ARTICLE

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Kinetics of oxidation of benzyl alcohols by the dication and radical cation of ABTS. Comparison with laccase–ABTS oxidations: an apparent paradox

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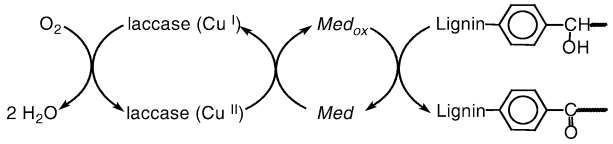
Laccase, a blue copper oxidase, in view of its moderate redox potential can oxidise only phenolic compounds by electron-transfer. However, in the presence of ABTS (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) as a redox mediator, laccase reacts with the more difficult to oxidise non-phenolic substrates, such as benzyl alcohols. The role of ABTS in these mediated oxidations is investigated. Redox interaction with laccase could produce *in situ* two reactive intermediates from ABTS, namely $ABTS^{++}$ or $ABTS^{\bullet+}$. These species have been independently generated by oxidation with Ce(IV) or Co(III) salts, respectively, and their efficiency as monoelectronic oxidants tested in a kinetic study towards a series of non-phenolic substrates; a Marcus treatment is provided in the case of $ABTS^{++}$. On these grounds, intervention of $ABTS^{++}$ as a reactive intermediate in laccase–ABTS oxidations appears unlikely, because the experimental conditions under which $ABTS^{++}$ is unambiguously generated, and survives long enough to serve as a diffusible mediator, are too harsh (2 M H_2SO_4 solution) and incompatible with the operation of the enzyme. Likewise, $ABTS^{\bullet+}$ seems an intermediate of limited importance in laccase–ABTS oxidations, because this weaker monoelectronic oxidant is unable to react directly with many of the non-phenolic substrates that laccase–ABTS can oxidise. To solve this paradox, it is alternatively suggested that degradation by-products of either $ABTS^{++}$ or $ABTS^{\bullet+}$ are formed *in situ* by hydrolysis during the laccase–ABTS reactions, and may be responsible for the observed oxidation of non-phenolics.

Introduction

The interaction of sterically demanding substrates with enzymes may be unfeasible whenever access to the active site is impeded. Under these circumstances some redox enzymes resort to mediators.[1] These small-sized compounds perform as electron shuttles and enable communication between the enzyme and a bulky substrate.[2] For example, lignin peroxidase (LiP) is a heme-enzyme endowed with a redox potential of 1.4 V *vs.* NHE,[3,4] and its natural substrate is the biopolymer lignin;[5] a direct enzyme–substrate interaction is prevented by the small-sized active site of LiP.[4b,6] Veratryl alcohol (3,4-dimethoxybenzyl alcohol; VA), a secondary metabolite of the LiP-producing *Phanerochaete chrysosporium* fungus, acts as a redox mediator.[7] VA is oxidised to radical cation by LiP, and then diffuses away from the active site and concurs to the oxidative biodegradation of lignin by enabling the transport of electrons between substrate and enzyme.[8–12]

Other examples of redox mediators can be found in the case of redox catalysis of electrochemical reactions,[13–15] where suitable species are selectively oxidised at the electrode and then oxidise the desired substrate in solution.[16,17] A successful redox mediator must have a redox potential compatible with the substrate it is aimed at, but it also needs to be sufficiently stable in its oxidised form to perform as an electron shuttle efficiently.

Besides LiP, white-rot fungi also excrete laccase, a family of 'blue copper' oxidases that catalyse the four-electron reduction of O_2 to $2H_2O$ by sequential one-electron uptake from a suitable reducing substrate.[1] Having a lower redox potential (0.6–0.8 V *vs.* NHE) than LiP,[1,18] the Cu(II)-containing laccase can oxidise uniquely the easily oxidisable phenolic subunits of lignin (phenoloxidase activity), whose abundance is merely 20% among the functional groups of the biopolymer.[19] Redox mediators can expand the versatility of laccase as a delignifying enzyme, because they enable the oxidation of other, more difficult to oxidise but more abundant (80%) functional groups of lignin, such as the benzyl alcohol subunits (Scheme 1).[14,20–24]



Scheme 1 The catalytic cycle of a laccase–mediator oxidising system.

Because laccase is more readily available and easier to manipulate than LiP or other delignifying enzymes, laccase–mediator catalytic systems begin to find biotechnological applications in selective organic transformations,[25–27] in textile dye bleaching,[28] in bioremediation of soils and water,[29,30] or also for an environmentally-benign delignification of wood pulp for paper manufacture.[20–22,31–34]

ABTS (*i.e.*, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) was the first to be employed among the mediators of laccase.[1,2,20,21,24] This water soluble compound undergoes one-electron oxidation to the relatively stable blue coloured radical cation $ABTS^{\bullet+}$,[20,24,35–37] at a redox potential (0.69 V *vs.* NHE) that almost matches that of laccase (Scheme 2). Indeed, the standard assay of the activity of laccase involves spectrophotometric monitoring of the generation of $ABTS^{\bullet+}$ with time.[38] Further one-electron oxidation of $ABTS^{\bullet+}$ to dication $ABTS^{++}$ occurs electrochemically at higher potential (1.1 V *vs.* NHE),[24,35–37] but this red-coloured species is less stable. Furthermore, oxidation of $ABTS^{\bullet+}$ to $ABTS^{++}$ is endoergonic for laccase.

