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Journal of Molecular Catalysis B: Enzymatic 22 (2003) 135–144
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Free radical versus electron-transfer routes of oxidation of hydrocarbons by laccase/mediator systems Catalytic or stoichiometric procedures

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Received 22 July 2002; received in revised form 28 January 2003; accepted 31 January 2003

Abstract

The oxidation of C–H bonds in alkylarenes can take place by dioxygen, under catalysis by the phenol-oxidase enzyme laccase, provided that suitable mediator compounds are added. The actual oxidation of the substrate is carried out by the oxidised form of the mediator, in a non-enzymatic step. The relative efficiency of four >N–OH-type mediators (HBT, HPI, VLA, NHA) has been evaluated, and compared with that of the structurally different mediator ABTS. Laccase/mediator catalysed oxidations of non-phenolic substrates can proceed via two different mechanisms. Either on monoelectronic oxidation, by the oxidised form of mediator ABTS, or, by abstraction of hydrogen atom, by a >N–O$^{\bullet}$ radical species derived from the >N–OH-type mediators. The former mechanism requires substrates with a low oxidation potential; the latter mechanism requires substrates with relatively weak C–H bonds. Electrochemical and thermochemical evidence is provided (i) to explain the failure in the oxidation of specific alkylarenes and (ii) in support to the rationalisation of the experimental findings. Particular emphasis is given to discuss the effect of the mediator-to-substrate molar ratio upon the efficiency of the oxidation procedure. In order to explain why, in previous literature studies, better results may have been obtained by using the mediator in more that stoichiometric amounts, we propose the concurrent formation of degradation products from the mediator, which could be responsible for the onset of alternative oxidation pathways. A better understanding of the natural role of laccase in the oxygen-dependent degradation of lignin in wood emerges from this study.

Keywords: Laccase; Mediators; Electron-transfer; H-atom abstraction; Hydrocarbons oxidation; Lignin degradation

1. Introduction

Oxidation of the C–H bonds of hydrocarbons is one of the most important, albeit difficult, synthetic task in organic chemistry [1]; it enables the transformation of raw components of oil into synthetically useful intermediate compounds. Either strong oxidants, in electron-transfer (ET) steps, or radical reagents, in H-abstraction steps, need to be applied in

Abbreviations: HBT, 1-hydroxybenzotriazole; HPI, *N*-hydroxyphthalimide; VLA, violuric acid; NHA, *N*-hydroxyacetanilide; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) salt

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1381-1177/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S1381-1177(03)00014-6

such transformations [1,2], due to obvious problems arising from the high redox potential of the hydrocarbon substrates or the high energy of their C–H bonds. However, it is not the purpose of the present paper to address this field of organic synthesis. In another context, the activation of molecular oxygen for insertion into suitable substrates is a problem of catalysis that has a fundamental importance for aerobic life. Some enzymes are able to carry out the oxidation of C–H bonds, notably among them being the cytochrome P450 enzymes [1,3]. Once again, either electron-transfer or radical routes can be followed by the enzymes, depending on structural properties of the substrate.

The case of the enzyme laccase presents distinct peculiarities. Laccase is among the ligninolitic enzymes that are excreted by white-rot fungi during the oxygen-dependent degradation of wood [4]. In view of its low redox potential (ca. 0.6–0.8 V), laccase is restricted to react with the easily oxidisable phenolic fragments of lignin (i.e. phenol-oxidase activity) [4–6]. However, the inclusion of low molecular-weight oxidisable substances [7], known as mediators, expands the catalytic activity of laccase towards different, more difficult to oxidise functional groups, including benzyl and allyl alcohols or ethers [8–12], which are largely represented in the structure of lignin. In few cases [13–15], the oxidation of alkylarenes and polycyclic aromatic compounds has been even reported, this providing a remarkable example of C–H bond oxidative functionalisation through laccase/mediator systems. We have taken inspiration from those investigations [13–15], and tried to expand the approach to additional substrates, as well as to provide a more satisfactory mechanistic rationale to the mediation phenomenon. Our results from an oxygen-dependent oxidation of a number of alkylarenes or polycyclic hydrocarbons, with laccase and its most efficient mediators [12], are herein described.

