Discussion Letter

On the mechanism of enzymatic lignin breakdown

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The recent discovery of an extracellular enzyme from *Phanerochaete chrysosporium* capable of degrading lignin model compounds has countered the view that reactive diffusible oxygen species are responsible for lignin biodegradation. In this paper we propose a mechanism which accounts for both the results obtained in enzymatic lignin degradation studies and in studies involving active oxygen species. The quintessence of the mechanism is initial one-electron oxidation of the lignin model compounds or of a specific lignin subunit followed by subsequent breakdown reactions via radical cation intermediates. The implication of this type of mechanism on the oxidative biodegradation of the natural lignin polymer is discussed.

Lignin degradation Phanerochaete chrysosporium *Single-electron transfer* *Radical cation* *Peroxidase compound I* *Active oxygen*

1. INTRODUCTION

Recent research using the white-rot fungus *Phanerochaete chrysosporium* has made rapid progress towards elucidating the molecular mechanism of lignin biodegradation. Efforts have been focussed both on the role of diffusible oxygen species such as hydroxyl radicals [1–6], superoxide anions [2,3] and singlet oxygen [2,5,6] and, following the isolation of a lignin-degrading H_2O_2-dependent oxygenase [7–11], on the influence of enzyme-bound active oxygen species [12]. This enzyme is reported to exhibit a broad specificity for aromatic substrates and will catalyse a variety of reactions in lignin models such as $C\alpha$-$C\beta$ cleavage, oxidation and hydroxylation of benzylic methylene groups, hydroxylation of olefinic groups in styrenes, oxidation of benzylic alcohols and oxidation of phenols leading to radical coupling [9]. In this paper we propose that oxidation of the substrate by initial one-electron transfer to yield radical cation intermediates provides a unifying explanation for the variety of reagents causing depolymerisation of model compounds and the wide variety of reactions catalysed by the lignin-degrading enzyme.

Abbreviations: CAN, ceric ammonium nitrate; $Fe(phen)_3^{3+}$, tris(phenanthroline)iron(III); PhIO, iodosylbenzene; TPP(Fe)Cl, tetraphenylporphyrinatoiron-(III)chloride

2. MECHANISM OF $C\alpha$-$C\beta$ CLEAVAGE IN LIGNIN MODEL COMPOUNDS. A PROPOSAL

Studies with the lignin-degrading enzyme have shown that the model compound 1,2-di(3,4-dimethoxyphenyl)-1,3-propanediol (1 in scheme I) is oxidised via $C\alpha$-$C\beta$ bond cleavage to yield veratraldehyde 2 and lesser amounts of phenylketol 4 and phenylglycol 3 [9]. In the course of oxidation, ^{18}O from dioxygen is incorporated at the $C\beta$ position, and deuterium is retained at the $C\alpha$ and $C\beta$ positions [9,13,14]. $C\alpha$-$C\beta$ cleavage has also been observed in related β-1 compounds which lack either the $C\alpha$ or the $C\gamma$ hydroxyl groups [9,15], in those with a benzylic ether substituent [14] as well as in β-*O*-4 [9,15] and dianisyl models [16].