ABTS reportedly mediates laccase in the catalytic oxidation of benzyl alcohols by oxygen, according to Scheme 1.[39] Table 1 gives the yield of carbonylic products that we obtained in laccase–ABTS oxidations of a few non-phenolic precursors.[24]

The yields are moderate, but laccase would never oxidise these substrates without the mediator. A precise assessment of the

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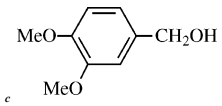
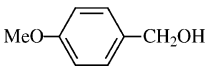
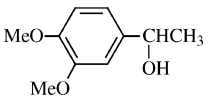
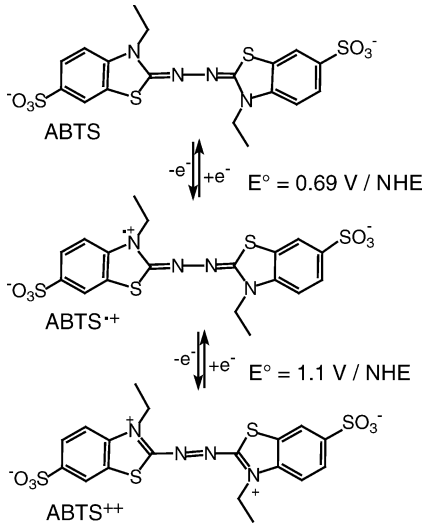
Table 1 Yield of oxidation of benzylic alcohols by laccase from *Poliporus pinsitus* and ABTS, under O_2[a]

Substrate	Yield of aldehyde or ketone (%)[b]
	37
	22
$PhCH_2OH$	2
	15

[a] From ref 24. [b] Reaction conditions: [Subst.] = 20 mM, [ABTS] = 6 mM, [laccase] = 3 U ml^{-1}, in buffered (pH 5) water solution at 25 °C for 24 h; O_2 initially purged in the solvent for 30 min. Product yields are calculated on the molar amount of substrate, the rest of mass balance being unreacted recovered substrate. [c] Veratryl alcohol, VA.



Scheme 2 The two oxidation steps of ABTS.

structure of the intervening Med_{ox} form (whether $ABTS^{\bullet+}$, or the stronger oxidant $ABTS^{++}$) has not been reported yet. The only *indirect* hint about $ABTS^{++}$ being the actual Med_{ox} form in laccase–ABTS reactions comes from a seminal study.[35] The $ABTS^{++}$ species was generated electrochemically, and shown able to oxidise the non-phenolic electron-rich veratryl alcohol (E^0 1.35 V *vs.* NHE),[11] whereas the weaker oxidant $ABTS^{\bullet+}$, analogously generated at the electrode, could not do so. The inference was then made that any *observed* oxidation of VA by the laccase–ABTS system (*cf.* Table 1) would *by necessity* involve the intermediacy of $ABTS^{++}$.[35] This inference was subsequently 'stretched' to uphold the alleged monoelectronic oxidation by laccase–ABTS of alkylbenzenes,[25] even though these are more difficult to oxidise than VA in electron-transfer (ET) routes, having redox potential in the 1.7–2.7 V *vs.* NHE range.[40] Consequently, although mediation of laccase by ABTS undoubtedly occurs with suitable non-phenolic substrates that the enzyme does not oxidise directly,[24,39] a comparable oxidation of other substrates (*e.g.* alkylbenzenes)[25,41,42] seems decidedly unfavourable from a redox standpoint. An insufficient knowledge of the mediation phenomenon by ABTS towards laccase clearly appears.[40]

For a more precise assessment of this point, it was deemed of first priority to generate both $ABTS^{++}$ and $ABTS^{\bullet+}$ independently and unambiguously, by resorting to appropriate chemical oxidants, and to explore their reactivity *per se* in the oxidation of a series of substrates spanning over a wide range of redox potentials. This could enable a 'redox potential boundary' to be determined for the substrate, beyond which a genuine ET oxidation is not possible by either $ABTS^{\bullet+}$ or $ABTS^{++}$. Subsequent comparison with the reactivity performances of the laccase–ABTS system towards the same substrates could disclose similarities between the oxidation patterns, and enable the form of the Med_{ox} involved to be pinpointed.

The results herein reported, besides underlining the experimental difficulties associated with an unambiguous generation of $ABTS^{++}$, allow us to fix some points. Oxidation of many non-phenolic substrates by $ABTS^{++}$ is indeed possible, but the generation of $ABTS^{++}$ requires conditions unsuited to the operation of an enzyme. In contrast, $ABTS^{\bullet+}$ is unable to oxidise many substrates that laccase–ABTS does instead oxidise. To solve this paradox, it is suggested that degradation by-products of the Med_{ox} species may be responsible for the oxidation of non-phenolics by laccase–ABTS.

Results and discussion

Generation of $ABTS^{++}$

The dication of ABTS (*cf.* Scheme 2) can be unambiguously generated by an oxidant of appropriate strength in a two-electron process.[36] We employed the cerium(IV) salt $Ce(SO_4)_2$ (hereafter *Ce*(IV); E^0 1.4 V *vs.* NHE)[43] and, by using a 1 : 2 ABTS : *Ce*(IV) molar ratio, observed a UV–vis spectrum (λ_{max} = 520 nm, ε = 3.6×10^4 M^{-1} cm^{-1}) (Figs. 1 and 2) complying with the literature description of the red-coloured dication (λ_{max} = 518 nm, $\varepsilon = 3.6 \times 10^4$ M^{-1} cm^{-1}) analogously generated by a Ce(IV) salt.[36] The absorption spectrum of $ABTS^{++}$ is not stable, however, because it suffers from hydrolytic cleavage.[36,44]

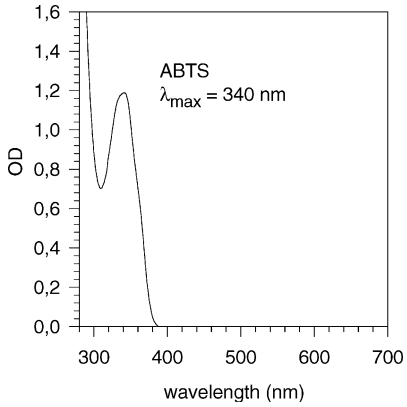


Fig. 1 [ABTS] 3.2×10^{-5} M, in 0.1 M citrate buffer at pH = 5.