2. Experimental procedures

2.1. Enzyme preparation

Laccase from a strain of *Trametes villosa* (viz. *Poliporus pinsitus*) (Novo Nordisk Biotech) was employed. It was purified by ion-exchange chromatography on Q-Sepharose by elution with phosphate buffer [12], and an activity of 9000 U/ml determined spectrophotometrically by the standard reaction with ABTS [16].

2.2. Materials

All the substrates and solvents were commercially available (Aldrich). NHA was synthesized according to a multistep reaction [17]; mp 63–64 °C (literature [17] 66–67 °C). Its ^{1}H NMR spectrum (200 MHz): δ in $CDCl_3$ 8.9 bs, 7.5–7.3 m., 2.1 s, matched the one reported [17].

2.3. Enzymatic reactions

The oxidation reactions were performed at room temperature in stirred water solution (3 ml), buffered at pH 5 (0.1 M in sodium citrate), that was purged with O_2 for 30 min prior to the addition of the reagents [12]. In general, 60 μmol of substrate (dissolved in 120 μl of MeCN) were incubated with 15 U of laccase and 20 μmol of mediator in 3 ml buffered water solution, giving the following initial concentrations: [substrate], 20 mM, [mediator], 6 mM, but different initial conditions were also tested. The reaction time was 24 h, an atmosphere of oxygen being kept throughout this time in the reaction vessel. A 1:1 aqueous-buffer:dioxane mixed solvent (always 3 ml in total volume) was alternatively used, in order to ensure a better solubility of the substrates. In some cases, the oxidation was carried out at 45 °C. At the end of the reaction, and following a conventional work-up with ethyl acetate, the molar amount of oxidation product was determined by GC analysis with respect to an internal standard (acetophenone or *p*-methoxyacetophenone), suitable response factors being determined from authentic products; the oxidation yields (Table 1) were calculated with respect to the initial amount of substrate. A VARIAN CP 3800 instrument, fitted with a 30 m × 0.25 mm methyl silicone gum capillary column, was employed in the GC analyses. The identity of the products was confirmed by GC–MS analyses, run on a HP 5892 GC, equipped with a 30 m × 0.2 mm 5% phenyl–methyl silicone gum capillary column, and coupled to a HP 5972 MSD instrument, operating at 70 eV, and by comparison with available authentic samples.

Table 1
Oxidations with five laccase/mediator systems: products and yields

Run	Substrate	Product(s)	Products yield (%) versus substrate[a]				
			HPI	HBT	VLA	ABTS	NHA
1	4-MeO-$C_6H_4CH_2CH_3$	4-MeO-$C_6H_4CH(OH)CH_3$	4 (74)[b]	8 (56)[b]	1 (68)[b]	0 (92)[b]	1 (81)[b]
		4-MeO-$C_6H_4C(O)CH_3$	11	20	1	0	2
2	$C_6H_5CH_2CHMe_2$	$C_6H_5CH(OH)CHMe_2$	1 (72)[b]	1 (72)[b]	0 (55)[b]	0 (87)[b]	0 (78)[b]
		$C_6H_5C(O)CHMe_2$	3	3	0	0	0
3	4-Me-$C_6H_4CHMe_2$	4-(Me_2CH)-C_6H_4CHO	1 (65)[b]	1 (65)[b]	0 (84)[b]	0 (84)[b]	0 (81)[b]
		4-Me-$C_6H_4C(OH)Me_2$	8	15	0	0	0
4	1-Me-naphthalene	1-CH_2OH-naphthalene	8 (38)[b]	2 (65)[b]	0 (95)[b]	–	–
		1-CHO-naphthalene	5	5	0		
5	Fluorene	9-Fluorenolo	2 (102)[b]	0 (105)[b]	–	–	1 (98)[b]
		9-Fluorenone	6	2			2
6	Anthracene	No product(s) found	(85)[b]	(95)[b]	(93)[b]	–	(90)[b]

Reaction conditions: [substrate], 20 mM, [mediator], 6 mM, in 0.1 M citrate buffer (pH 5) containing 4% MeCN, with 15 U laccase at room temperature for 24 h.

