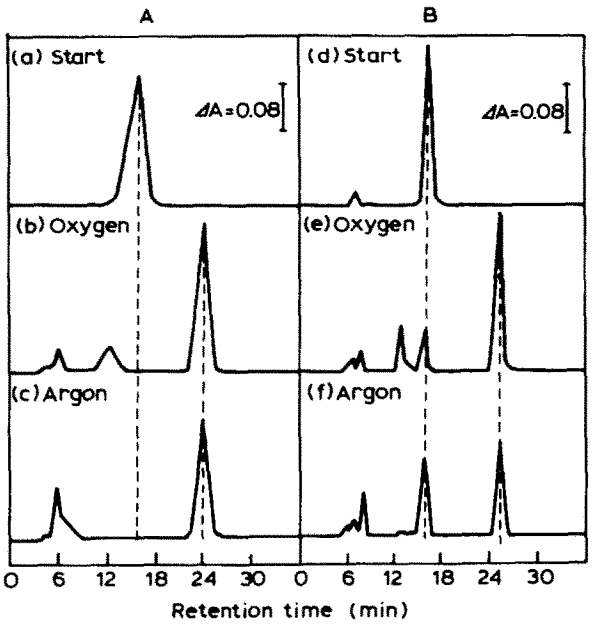


Fig.2. HPLC analyses of the products of the reaction between 1,2-di(3,4-dimethoxyphenyl)-1,3-propanediol and (A) $Fe(phen)_3^{3+}$, (B) the lignin-degrading enzyme, monitored at 230 nm. In (A) the reactants (1 mM in 100% acetonitrile) were incubated at 25°C under the appropriate gas phase and then analysed, in (B) 0.04 units of enzyme, 0.4 mM substrate and 0.3 mM $H_2O_2$ in 20 mM acetate pH 3 were incubated at 37°C under appropriate gas phases, then analysed.

Recently $\alpha$-$\beta$-cleavage of a diarylpropane substrate has been demonstrated using the metalloporphyrin tetraphenylporphyrinatoiron(III) chloride in the presence of *tert*-butyl hydroperoxide or iodosylbenzene [14]. This system is considered to model the oxy-ferryl complexes of the monooxygenase cytochrome P-450 and of peroxidases [8]. Since the data presented here show that the high redox complex $Fe(phen)_3^{3+}$ also brought about $\alpha$-$\beta$ cleavage without involvement of oxygen, we consider that the essential feature of the oxy-ferryl complex in the lignin-degrading enzyme is that of a high redox potential, sufficient to bring about single-electron transfer. We propose that when oxidizing diarylpropane substrates, the enzyme extracts a single electron from the aromatic ring of the substrate via the high potential oxy-ferryl complex created with $H_2O_2$. A cationic radical is produced in the substrate, which consequently cleaves at the $\alpha$-$\beta$ bond to yield a cation from the $\alpha$-carbon moiety and a benzyl free radical in the other half of the molecule. The cation immediately deprotonates to yield the aldehyde as one product. The radical ($\beta$-carbon) would react at a diffusion-controlled rate [15] with molecular oxygen, if present, to produce a peroxy radical; this explains the observed insertion of $^{18}O$ from labelled dioxygen onto the $\beta$-carbon [3,4]. The fate of the peroxy radical can be complex. Two peroxy radicals could, however, interact to produce one molecule of phenylglycol and one molecule of ketol [16], both of which have been detected as degradation products from the $\beta$-1 model compounds [1–5].

In conclusion we believe that the lignin-degrading enzyme mediates single-electron transfer between aromatic rings and a high potential oxy-ferryl haem complex in the enzyme. This process produces a cationic radical in the substrate; the nature of subsequent products will depend on the

nature of the substituents in the aromatic ring. This protein could act as an endo-ligninase and its mode of action could account for many of the products produced during the biodegradation of lignin. The chemical justification for this view is presented in the accompanying Discussion Letter [6].

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