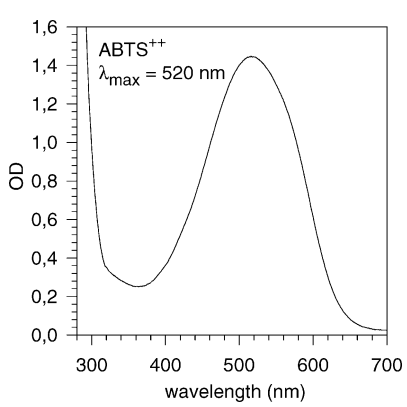


Fig. 2 [$ABTS^{++}$] 4×10^{-5} M, in 2 M H_2SO_4.

The rate of hydrolysis considerably decreases (but does not stop) in strongly acidic media.[36] For example, by using a 2 M H_2SO_4–MeCN 5 : 1 mixed solvent, the half-life of $ABTS^{++}$ is 90 s. In contrast, in the pH 5 buffered water solution typical of laccase–ABTS oxidations,[24] no absorption band unambiguously pertaining to $ABTS^{++}$ could be detected on mixing *Ce*(IV) and ABTS solutions, not even after the very short observation time accessible to the stopped-flow spectrophotometer (15 ms). Then, the 2 M H_2SO_4–MeCN 5 : 1 mixed solvent, where the half-life of $ABTS^{++}$ is sufficiently long, is the standard solvent in this study, and the related $k_{(decay)}$ of the dication (*i.e.*, 8×10^{-3} s^{-1}, at 25 °C) represents the background reactivity in this particular medium. The kinetic determinations were run with the stopped-flow device, and $ABTS^{++}$ was generated by fast mixing [*Ce*(IV)] 3×10^{-4} and [ABTS] 1.2×10^{-4} M solutions. Although the redox data of ABTS (Scheme 2) were obtained in either acetate or citrate buffer solutions at pH 5,[24,35,36] no major change in the E^0 of $ABTS^{++}$ (*i.e.*, 1.1 V *vs.* NHE) is documented on increasing the acidity up to 1.5 M $HClO_4$.[36] We observe that in a previous study $ABTS^{++}$ was reported to be generated by oxidation of ABTS with $S_2O_8^{2-}$ in 20% MeCN–H_2O solution,[45] isolated as a red-brown brittle precipitate, and re-suspended in 20% MeCN for oxidation experiments. In view of the fast hydrolysis of $ABTS^{++}$ in solutions that are not strongly acidic, the true nature of the oxidising species in that investigation[45] appears suspicious.

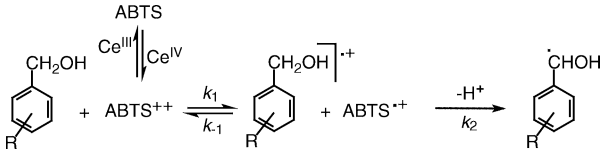
Kinetic study of the ET step. The monoelectronic oxidation of a series of substituted benzyl alcohols was kinetically investigated by generating $ABTS^{++}$ with *Ce*(IV) in the 2 M H_2SO_4–MeCN 5 : 1 mixed solvent, aiming at determining the electron-transfer rate constants k_1 (in Scheme 3). In fact, according to the steady-state approximation,

$$v = -\mathrm{d}[ABTS^{++}]/\mathrm{d}t$$
$$= k_1\, k_2\, [ABTS^{++}][\mathrm{Subst.}]/(k_{-1}[ABTS^{\bullet+}] + k_2)$$

the kinetic system for Scheme 3 becomes:

$$v = k_1[ABTS^{++}][\mathrm{Subst.}] \text{ whenever } k_2 \gg k_{-1}\,[ABTS^{\bullet+}].$$

The kinetic study was performed at 25 °C with a stopped-flow apparatus where pre-mixing of ABTS and *Ce*(IV) (1 : 2.5 molar ratio) quantitatively generated $ABTS^{++}$ (at the initial concentration of 1.2×10^{-4} M reported above), and then this solution was quickly added to the chosen substrate at an initial concentration ([Subst.] = 1.2×10^{-3} M or higher) that would enable a pseudo-first-order treatment of the data. The decrease in the A_{520} value of $ABTS^{++}$, during the one-electron reduction to $ABTS^{\bullet+}$ by the substrate was time monitored.



Scheme 3 Kinetic scheme of the oxidation by $ABTS^{++}$.

It must be emphasized that $ABTS^{++}$ was quantitatively generated from ABTS by using an almost stoichiometric amount (2.5 : 1 *Ce*(IV)–ABTS molar ratio) of the monoelectronic oxidant (a 2-electron process, with ΔE^0 *ca.* 0.3 V), thereby avoiding any excess of *Ce*(IV) that could oxidise the non-phenolic substrate *directly*. This was not the case in previous studies, where $ABTS^{++}$ was generated by using a 100 fold amount of the oxidant (peroxodisulfate).[45,46]

In order to check the reliability of our kinetic approach, veratryl alcohol (VA) was investigated. The observed pseudo-first-order rate constants of oxidation by $ABTS^{++}$ ($k_{obs} = k$ [VA]), with [VA] in the 1–4 $\times 10^{-3}$ M range, were 40–130 times faster than the spontaneous decay of $ABTS^{++}$ in the 2 M H_2SO_4–MeCN 5 : 1 mixed solvent, so to be unambiguously distinct. From the k_{obs} *vs.* [VA] plot (Fig. 3), the second order rate

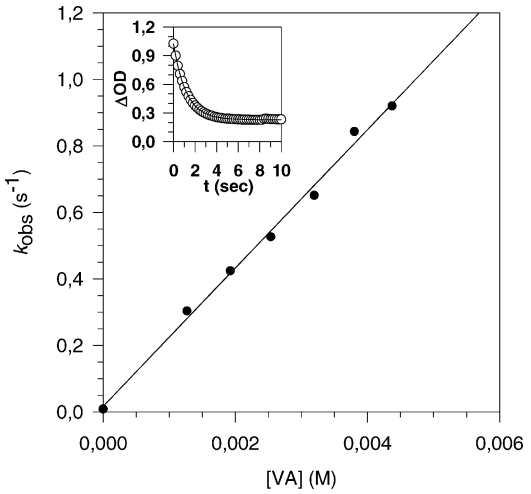


Fig. 3 Values of k_{obs} for the decay of $ABTS^{++}$ as a function of [VA]. Inset: example of the decrease in A_{520} of $ABTS^{++}$ (0.13 mM) in the presence of VA (3.9 mM); the open circles are the experimental points, and the line shows the computed first-order decay for $k_{obs} = 0.843$ s^{-1}.