[a] Typical uncertainty of the GC determinations: ±4%.

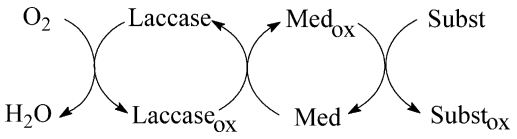
[b] Substrate recovered.

3. Results

3.1. Reactivity of substrate

Alkylarene precursors, not exclusively phenyl derivatives, featuring not only primary benzylic C–H bonds [13,14] but also secondary, tertiary as well as aliphatic C–H bonds, were chosen for our laccase-catalysed oxidations, besides two polycyclic aromatic hydrocarbons [15]. A first set of experiments was performed with HBT (1-hydroxybenzotriazole), HPI (N-hydroxyphthalimide), and VLA (violuric acid) as mediators: all these N–OH-type compounds had performed efficiently with laccase in the oxidation of *p*-methoxybenzyl alcohol, taken as a simple model for α-oxidation of the lignin structure [12]. Another N–OH-type mediator investigated here was NHA (N-hydroxyacetanilide) [18,19], that needed to be synthesized. Also employed was ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) salt), which is not a N–OH derivative but a well known mediator of laccase [9,20–22]; it had performed less well in the benchmark oxidation of *p*-methoxybenzyl alcohol [12]. The experimental conditions investigated, namely, [substrate], 20 mM, [mediator], 6 mM, with 5 U/ml of laccase at room temperature for 24 h, were such as to comply with the 'ideal' scheme of a mediated oxidation [23] (Scheme 1). The mediator, in deficiency with respect to the substrate, turns over between its natural and oxidised (Med_{ox}) states, due to the intervention of laccase and oxygen, while carrying out the oxidation of the substrate in a catalytic cycle.

According to Scheme 1, the role of the enzyme is to oxidise the mediator, whereas the actual oxidation of the substrate takes place in a subsequent non-enzymatic step by the action of the Med_{ox} species. It is therefore of primary importance to understand the nature of the reaction mechanism operating in the oxidation of a substrate by the Med_{ox} species derived from the corresponding mediator investigated. In the laccase-depending oxidation of non-phenolic substrates, such as the benzyl alcohols or ethers, previous evidence suggests an electron-transfer (ET) mechanism [18,19,23] with mediator ABTS [9,20,21], towards substrates having a low oxidation potential. Alternatively, a radical hydrogen atom transfer (HAT) route may operate with N–OH-type mediators [10,12,24], if weak C–H bonds are present in the substrate. Could this dichotomy oc-



Scheme 1.

cur also in the oxidation of alkylarenes as substrates by laccase/mediator systems?

Blank experiments were performed (not shown) with the alkylarenes reported in Table 1, and confirmed the expected lack of reactivity of laccase alone with these non-natural substrates [5], but also the lack of oxidation with the mediator alone. In these cases, the substrate was quantitatively recovered (>97%), showing that no auto-oxidation processes [25] concur to the formation of products in the laccase-catalysed reactions. The yield of oxidation product(s) and the amount of substrate recovered in the laccase-catalysed experiments are reported in Table 1; no other oxidation products, besides those indicated, were detected.

Inspection of Table 1 shows that only HPI and HBT are effective mediators of laccase in the oxidation of the substrates. The best results are obtained with 4-ethylanisole and *p*-cymene (*p*-methylisopropylbenzene) when HBT is used as mediator. We do not confirm the oxidation of the alkyl group [13] of alkylarenes by the laccase/ABTS system (runs 1 and 3 in Table 1), nor that of anthracene [15].

3.2. Effect of 'natural' mediators

Some 'natural' compounds, such as phenol and aniline derivatives, were recently reported to mediate laccase efficiently in the oxidation of a number of aromatic hydrocarbons, including fluorene and anthracene [15]. A key point is that the mediator was not employed in deficiency, at variance with our present case, but mediator-to-substrate molar ratios as high as 40, or even 200, were used [15]. We wanted to repeat some of those experiments under our 'ideal' mediation conditions, taking *p*-cymene as the reference substrate, because it had reacted satisfactorily with the previous set of mediators (Table 1), whereas fluorene and anthracene had reacted poorly. The results are presented in Table 2.