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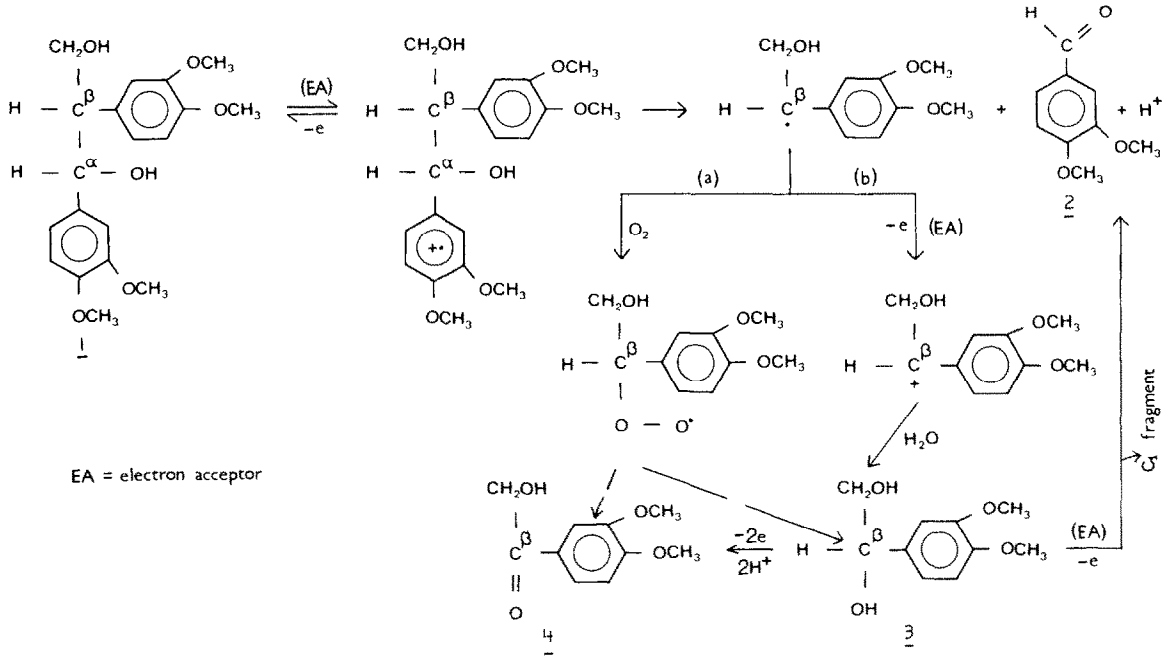
In the accompanying paper [17] we show that the one-electron oxidants CAN and $Fe(phen)_3^{3+}$ will oxidise model 1 to yield the same products as those observed for the enzyme-catalysed reaction. We propose that the initial reaction leading to the degradation of this model is a single-electron transfer from the methoxylated aromatic ring to a high redox potential centre (EA in scheme I), yielding a radical cation in the substrate. Chemical studies concerned with the oxidation of aromatic side chains and with the chemistry of radical cations [18–23] predict that the radical cation intermediate of model 1 would undergo C-C bond cleavage to yield radical and cationic fragments (scheme I). Veratraldehyde 2 is obtained after deprotonation of the Cα-derived cation fragment. The benzylic radical from the Cβ moiety can undergo a variety of reactions and in scheme I we present two possibilities. The first of these (pathway a) illustrates the reaction with dioxygen to produce a peroxy radical [24] which subsequently gives rise to phenylglycol 3 and/or phenylketol 4. The second (pathway b) shows further one-electron oxidation to yield a cation and subsequent hydration with water to produce phenylglycol 3. The phenylglycol has side chains which are electron supplying, therefore further one-electron oxidation to produce a radical cation is predicted [22]. When possible, the most characteristic reaction of alkyl aromatic radical cations is that of proton loss to yield a benzylic radical. However, C-C bond cleavage may occur if this will lead to relatively stable radical and cationic fragments [22]. We propose, therefore, that the phenylglycol radical cation undergoes two competing reactions of both proton loss and C-C bond cleavage. Proton loss ensues in production of the phenylketol 4; C-C bond cleavage yields a second molecule of veratraldehyde 2 and a C1 fragment. This scheme, involving one-electron oxidation processes and production of radical cations, would thus predict the outcome of products observed and would also allow for the observed labelling patterns [9,13,14].

Photosensitising riboflavin [6] and the one-electron oxidants CAN and $Fe(Phen)_3^{3+}$ [17] would act as suitable electron acceptors (EA) in scheme I. The redox potential of the $Fe(Phen)_3^{3+}$ complex is, however, less than that of the methoxylated



Scheme I.

aromatic ring [25], but irreversible C-C cleavage, which follows electron abstraction, would shift an unfavourable equilibrium towards the formation of products allowing the reaction to proceed to completion [17]. More powerful oxidants such as hydroxyl radicals will also induce formation of aromatic radical cations [20]: hydroxyl radicals have been shown to degrade model 1 to yield, in addition to various other unidentified products (these additional unidentified products could be generated by direct attack by hydroxyl radicals on the aromatic substrate via path e in scheme II, and path d in scheme III), the same products as those of the enzyme-mediated reaction and the same labelling patterns [14,26].

Recently, Shimada et al. [12] have shown that an oxy-ferryl complex (FeIV-O·) generated from TPP/PhIO will also bring about $C\alpha$-$C\beta$ cleavage in non-phenolic β-1 model compounds. Data presented in the accompanying experimental paper [17] show spectral evidence for the formation of an oxy-ferryl centre in the active site of the lignin-degrading enzyme. However, the enzyme degraded model 1 without the use of dioxygen. Since $Fe(phen)_3^{3+}$ also degraded model 1, but without the necessity for either H_2O_2, O_2 or H_2O, it is apparent that an oxygenated iron centre is not a prerequisite for the reaction to occur. Therefore we propose that the FeIV-O· centre in the enzyme acts as a one-electron transfer reagent, as in peroxidases, rather than as an activated oxygen transfer reagent as in oxygenases. Thus we argue that hydroxyl radicals, one-electron oxidants and the active site of the enzyme all degrade lignin through the common mechanism of single-electron transfer from the substrate to create a radical cation.