constant was obtained as 210 (±6) M^{-1} s^{-1}. This value confirms the previously determined rate constant of oxidation of VA (170 M^{-1} s^{-1}) by the electrochemically generated $ABTS^{++}$.[35] The intercept in Fig. 3 is almost negligible, supporting the $k_2 \gg k_{-1}$ [$ABTS^{\bullet+}$] assumption.

The kinetic study was extended to a series of substituted benzyl alcohols and ethers, presenting a range of oxidation potential broad enough to appreciate the efficiency of electron-abstraction by $ABTS^{++}$ as a function of the electron-donor propensity of the substrates. A few methoxy-substituted aromatic compounds were also investigated, in order to cover an even wider range of redox potential of the substrate. The results are reported in Table 2. Unfortunately, precise rate constants could be determined only for a few compounds. In fact, the rate of oxidation of very electron-rich substrates by $ABTS^{++}$ was too fast to determine with our stopped-flow device. On the contrary, the 'apparent' rate constant for electron-poor substrates was so slow to match the $k_{(decay)}$ of $ABTS^{++}$. Despite this, the reasonable expectation that the ET rate (k_1) is larger for the easier to oxidise substrates is qualitatively met. A more quantitative relationship between the rate of ET oxidation and thermodynamic driving force to the ET between donor substrates and acceptor $ABTS^{++}$ was sought within the Marcus theory framework[47] and ensuing eqn. (1).[48]

$$\Delta G^{\neq} = \lambda/4\,(1 + \Delta G^{0\prime}/\lambda)^2 \qquad (1)$$

Unfortunately, the redox potentials of the substrates were available in different solvents, so that a homogeneous assessment of their redox power was largely hampered.[47] More than that, irreversible E^P values were often accessible because the radical cations of benzylic compounds deprotonate very fast (k_2 in Scheme 3),[47,49–53] thereby preventing the determination of reversible E^0 values without sophisticated techniques. Microelectrodes and fast sweep scans of the potential were unavailable to us,[54,55] and therefore it was impossible to determine the missing E^0 data directly. We solved this problem by determining the charge-transfer absorption band ($h\nu_{CT}$) of the substrates.[56]

Determination of $h\nu_{CT}$ and E^0 data. Because the tendency of a substrate to transfer an electron to an electrode is expected to match the tendency to form an electron donor–acceptor complex with a reference acceptor compound, the determination of $h\nu_{CT}$ data may enable to circumvent the unavailability of E^0 redox potentials.[56–58] The charge-transfer (CT) absorption band (*i.e.*, the $h\nu_{CT}$ band, obtained *vs.* tetracyanoethylene, TCNE) of benzylic derivatives structurally comparable to our ones was

Table 2 Rate constants of oxidation (k_1) of the substrates by $ABTS^{++}$ at 25 °C, in 2 M H_2SO_4–MeCN 5 : 1 mixed solvent. Literature redox potentials (V *vs.* NHE),[11,51,57,59–61] experimental $h\nu_{CT}$ data (eV *vs.* TCNE), and extrapolated E^0 data (V, in MeCN and in H_2O) of the substrates are reported. Knowledge of the reduction potential of $ABTS^{++}$ (1.1 V, in H_2O)[24,35,36] enabled to calculate the values in the ΔE^0 column, and correction for charges solvation gave the values in the $\Delta G^{0\prime}$ column (see text)

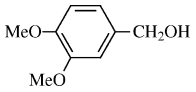
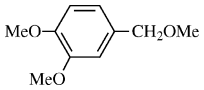
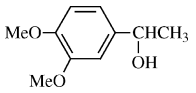
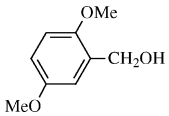
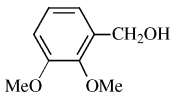
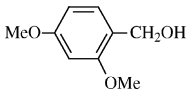
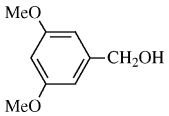
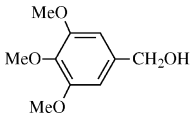
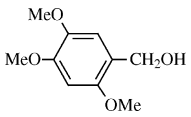
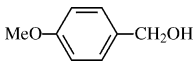
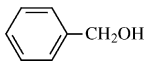
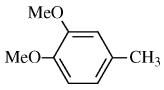
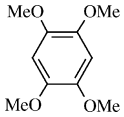
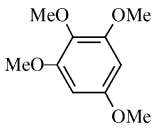
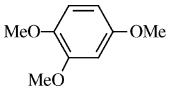
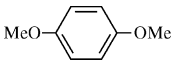
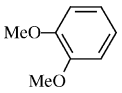
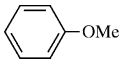
Substrate	k_1 /M^{-1} s^{-1}	E^0exp/v *vs.* NHE		$h\nu_{CT}$/eV in MeCN *vs.* TCNE	E^0extrap/V *vs.* NHE in MeCN	E^0extrap/V *vs.* NHE in H_2O	ΔE^0/V in H_2O	$\Delta G^{0\prime}$/kcal mol^{-1} in H_2O
		in H_2O	in MeCN					
	210	1.36	—	2.16	1.60	—	−0.26	6.3
	14	1.36	—	2.19	1.63	—	−0.26	6.3
	77	(*ca.* 1.4)	—	2.19	1.63	1.36	−0.26	6.3
	66	1.33	—	2.02	1.48	—	−0.23	5.6
	—	1.39	—	2.68	—	—	−0.29	7.0
	—	1.38	—	2.22	1.66	—	−0.28	6.8
	129	1.42	—	2.58	1.97	—	−0.32	7.7
	13	(*ca.* 1.4)	—	2.48	1.89	1.39	−0.29	7.1
	(≫ 10^4)	(*ca.* 1.1)	—	1.74[b]	*ca.* 1.2	*ca.* 1.3	*ca.* −0.2	*ca.* 5
	54	—	1.98	2.49	—	1.39	−0.29	7.0
	—[a]	—	2.68	3.45	—	1.49	−0.39	9.3
	17	—	(*ca.* 1.54)	2.06	1.52	1.35	−0.25	6.1
	(≫ 10^4)	—	—	1.61	1.11	1.30	−0.2	4.9
	—	—	1.05	1.61	—	1.30	−0.2	4.9