4-Hydroxybenzyl alcohol had been described as an effective mediator [15], and 3-hydroxyanthranilic acid (HAA), that is structurally both a phenol and an aniline, had been reported to mediate the oxidation of non-phenolic lignin structures by laccase from the fungus *Pycnoporus cinnabarinus* [26]. Our results showed that neither of the two laccase/mediator systems provides any appreciable oxidation of substrate *p*-cymene. Equally ineffective were the two anilines [15] of Table 2, including natural compound 2-amino-purine. We suggest that what made these or similar phenols/anilines to behave as mediators of laccase was the large mediator-to-substrate ratio adopted [15] (*vide infra*), rather than the fact of being 'natural' substances.

3.3. Effect of the substrate:mediator ratio

To address this crucial point, HBT was selected as the mediator, being it the most efficient among those reported in Table 1. It was tested in a 1:1 molar ratio with substrates 4-ethylanisole or *p*-cymene, in combination with laccase. The solvent was the 0.1 M citrate buffer (pH 5) containing 4% MeCN. Because problems of solubility began to appear, we shifted to the 1:1 aqueous-buffer:dioxane mixed solvent (hereafter, 50%-dioxane), where we could extend the study to additional substrates because solubility became good. Independent information ensures that the activity of laccase is preserved in this mixed solvent [10,27]. A few oxidations were also carried out [15] by employing laccase with a 40:1 HBT:substrate molar ratio, in the 50%-dioxane mixed solvent. The results are summarized in Table 3. The yields are expressed in mol% per substrate.

A comparison of the results in Tables 1 and 3 shows that only minor differences are observed between the oxidations in two mixed solvents, besides the solubility advantage in 50%-dioxane. It can be also observed that the change in mediator:substrate molar ratio, from 1:3 (in Table 1) to 1:1 (Table 3) causes an appreciable increase in the total yield of oxidation products, e.g., from 28 to 71 mol% (combined yields of alcohol and ketone) for the oxidation of 4-ethylanisole, and from

Table 2
Oxidations of *p*-cymene with laccase and 'natural' mediators

Mediator	Product	Substrate recovered (%)
4-Hydroxybenzyl alcohol	–	97
3-Hydroxyanthranilic acid	–	96
4-*t*-Butyl-aniline	–	97
2-NH_2-purine	–	97

Reaction conditions: [substrate], 20 mM, [mediator], 6 mM, in 0.1 M citrate buffer (pH 5) containing 4% MeCN, with 15 U laccase at room temperature for 24 h. Experimental error of GC determinations: ±4%.

Table 3
Oxidations with the laccase/HBT system, at both 1:1 and 40:1 mediator:substrate molar ratios, in two different mixed solvents

Run	Substrate	Product(s)	Yield of products (%)		
			1:1[a,b]	1:1[a,c]	40:1[a,c]
1	4-MeO-$C_6H_4CH_2CH_3$	4-MeO-$C_6H_4CH(OH)CH_3$	16 (8)[d]	10 (38)[d]	0 (0)[d]
		4-MeO-$C_6H_4C(O)CH_3$	55	54	79
2	4-Me-$C_6H_4CHMe_2$	4-(Me_2CH)-C_6H_4CHO	1 (42)[d]	3 (84)[d]	0 (71)[d]
		4-Me-$C_6H_4C(OH)Me_2$	25	18	5
3	$C_6H_5CH_2CHMe_2$	$C_6H_5CH(OH)CHMe_2$	–	1 (87)[d]	–
		$C_6H_5C(O)CHMe_2$	–	2	–
4	1-Me-naphthalene	1-CH_2OH-naphthalene	–	0 (86)[d]	0 (63)[d]
		1-CHO-naphthalene	–	10	35
5	Fluorene	9-Fluorenol	–	0 (43)[d]	0 (0)[d]
		9-Fluorenone	–	58	93
6	Anthracene	Anthraquinone	–	3 (98)[d]	54 (33)[d]

[a] HBT:substrate molar ratio.
[b] In buffered water containing 4% MeCN.
[c] In the 50%-dioxane mixed solvent.
[d] Recovered substrate.

