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Short communication

Radical or electron-transfer mechanism of oxidation with some laccase/mediator systems

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The use of appropriate low molecular-weight compounds (viz. mediators) in combination with fungal laccase enables this enzyme to catalyse the oxidation of 'non-natural' non-phenolic substrates [1,2], such as benzyl alcohols, according to the reaction scheme outlined below (Scheme 1) [3].

The mediator needs to be easily oxidised by laccase to the Med_{ox} status: the structure of the latter is then crucial for the mechanism of the ensuing non-enzymatic oxidation of the substrate. 1-Hydroxybenzotriazole (HBT) [4–6] and *N*-hydroxyphthalimide (HPI) [7,8] are two N–OH compounds that are known to mediate the activity of laccase, driving it towards non-phenolic substrates, and the relative efficiency of these mediators has been recently evaluated [9]. Consensus has been reached upon the preliminary monoelectronic oxidation of these N–OH mediators by laccase to the corresponding *N*-oxyl radical (N–O$^{\bullet}$) forms [3,6,8–11], through the fleeting intervention of their radical cations, HPT$^{\bullet+}$ or HPI$^{\bullet+}$, respectively. Two different hypotheses have been proposed to explain the subsequent oxidation of the substrate. Either these N–O$^{\bullet}$ radicals perform a one-electron oxidation (ET) of the substrate to a radical cation [3–5,11], or they abstract a H-atom from the substrate (HAT), converting it into a radical [9,10,12]. The end-products of oxidation would be formed from either one of these two short-lived intermediates of the substrate. We favour the HAT mechanism on the basis of evidence previously described [9], but wanted to address this key issue once more.

A probe substrate

For a reliable assessment of the oxidation mechanism with the laccase/HBT and laccase/HPI systems, we resorted to the probe substrate **1**. Besides being very comparable in structure with other widely-employed lignin model compounds [4,12], **1** presents the distinct feature of giving rise to two diverse end-products depending on the oxidation mechanism (Scheme 2).

In fact, under genuine ET conditions with chemical oxidants [13], **1** gives the transient **1**$^{\bullet+}$ intermediate that cleaves at the C_{α}–C_{β} bond, to produce veratryl aldehyde **2** and *tert*-butyl radical. This reaction is driven by steric and stereoelectronic factors [14]. Conversely, under bona fide radical HAT conditions, **1** undergoes cleavage of the C_{α}–H benzylic bond, and produces ketone **3** [13,15]. This clear-cut behaviour makes **1** a useful probe, enabling it to assess the oxidation mechanism from product analysis [13]. In the present study, besides HBT and HPI, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (viz. ABTS) was also used for comparison, since it is regarded as a laccase redox mediator responsible for one-electron oxidation pathways [4,16,17].

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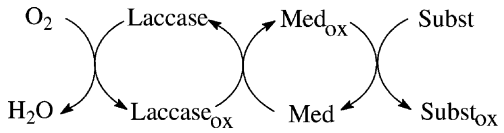
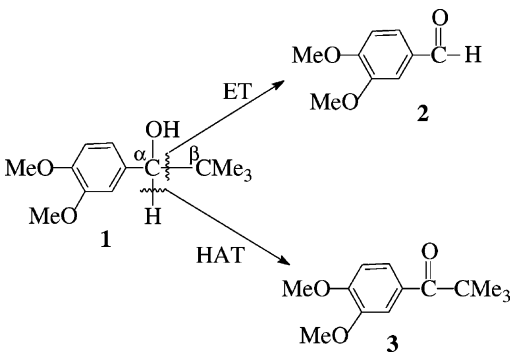


Scheme 1. The role of a mediator of laccase activity.



Scheme 2. Competing oxidation routes with **1**.

Enzymatic oxidations of **1** (20 mM) were carried out with 3 U/ml of purified laccase in 3 ml 0.1 M citrate buffer at pH 4.5, with 6 mM of mediator, dioxygen being purged in the solvent for 30 min prior to the beginning of the reaction [9]. All reactions were run at rt for a fixed time (24 h), and the reaction products analysed by gas chromatography (Table 1) [9]. The results obtained with the three laccase/mediator systems were compared with the results obtained with a genuine ET oxidant, such as potassium 12-tungstocobaltate(III) (viz. $Co^{III}W$; E° 1.4 V) [18], or with $ABTS^{++}$ (E° 1.1 V) [9], independently generated by use of the strong oxidant $(NH_4)_2Ce^{IV}(NO_3)_6$ (viz. CAN; E° 1.5 V) [13]. Table 1 shows that the aldehyde **2** was produced only from the reaction of $Co^{III}W$ as well as from the laccase/ABTS system, whereas, the laccase/HBT and laccase/HPI systems induced *only* the formation of ketone **3**.