Table 2 (*cont.*)

Substrate	k_1 /M^{-1} s^{-1}	E^0exp/v *vs.* NHE in H_2O	E^0exp/v *vs.* NHE in MeCN	hν_{CT}/eV in MeCN *vs.* TCNE	E^0extrap/V *vs.* NHE in MeCN	E^0extrap/V *vs.* NHE in H_2O	ΔE^0/V in H_2O	$\Delta G^{0\prime}$/kcal mol^{-1} in H_2O
	($\gg 10^4$)	—	1.36	1.91	—	1.33	−0.23	5.6
	—[a]	—	1.58	2.09	—	1.35	−0.25	6.1
	—	—	1.70	2.24	—	1.37	−0.27	6.5
	—[a]	—	2.00	2.59	—	1.40	−0.30	7.3

[a] The observed pseudo-first-order decay matched the value of k_{decay} of $ABTS^{\cdot+}$, *i.e.* 8×10^{-3} s^{-1}. [b] Solubility problems.

already found to correlate linearly with the reversible E^0 redox potentials (eqn. (2)).[57]

$$E^0 = 0.862 \times h\nu_{CT}\ (eV) - 0.012 \quad (2)$$

We have extended these measurements to our substrates *vs.* TCNE in MeCN solution, because experimental problems prevented the use of the 2 M H_2SO_4–MeCN 5 : 1 mixed solvent. The obtained hν_{CT} data (as $h\nu_{CT} = hc/\lambda_{max}$; data in Table 2) were plotted *vs.* the E^0 values available in MeCN for some of the substrates,[11,51,57,59] and provided the experimental relationship (eqn. (3); plot in Fig. 4):

$$(E^0)_{MeCN} = 0.881 \times h\nu_{CT}\ (eV) - 0.299 \quad (3)$$

From it, the missing E^0 potentials for other substrates of the study could be extrapolated in MeCN (values given in Table 2).

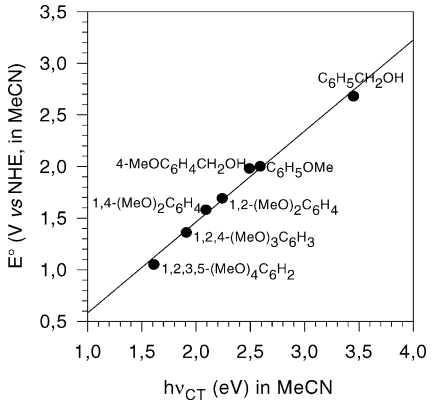


Fig. 4 Correlation of E^0 (in MeCN solution) *vs.* hν_{CT} for benzyl alcohols and polymethoxybenzenes.

The hν_{CT} data were also plotted *vs.* E^0 values available in H_2O for some of the compounds,[60,61] providing another experimental relationship (eqn. (4); plot in Fig. 5):

$$(E^0)_{H_2O} = 0.102 \times h\nu_{CT}\ (eV) + 1.14 \quad (4)$$

Once again, missing E^0 potentials in H_2O could be extrapolated from this correlation line. In the end, two sets of extrapolated E^0 data, one in MeCN and the other in H_2O (see Table 2), were gathered. Since the E^0 of $ABTS^{\cdot+}$ is obtained in water solution,[20,24,36] and the mixed solvent of the kinetic study approximates water better than MeCN, the E^0 data in H_2O (both experimental and extrapolated ones) were preferred for use in the Marcus treatment.

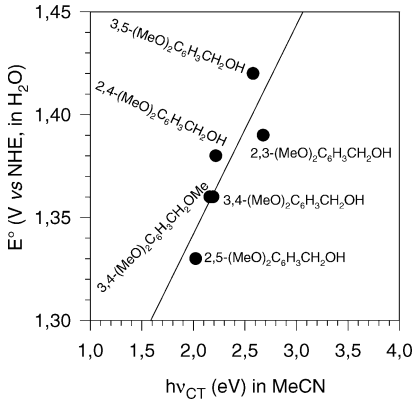


Fig. 5 Correlation of E^0 (in H_2O solution) *vs.* hν_{CT} for benzyl alcohols.

Marcus treatment[47,48]. The electrochemical driving force to the ET between acceptor $ABTS^{\cdot+}$ and each donor substrate, calculated as ΔE^0 (in V; see Table 2) from the individual E^0 values in H_2O, was converted into ΔG^0 (1 V = 23.06 kcal mol^{-1}) data. From the relationship:

$$\Delta G^{0\prime} = \Delta G^0 + (Z_1Z_2)/(Dr_{12})e^2f \quad (5)$$

the final $\Delta G^{0\prime}$ data were obtained (given in Table 2). The contribution from the electrostatic interaction term $(Z_1Z_2)/(Dr_{12})e^2f$ (overall value: +0.3 kcal mol^{-1}) was reckoned as follows.[59] Z_1 is the charge of the reduced oxidant (*i.e.*, a radical anion; see charge of $ABTS^{\cdot+}$ in Scheme 2),[37] Z_2 is the charge of the oxidised substrate (*i.e.*, a radical cation), the dielectric constant D was taken equal to 78, while a value of −23 was used for the ratio e^2f/r_{12}, in keeping with the assumptions (*i.e.*, $f = 0.57$, $r_{12} = 7$ Å) adopted in the monoelectronic oxidation of structurally comparable $ArCH_2Z$ derivatives.[59] The $\Delta G^{0\prime}$ data were finally used to numerically solve the Marcus equation (eqn. (1)), on the basis of an appropriate value for the reorganisation barrier (λ) of the reaction, obtained as follows. Being the intrinsic barrier λ_{ox} for the $ABTS^{\cdot+}/ABTS^{+}$ couple reported as 27 kcal mol^{-1},[36] and being λ_{red} for a $ArCH_2Z^{\cdot+}$–$ArCH_2Z$ couple available as 55 kcal mol^{-1},[59] a mean value of 41 kcal mol^{-1}, as $(\lambda_{ox} + \lambda_{red})/2$, gave the λ value of the reaction.[47,48] The $\Delta G^{\neq}$ data accordingly calculated from (eqn. (1)) are plotted as a solid line *vs.* $\Delta G^{0\prime}$ (Fig. 6). On this plot, the experimental rate constant values (k_1, in Table 2), suitably converted into $\Delta G^{\neq}$ data according to the relationship $k = 6 \times 10^{11}$ exp($-\Delta G^{\neq}/RT$), are represented as black circles (●). These circles are indeed interpolated by the curve calculated

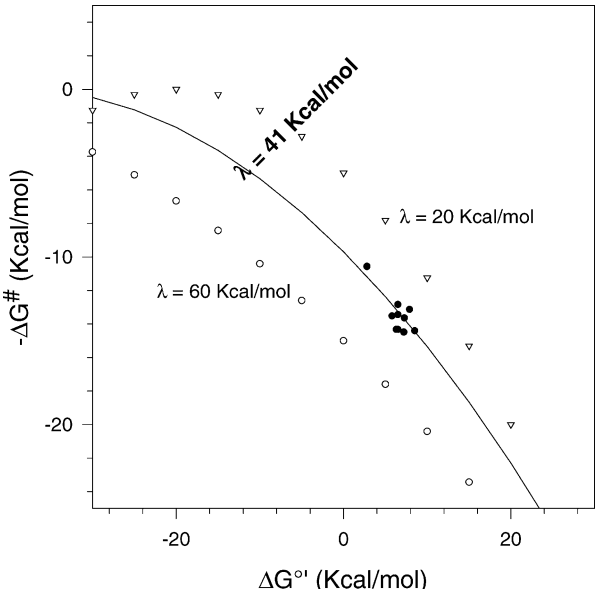


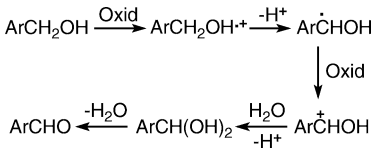
Fig. 6 Marcus plot for the electron-transfer oxidation of non-phenolic substrates by $ABTS^{++}$.

according to the $\lambda = 41$ kcal mol^{-1} barrier. For comparison, two other profiles are given in Fig. 6, being calculated from a higher (*i.e.*, 60 kcal mol^{-1}; ○ symbols) and lower (*i.e.*, 20 kcal mol^{-1}; ▽ symbols) λ value; it clearly appears that not any curve will fit the experimental data correctly. The much better agreement with the $\lambda = 41$ kcal mol^{-1} curve confirms the appropriateness of the chosen Marcus parameters.

A few considerations are possible on the basis of the solid plot of Fig. 6. For the substrates represented as black circles, rate-determining monoelectronic oxidation by $ABTS^{++}$ is viable, even if slightly endoergonic, and the k_1 values are in Table 2; these substrates have E^0 in the 1.3–1.4 V range. Substrates endowed with $E^0 < 1.3$ V would have $\Delta G^{\neq}$ values > -10 kcal mol^{-1} and $k_1 > 10^4$ M^{-1} s^{-1}, therefore *too fast* for our experimental technique, as confirmed by the tetramethoxy- or trimethoxy-derivatives in Table 2. On the other extreme, substrates endowed with $E^0 > 1.4$ V would have $\Delta G^{\neq}$ values < -16 kcal mol^{-1} and $k_1 < 1$ M^{-1} s^{-1}, thereby *slower* than the decay rate constant of $ABTS^{++}$ (*cf.* $PhCH_2OH$ in Table 2). Monoelectronic oxidation of such electron-poor substrates by $ABTS^{++}$ is therefore doubtful. This is an important point, because the laccase–ABTS oxidation of benzyl derivatives[25] and polycyclic aromatic hydrocarbons,[41] similarly endowed with redox potential well *above* 1.6 V,[40] was instead assumed to take place by ET through the $ABTS^{++}$ intermediate.

Product analyses with $ABTS^{++}$ as the oxidant. These were carried out under kinetic conditions with six significant substrates (see Experimental section for details).

Because the formation of the product requires a second oxidation of the intermediate benzyl radical to a cation that reacts with

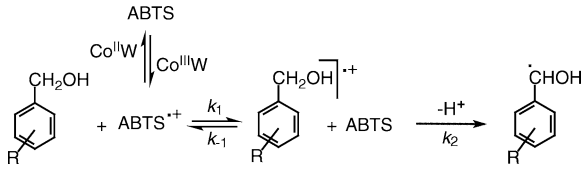


Scheme 4 Mechanism of the oxidation reaction.

water (Scheme 4), a 50% yield indicates quantitative conversion; this is reasonably well verified (Table 3). It is concluded that $ABTS^{++}$ truly converts appropriate substrates into oxidation products. Even in the presence of a 'moderately endoergonic' ($\leq$ 0.3 V) ET step, the subsequent and irreversible deprotonation of $Subst^{\bullet+}$ drives the thermodynamically unfavourable oxidation forward. However, this does not hold for $PhCH_2OH$, which is recovered unreacted because its redox potential makes the ET step *too* endoergonic.