2 to 58 mol% for the oxidation of fluorene, depending on the optimum reaction conditions. In the oxidation of 4-ethylanisole, the corresponding α-carbonyl product is predominantly obtained over the corresponding α-hydroxyl product. This is reasonable, since the latter is the product of the first oxidation step, and is more readily oxidisable than the CH_2 group in precursor 4-ethylanisole [1,28]. In the oxidation of *p*-cymene (run 2), in contrast, the corresponding tertiary α-hydroxyl compound is preferentially produced over the corresponding benzaldehyde derivative. Evidently, the α-CH group in the 4-isopropyl group is appreciably more susceptible to oxidation by the laccase/HBT system than the α-CH_3 group of the substrate. Moving to the 40:1 HBT:substrate molar ratio, the oxidation of fluorene and 4-ethylanisole becomes nearly quantitative, and the oxidation yields of 1-methylnaphthalene and anthracene (runs 4 and 6) are substantially increased. Thus, these results verify the claim of Johannes and Majcherczyk [15]. A possible explanation of this finding will be advanced later. Moreover, two additional oxidation experiments were performed using laccase and 'natural' mediator HAA, with the mediator:substrate molar ratio of 40:1 in 50%-dioxane. The substrate was 4-ethylanisole for the first reaction, and *p*-cymene for the second. None of the expected oxidation products were detected (compare with runs 1 and 2 in Table 3, respectively), in spite of an high consumption of the substrate (7–8% recovery). Because in many experiments performed under high mediator:substrate molar ratio conditions [15], the only given evidence for the progress of the oxidation reaction was the amount of recovered substrate, and no information on product(s) structure nor yield was added, it is possible to advance the hypothesis that the substrate was consumed in undesired side-reactions of unknown nature.

3.4. Effect of the pH and of the reaction temperature

Laccase-catalysed oxidations proceed preferentially in a medium of moderate acidity [5]. For the laccase/HBT system, a search for the optimum pH was made in the oxidation of *p*-cymene in 50%-dioxane. The reaction time was extended from 24 to 72 h, and the results are reported in Table 4, where 'alcohol' stands for the tertiary alcohol 4-Me-$C_6H_4C(OH)Me_2$ and 'aldehyde' for 4-(Me_2CH)C_6H_4CHO; the substrate recovered is also given. The optimum pH for the oxidation of this non-phenolic substrate was around 5, in keeping with other literature data [18]. When the pH is over 7, the laccase activity is drastically decreased [5,29]. Therefore, oxidations with laccase/mediator systems should not be carried out under alkaline conditions [30].

Table 4
Effect of a change of pH in the laccase/HBT oxidation of *p*-cymene in 50%-dioxane: yield of products

Substrate[a]	Yield of products at		
	pH = 3.5	pH = 5	pH = 7
p-Cymene	Alcohol, 18% Aldehyde, 3% Recovered substrate, 65%	Alcohol, 26% Aldehyde, 3% Recovered substrate, 62%	Alcohol, 0% Aldehyde, 0% Recovered substrate, 81%

[a] In a 1:1 molar ratio with mediator HBT. The yields are calculated versus the initial amount of substrate.

Table 5
Oxidation of 4-ethylanisole at 45 °C with the laccase/HBT system: yield of products

Substrate[a]	Yield of product in	
	50% Dioxane	50% Dioxane[b]
4-Ethylanisole	Alcohol, 2% Ketone, 7% Recovered substrate, 90%	Alcohol, 1% Ketone, 3% Recovered substrate, 98%

[a] In a 1:1 molar ratio with mediator HBT; 27 U of laccase were used. The yields are calculated versus the initial amount of substrate.
[b] With 15 U of laccase.