Table 1
Comparison of laccase-mediated oxidations of **1**, and of kinetic isotope effect determinations with **4**, by using some mediators or chemical oxidants

Oxidising system	Substrate recovered (%)	Product (yield%)[a]	k_H/k_D ratio[b]
Laccase/ABTS	**1** (98)	**2** (2)	–
Laccase/HBT	**1** (67)	**3** (30)	–
Laccase/HPI	**1** (40)	**3** (50)	–
$Co^{III}W$[c]	**1** (80)	**2** (15)	–
$ABTS^{++}$[d]	**1** (75)	**2** (4)	–
$Co^{III}W$[c]	**4** (72)	**2** + **2D** (12)	3.8
$ABTS^{++}$[d]	**4** (83)	**2** + **2D** (13)	3.7
Laccase/ABTS	**4** (70)	**2** + **2D** (25)	3.6
Laccase/HBT	**4** (65)	**2** + **2D** (23)	6.4
Laccase/HPI	**4** (10)	**2** + **2D** (76)	6.2

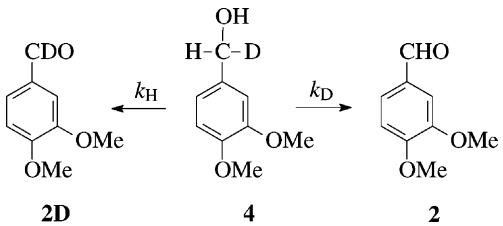
[a] With respect to the molar amount of substrate. Reaction conditions are given in the text.
[b] Determined by GC–MS analysis.
[c] At a $[Co^{III}W]$:[substrate] 1:1 ratio.
[d] With a 3:2:1 substrate:CAN:ABTS molar ratio.

Blanck reactions ensured that products **2** and **3** are stable under the reaction conditions. This result unambiguously confirms the radical HAT route of oxidation of benzyl alcohol **1** with the two N–OH mediators [9], whereas, it supports the ET route with laccase/ABTS through the likely formation of $ABTS^{++}$ [19]. In fact, reaction of **1** with $ABTS^{++}$, independently generated by oxidation with CAN without any enzyme added, similarly converted **1** into **2**. We anticipate that an analogous reactivity picture emerges from the reactions of a derivative of **1** that has only one methoxy substituent on the aromatic ring.

Kinetic isotope effect

As a further proof of this conclusion, we determined the intramolecular kinetic isotope effect in the laccase-mediated oxidation of a suitably synthesised [20] monodeuteriated veratryl alcohol (**4**). For comparison purposes, we have also run this reaction with $Co^{III}W$ and $ABTS^{++}$.

Determination of the relative amount of the Ar–CHO (**2**) and Ar–CDO (**2D**) oxidation products (Scheme 3) was done by GC–MS analyses after a 5 h reaction time, and this enabled to reckon the k_H/k_D ratios. The results are reported in Table 1. The k_H/k_D



Scheme 3. Determination of the kinetic isotope effect in the laccase-mediated oxidation of **4**.

ratios with laccase/HBT and laccase/HPI are clearly equal and *large* in value, as it is expected for an HAT oxidation route where H- or D-abstraction from the α C–H (or C–D) bond of benzyl alcohol **4** is rate determining [10]. In contrast, the k_H/k_D ratio with laccase/ABTS is decidedly *smaller* in value and practically coincident with that of the bona fide ET agents $Co^{III}W$ and $ABTS^{++}$ (independently generated). A smaller k_H/k_D ratio is expected for an ET oxidation route of monodeuteriated probe **4**, because the rate determining step is likely to be the abstraction of electron, followed by fast deprotonation or dedeuteriation of intermediate $\mathbf{4}^{\bullet+}$ to **2D** or **2**, respectively [13]. The radical HAT oxidation mechanism with laccase/HBT and laccase/HPI is, therefore, firmly confirmed, whereas, an ET route by laccase/ABTS is supported [17,19].

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