3. OTHER REACTIONS RESULTING FROM SINGLE-ELECTRON TRANSFER IN LIGNIN MODEL COMPOUNDS

The formation and reactions of aromatic radical cations in solution have received keen attention and a reasonably coherent picture has now emerged of the various paths by which aryl side chains may be degraded via initial radical cation intermediates [22]. By extending our model, we can provide a rational explanation for the variety of different chemical reactions catalysed by the lignin-degrading enzyme.

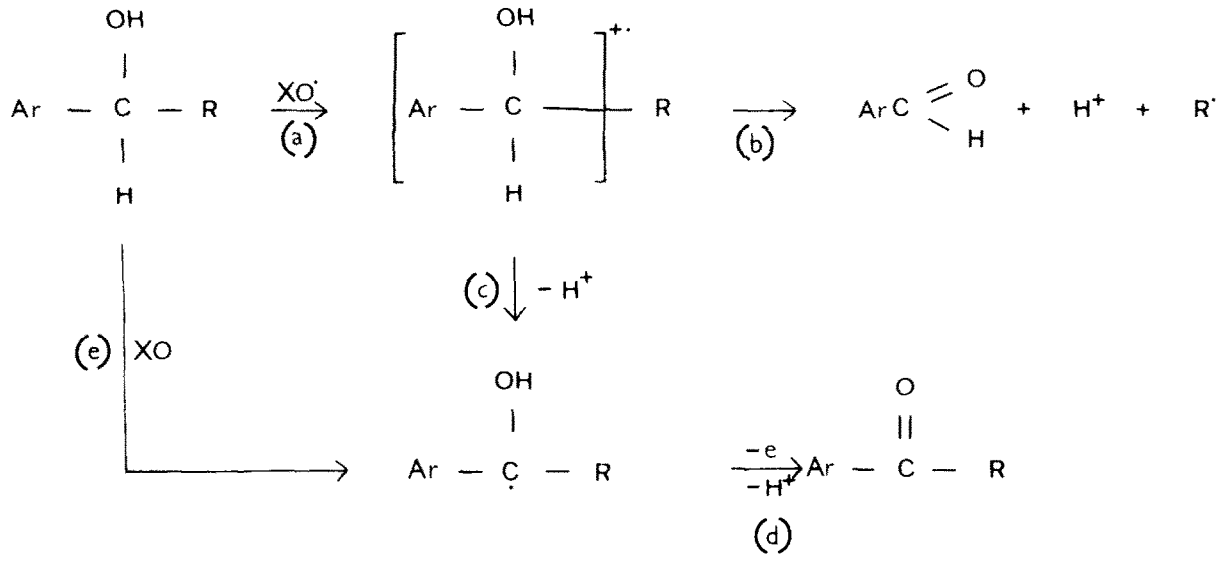
3.1. *Oxidation and hydroxylation of the benzylic carbon*

From studies of the oxidation of aryl-alkyl and aryl-benzyl carbinols [18,20,21,23], it has been observed that the radical cation intermediates of these compounds will fragment via two pathways (see scheme II), depending on the stability of the radicals and ions formed, as well as on reaction conditions. When R is secondary, tertiary or benzylic, C-C bond cleavage is predominant (pathway b) because relatively stable ions and/or neutral molecules and radical fragments can be formed. When R is primary, however, proton loss is favoured (pathway c). These two alternative pathways explain why the oxidation of β-*O*-4 ether models yields both products derived from $C\alpha$-$C\beta$ cleavage as well as a ketone derivative from $C\alpha$ oxidation [9]; they also explain why the oxidation of veratryl alcohol yields veratraldehyde, a reaction frequently used as an assay for the enzyme [9].