Similarly, the effect of the reaction temperature was tested in the laccase/HBT oxidation of 4-ethylanisole at 45 °C (Table 5), employing a 1:1 mediator:substrate molar ratio and the 50%-dioxane mixed-solvent. A larger amount of laccase (27 U) was needed, because a poorer conversion was obtained with the usual amount (15 U). Once again 'alcohol' stands for product 4-MeO-$C_6H_4CH(OH)CH_3$ and 'ketone' for 4-MeO-$C_6H_4C(O)CH_3$. The increase of the temperature appears to have an adverse effect upon the efficiency of the oxidation, when compared with the 60–70% conversion obtained at room temperature (Table 3, run 1). Independent determination gives a substantial decrease of the activity of laccase in mixed solvents at 45 °C, determined by the use of HAA rather than ABTS as the chromogen [27].

4. Discussion

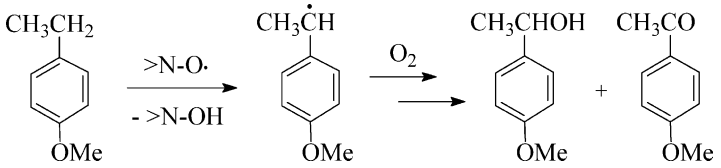
4.1. Mechanistic considerations

The results of our investigation show the difficulty of a selective oxidation of the alkyl group in alkylarene substrates. Oxidation with laccase/mediator systems rarely affords yields of products higher than 15–20% with the mediator:substrate 1:3 molar ratio (Table 1). However, it is worth to mention that the yield is expressed in mol% per substrate, whose molar amount is three times that of the mediator. It is important that a deficiency of both the laccase and mediator is used, if one aims to substantiate the catalytic cycle delineated in Scheme 1.

Other experimental parameters investigated were pH, reaction temperature and the solvent system. The optimum pH was determined to be around 5 (Table 4). The optimum reaction temperature was found to be 25 °C, while an increase to 45 °C did not improve the efficiency of the oxidation (Table 5). Two water–organic solvent systems have been tested, and 50%-dioxane resulted very valuable, because it dissolved hydrophobic substrates without endangering the enzymatic activity [27].

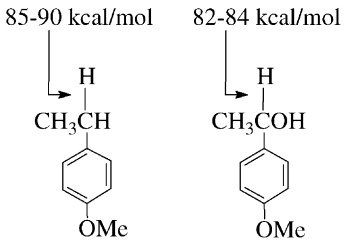
Among the mediators investigated, HBT is found to be the most effective (Table 1), whereas the effectiveness of some 'natural' mediators is not verified under our 'ideal' conditions (Table 2). In previous studies [12,24,31], we have suggested that the oxidised form of HBT (Med_{ox}, in Scheme 1), generated in an initial laccase-catalysed oxidation of HBT [18], oxidises the substrate via a radical HAT mechanism [32]. The Med_{ox} species in this case, as well as for the other >N–OH-type mediators, is a nitroxyl radical (>N-O$^{\bullet}$) species [18], capable of abstracting H-atom from the substrate [32,33] to give a benzylic radical in the rate determining step. The benzylic radical is then transformed into oxidation product(s) by interaction with oxygen [28,33] (Scheme 2).

The proposed radical mechanism of Scheme 2 is supported by the effect of substituents (Hammett correlation) [31], by the value of the kinetic isotope effect [24], and by the product distribution with

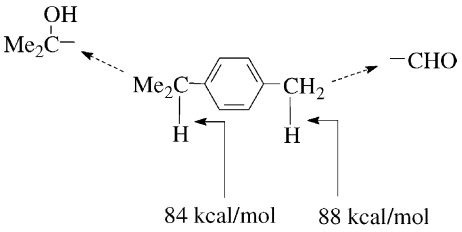


Scheme 2. The HAT pathway.