Oxidation by $ABTS^{\bullet+}$

Oxidation of phenols by $ABTS^{\bullet+}$ had already been investigated kinetically,[64] but no analogous study was available for non-phenolics (Scheme 5). By using a *stoichiometric* amount of the monoelectronic oxidant potassium 12-tungstocobaltate(III) (*viz.*, Co(III)W; E^0 1.1 V),[57] we generated $ABTS^{\bullet+}$ quantitatively as confirmed by the absorption spectrum ($\lambda_{max} = 420$ nm, $\varepsilon = 3.5 \times 10^4$ M^{-1} cm^{-1}; Fig. 7).[36,37]



Scheme 5 Kinetic scheme of the oxidation by $ABTS^{\bullet+}$.

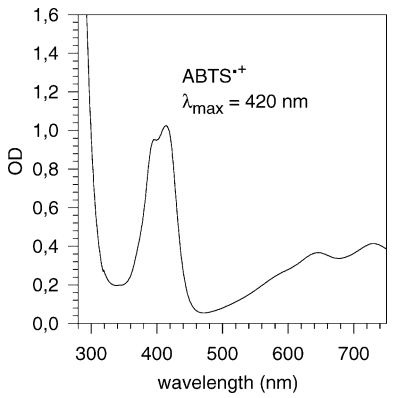


Fig. 7 $[ABTS^{\bullet+}]$ 3×10^{-5} M, in 0.1 M citrate buffer at pH = 5.

$ABTS^{\bullet+}$ survives in MeCN or in buffered (pH 5) water solutions much longer (half-life of 90 min; k_{decay} 1.3×10^{-4} s^{-1}) than $ABTS^{++}$ did.

Table 3 Product analysis in the oxidations with $ABTS^{++}$, in 2 M H_2SO_4–MeCN 5 : 1 mixed solvent at room temperature. Conditions: [Subst.] = 4, [ABTS] = 0.4, [*Ce*(IV)] = 1 mM

Substrate	Oxidation product	Yield (%)[a]
3,4-Dimethoxybenzyl alcohol (VA)	Veratryl aldehyde	50
Methyl ether of VA	Veratryl aldehyde	36[b]
4-Methoxybenzyl alcohol	4-Methoxybenzaldehyde	40
3,4,5-Trimethoxybenzyl alcohol	3,4,5-Trimethoxybenzaldehyde	46
1,2,4-Trimethoxybenzene	Quinone(s)[c]	n.d.
Benzyl alcohol	—	0

[a] Calculated *vs.* the mmol of $ABTS^{++}$. [b] Minor amounts of the corresponding methyl ester are also formed (see, ref. 62). [c] This substrate, lacking benzylic C–H bonds, gave a mixture of products, most likely quinones (according to GC–MS inference; see ref. 63), which was not further investigated.

Once again, VA was the first substrate examined under the conditions: [ABTS] 7×10^{-5}, [Co(III)W] 9×10^{-5}, [VA] $7 \times 10^{-3} - 7 \times 10^{-2}$ M, in buffered water solution (pH 5) at 25 °C. Bleaching of $ABTS^{\bullet+}$ in the presence of VA was monitored in the 690–730 nm range, where the spectrum of $ABTS^{\bullet+}$ presents a significant shoulder and there is minimal interference from spurious absorption bands. The drop of $ABTS^{\bullet+}$ absorption was slower than with the stronger oxidant $ABTS^{++}$, so that a conventional and not stopped-flow spectrophotometer could be used. However, no simple pseudo-first-order kinetic behaviour was observed, in spite of a 100 : 1 or even 1000 : 1 ratio in the [VA] : [$ABTS^{\bullet+}$] initial concentrations.

In kinetic studies of consecutive reactions (as in Scheme 5), where the ET step is analogously endoergonic, it is necessary to take into account the *full* equation resulting from the steady state approximation:[59,65]

$$v = -\mathrm{d}[ABTS^{\bullet+}]/\mathrm{d}t = k_1k_2\,[ABTS^{\bullet+}][\mathrm{Subst.}]/(k_{-1}[ABTS] + k_2)$$

By following the mathematical analysis provided by Kochi *et al.*,[65] and by a non linear fit of the $x = [ABTS^{\bullet+}]/[ABTS^{\bullet+}]_0$ *vs.* t data, according to the complex biparametric equation:

$$-\log x - a(1 - x) = bt$$

where $a = (k_{-1}C)/(k_{-1}C + k_2)$; $b = k_1k_2\,[\mathrm{Subst.}]/(k_{-1}C + k_2)$

$$C = [ABTS^{\bullet+}]_0 + [ABTS]_0$$

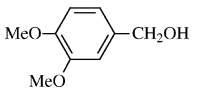
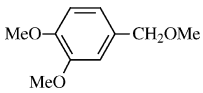
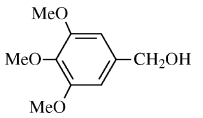
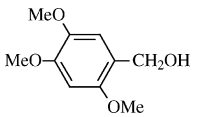
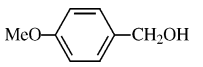
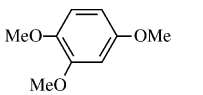
the values of k_1 and k_{-1}/k_2 could be attained (Table 4), even though this was not possible for all the substrates tested in Table 2.

The successful substrates follow a limit kinetic behaviour where deprotonation of the radical cation (k_2 in Scheme 5) is the slow step, being the back-ET step faster (*i.e.*, $k_{-1}\,[ABTS] \gg k_2$) and close to the diffusion limit,[59] owing to the unfavourable thermodynamic driving force ($\Delta E > 0.7$ V) of the ET step. Product analyses under kinetic conditions were also carried out, and the yields are given in Table 4.