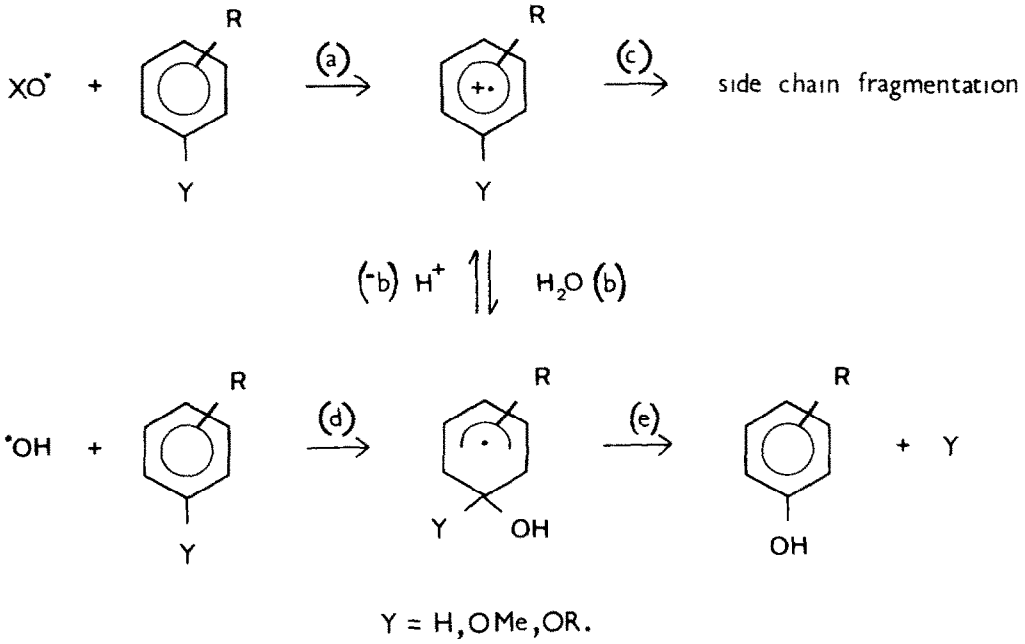
Additional studies [19,23] have shown that whilst substituents such as the hydroxyl group in scheme II greatly accelerate the C-C cleavage reaction, radical cations of compounds in which the α-hydroxyl group is replaced with an α-hydrogen will fragment mainly by proton loss (pathway c) to yield a relatively stable benzyl radical. Further one-electron oxidation of the radical followed by hydration with water results in hydroxylation at the benzylic position, another reaction the enzyme will catalyse [9,15].

3.2. *Formation of free phenolics and demethoxylation*

Walling et al. [27] have shown that radical cations of a variety of aromatic molecules may also reversibly hydrate (pathway b in scheme III): in the presence of suitable oxidants, phenolic products may be obtained (pathway e in scheme III). Direct hydroxylation via hydroxyl radicals will also yield phenolic products (pathway d). Since Y can be a methoxy, or an aryl glycerol group, this scheme provides a mechanism for the demethoxylation [28,29] and ether cleavage [30] reactions observed with either the enzyme or hydroxyl radicals. The enzyme acts like a peroxidase with phenolic substrates [9], therefore it will also catalyse oxidative coupling, dimerisation [9], $C\alpha$-arene cleavage [31] and decarboxylation [32] of any phenolic products obtained.



Scheme II.



Scheme III.

At this point it is pertinent to note that when oxidising β-1 models, there is little evidence for the formation of free phenol groups. However, various authors [20,22,23,27] have observed that phenolics could not be obtained via aromatic radical cations of either methoxylated benzyl alcohols or of alkyl-substituted aromatics in acidic media. This suggests that the failure to yield free phenols may be an intrinsic property of the system being studied. Such a view is supported by the data

of Shimada et al. [12], where no evidence was obtained for the formation of phenolics during the breakdown of a non-phenolic β-1 dimer by the TPP(Fe)Cl/PhIO system, despite the fact that this system, considered to model the mono-oxygenase cytochrome P-450, will demethylate anisole to phenol [33].

4. ROLE OF RADICAL CATIONS IN LIGNIN DEGRADATION

Aromatic radical cations may also act as electron transfer agents [23]. We propose, therefore, that lignin degradation products in the form of radical cations, produced either enzymatically or by direct interaction with hydroxyl radicals, may act as electron transfer agents to induce the formation of radical cations in the 'remote' lignin structure, thus causing degradation in polymers not accessible to the large enzymes located on the hyphal surface.

5. CONCLUSION

It is apparent that one-electron oxidation of lignin model compounds by a high redox potential centre to form radical cations, followed by side chain fragmentation, can adequately account for most of the complex reactions associated with lignin biodegradation. This proposal unifies the complex data already available and forms the basis for the design of further experiments on the catalytic potential of the enzyme and the reaction mechanism of haem proteins such as peroxidases and cytochrome P-450 [34].

It is to be hoped that this proposal will lead to future research which will provide more detailed information concerning the actual mechanism of lignin breakdown and enable us to put the insight gained into practical use. We are actively pursuing the ramifications of this proposal.

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