a suitable probe substrate [24]. The HAT radical pathway explains various findings. First of all, the occurrence of the oxidation of substrates not particularly electron-rich, provided that moderately weak C–H bonds are present. In fact, benzylic substrates, that have bond energies ($BDE_{C–H}$) in the range of 85–90 kcal/mol for the benzyl C–H bonds, are easier to oxidise than those having non-benzyl aliphatic ($BDE_{C–H}$ ca. 94–99 kcal/mol) or aromatic C–H bonds ($BDE_{C–H}$ ca. 110–114 kcal/mol) [34]. This also explains the predominance of producing α-carbonyl derivatives over the corresponding α-hydroxyl derivatives, in the oxidation of 4-ethylanisole and isobutylbenzene (Tables 1, 3–5), because the α-hydroxyl derivative formed in the first oxidation step is easier to oxidise than the starting material [28], in view of a $BDE_{C–H}$ of ca. 82–84 kcal/mol for the benzyl C–H bond geminal to the C–O bond [33,34], as compared to the $BDE_{C–H}$ of ca. 85–90 kcal/mol for the C–H bond in a benzyl CH_2 group.



Additionally, the HAT pathway is in agreement with the intramolecular selectivity observed in the oxidation of *p*-cymene with the laccase/mediator systems, where homolytic cleavage of the tertiary benzyl C–H bond ($BDE_{C–H}$ of 84.4 kcal/mol) prevails over that of the primary benzyl C–H bond ($BDE_{C–H}$ of 88.0 kcal/mol) (Tables 1, 3 and 5), as expected [35]. For example, k_{tert}/k_{prim} is 45 and 24 for laccase/HBT and laccase/HPI systems, respectively, on the basis of the yield data in Table 1 (run 3).



Finally, the polarity of the >N–O• radical species [31,33] derived from HBT explains the good conversions obtained with 4-ethylanisole [14], an electron-rich substrate due to the resonance effect of the methoxy-group. Two cases, taken from the previous literature, will now be discussed in details.

4.2. The case of ABTS

We could not confirm (Table 1) the oxidation of aromatic alkyl groups by the laccase/ABTS system [13]. Admittedly, our laccase originates from *Trametes villosa*, that is a slightly less good an oxidant (0.75–0.79 V) than laccase from *Coriolus versicolor* (≥0.8 V) [18,36,37] employed in the quoted paper [13]. Although the efficiency of laccase from various species of fungi may differ, the laccase preparations from these two particulat fungi are reported to have the same Type 1 (T1) copper-site [38], that is involved in the interaction with the substrate [5]. In addition, the role of the enzyme in the oxidation of non-phenolic substrates with a laccase/mediator system (Scheme 1) is to oxidise the mediator, but not the substrate. It is the Med_{ox} that in turn oxidises the substrate by way of an oxidation mechanism which may differ from that of the enzyme-catalysed oxidation. This is the reason why non-phenolic substrates become oxidisable with a laccase/mediator system.

Because all laccase enzymes can oxidise ABTS to $ABTS^{•+}$ (0.61 V) [16,21], or perhaps even to $ABTS^{++}$ (1.1 V) [23], a question then arises. Can

$ABTS^{\bullet+}$, or $ABTS^{++}$, oxidise alkylarenes by electron abstraction? The results in Table 1 show clearly that this oxidation is not feasible [12]. Since the alkylarenes have redox potentials in the range of 1.5–2.5 V [39–41], the required electron-transfer steps are too endoergonic to occur.

If any oxidation product is formed from alkylarenes through the laccase/ABTS system, we suggest that this is likely to be due to the intervention of by-products of the Med_{ox} species. It is known that $ABTS^{++}$ is not stable in aqueous solutions that are not strongly acidic [24]. By-products of degradation/hydrolysis can be formed from the Med_{ox} in laccase/ABTS procedures [27]. These by-products could be radical species, and therefore lead to HAT oxidation pathways of the substrate. Furthermore, $ABTS^{++}$ (or $ABTS^{\bullet+}$) is reported to undergo degradation and form diazonium-like products [42]. Interestingly, one of these products has a redox potential of 1.2 V, which is higher than that of $ABTS^{++}$. Thus, the non-phenolic substrate could be actually oxidised through the incursion of by-products of the mediator, in routes that could be even of electron-transfer nature. The presence of excess amounts of laccase, as it was the case in the quoted investigation [13], would concur to make this incursion more feasible.

4.3. The substrate:mediator ratio

The feasibility of the oxidation of (polycyclic) aromatic compounds by the laccase/HBT system [15] is indeed confirmed by our data. However, the oxidation requires stoichiometric (or, over-stoichiometric) amounts of the mediator, the mediator:substrate molar ratio being 40:1 or even 200:1 [15]. This certainly limits the practical value of any synthetic procedure of this sort, in view of the necessity to separate the desired product from the overwhelming amount of mediator; however, our major concern about this procedure is conceptual. What is the point of using such a large excess of mediator? The catalytic cycle of the laccase/mediator system (Scheme 1) suggests a turnover of the mediator. Therefore, the presence of nearly catalytic amounts of mediator ought to be sufficient. Amino-oxyl radical species (>N–$O^{\bullet}$) derived from >N–OH-type mediators are known to inhibit free radical autoxidation processes, by acting as radical scavengers [25,33,43]: they remove alkylperoxy radical species from the oxidation system, and are consumed. This cannot justify the use of >N–OH-type mediators in great excess, because our blank experiments show that no appreciable autoxidation of the substrate occurs when the mediator is used without laccase.

An important experimental information is that the cyclic voltammetry oxidation curves of many >N–OH-type mediators are irreversible [23,44], at sweep scans slower than 1 or 2 V/s [12], indicating that the Med_{ox} species undergoes a relatively fast decomposition. In the oxidation of non-phenolic substrates with laccase/mediator systems, decomposition products of the Med_{ox} species could perform side-reaction(s) with the substrate. As an example of side-reaction, Greci [45] showed that the >N–$O^{\bullet}$ species may add to its parent, the >N–OH species, producing the corresponding reduced >N–H species. Such a bimolecular addition reaction would occur more easily at high, or very high, initial concentration of the mediator. Potthast et al. [22] recently pointed out that at high concentration of HBT, the reaction mechanism for the oxidation with the laccase/HBT system changes from a two-electron- to a three-electron-transfer process. Thus, it is possible that side-reactions of the oxidised mediator, such as dimerisation [45], do occur. These processes could lead to the formation of new reactive (radical) intermediates [9,46]. Even though by-products derived from the mediator were generated in trace amounts in the oxidation with a laccase/mediator system, the high initial concentration of mediator [15] would cause them to build up in the course of the reaction in amounts comparable to those of the substrate. This could lead to new oxidation pathway(s) for the substrate.

In a recent review, ten Have and Teunissen [4] suggested that phenoxy radical species, generated by laccase-catalysed dehydrogenation of the phenolic moieties in lignin, are responsible for the degradation of lignin subunits by way of radical routes. These phenoxy radical species could be persistent radical species, and as such they might act as mediators for the laccase-catalysed oxidation of lignin. As a matter of fact, preliminary results from our laboratory show that a laccase-catalysed oxidation of a recalcitrant phenol becomes feasible whenever mediated by another phenol that possibly produces a persistent phenoxy radical species on interaction with laccase

[47]. A likely biogenic hypothesis thus emerges, to explain the finding that some ligninolytic fungi do not excrete the stronger oxidising enzymes Lignin peroxidase or Manganese peroxidase [48,49], and yet carry out the oxidative degradation of lignin by only relying on laccase [4]. The fungi could take advantage from the occasional formation of (persistent) radical species during the phenol-oxidase activity of laccase, or also during lipid peroxidation [49,51]. For example, phenanthrene was efficiently oxidised (73%) by laccase in the presence of HBT and unsaturated lipids [51]. The role of these radical intermediates might be that to break down the covalent structure of lignin [48–50], including the recalcitrant alkylarene or biphenyl moieties [15]. Analogously, but in the field of laccase/mediator systems, radical by-products [27,42] generated by the degradation of the Med_{ox} species could be the true responsible for the oxidation of non-phenolic substrates under non-catalytic conditions [13,15].

Acknowledgements

Thanks are due to Novo Nordisk Biotech (Denmark) for a generous gift of laccase. We also thank the EU project OXYDELIGN (grant QLK5-CT-1999-01277) for financial support.

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