The kinetic data come out uniformly slower than those with $ABTS^{++}$ by at least two powers of ten, but no simple relationship emerges between k_1 and E^0 data. Product analysis with the electron-rich compounds (Table 4) supports oxidation to an acceptable extent. In the case of less electron-rich substrates, though, the drop in $ABTS^{\bullet+}$ absorption *vs.* time was as slow as the spontaneous decay of this oxidant, thus precluding determination of meaningful k_1 values. Consistently, product analysis gave no evidence of oxidation of 4-methoxybenzyl alcohol, nor of dimethoxytoluene or benzyl alcohol by $ABTS^{\bullet+}$ (not given in Table 4).

Oxidations with the laccase–ABTS system. Recent X-ray studies provide important structural details on laccase from *Trametes versicolor*,[66a,b] and even for an adduct between ABTS and laccase from *Bacillus subtilis*.[66c] The substrate binding site is a negatively charged depression on the surface of the enzyme near the cavity where copper T1, the primary electron acceptor, lies.[1,2,66,67] The electron removed from the substrate is shuttled from T1 to the T2/T3 trinuclear Cu(II) cluster, the site of oxygen binding and reduction, in order to regenerate Cu(II) at T1, and enable the oxidation of a second molecule of substrate. The negative charge in the substrate binding site is expected to stabilise the product of oxidation.[66a] This may be a crucial point. We have determined k_{cat} (510 s^{-1}) and K_M (1.1×10^{-5} M^{-1} s^{-1}) for laccase from *Poliporus pinsitus* and ABTS (see Experimental); the data confirm a substantial enzyme–mediator affinity, and indeed laccase-generated $ABTS^{\bullet+}$ is clearly detectable by spectrophotometry. However, production of $ABTS^{++}$ by laccase would require that the initially formed $ABTS^{\bullet+}$ remains in the active site, in order to undergo the second monoelectronic oxidation, instead of being turned out in solution to enable the oxidation of a new molecule of ABTS. In fact, enzymatic generation of $ABTS^{++}$ from the tiny amounts of $ABTS^{\bullet+}$ produced in the catalytic cycle and released in solution, seems improbable. Charge stabilisation might tie $ABTS^{\bullet+}$ in the

Table 4 Rate constants (k_1)[a] and preparative yields[b] for the oxidation of the substrates by $ABTS^{\bullet+}$, at 25 °C in buffered water solution (pH 5)

Substrate	$k_1/M^{-1}\,s^{-1}$	$k_{-1}/k_2/M^{-1}$	E^0/V *vs.* NHE in H_2O[c]	Yields[d] (%)
	0.6	2×10^7	1.36	50
	5.2×10^{-3}	3.4×10^5	1.36	11
	1.3×10^{-2}	4.2×10^5	*1.39*	26
	75	2.2×10^7	*ca.* 1.3	40
	0.13	1.8×10^8	*1.39*	0
	115	5.7×10^7	*1.33*	quinones[e]

[a] Conditions: [ABTS] 7×10^{-5}, [Co(III)W] 9×10^{-5}, [Subst.] $7 \times 10^{-3} - 7 \times 10^{-2}$ M in buffered (pH 5) water solution. See Scheme 5. [b] Conditions: [Subst.] = 2.5, [ABTS] = 0.25, [Co(III)W] = 0.30 mM in buffered water solution containing 4% MeCN for solubility reasons. [c] From Table 2; extrapolated values in italics. [d] Of carbonylic product *vs.* the molar amount of ABTS (*viz.* $ABTS^{\bullet+}$). [e] Qualitative result by GC-MS.

Table 5 Yields in the oxidation by laccase–ABTS, in 3 mL 0.1 M citrate buffer (at pH 5) containing 4% MeCN. Conditions: [Subst.] = 5 mM, [ABTS] = 0.5 mM, laccase 3 U ml^{-1}, for 24 h at rt, under O_2

Substrate	Oxidation product	Yield (%)[a]
3,4-Dimethoxybenzyl alcohol (VA)	Veratryl aldehyde	300
4-Methoxybenzyl alcohol	4-Methoxybenzaldehyde	70
Methyl ether of VA	—	0
3,4,5-Trimethoxybenzyl alcohol	3,4,5-Trimethoxybenzaldehyde	65

[a] The yields are calculated on the amount of ABTS.

active site, enabling the second step of oxidation to $ABTS^{++}$ *within* the enzyme. On the whole, ABTS has the charge of a dianion at pH 4–5, whereas $ABTS^{\bullet+}$ is equivalent to a radical anion and $ABTS^{++}$ is electrostatically neutral (*cf.* Scheme 2), thus supporting an increasing affinity of these oxidised states for the negatively charged enzymatic active site.

Whereas on mixing suitable amounts of laccase and ABTS we detect no absorption bands pertaining to $ABTS^{++}$, the two-electron oxidation of ABTS to $ABTS^{++}$ is instead accomplished by using a two-equivalent amount of the monoelectronic oxidant *Ce*(IV) (or by anodic oxidation).[35] However, this occurs only in very acidic solution (2 M H_2SO_4), where $ABTS^{++}$ survives with an half-life of 90 s. In buffered water solution at pH 5, instead, no bands of $ABTS^{++}$ are detected; consequently, its half-life ought to be shorter than 15 ms, otherwise it had to be detected on mixing *Ce*(IV) and ABTS in a 2 : 1 molar ratio in the stopped-flow spectrophotometer.

In Table 1 the yields of oxidation of a few benzylic alcohols by laccase–ABTS were given.[24] The feasibility of a monoelectronic oxidation of substrates endowed with $E^0 < 1.4$ V (*i.e.*, excluding $PhCH_2OH$) is confirmed by the present kinetic study (Table 2). Therefore it would seem plausible to suggest that, in the oxidations with the laccase–ABTS system, $ABTS^{++}$ is the reactive intermediate (Med_{ox}). We could assume that, following the preliminary interaction with laccase, $ABTS^{\bullet+}$ is kept within the enzyme and further oxidised to $ABTS^{++}$. Electrostatic interactions with amino acidic residues in the binding site could stabilise both $ABTS^{\bullet+}$ and particularly $ABTS^{++}$. This interaction would protect the latter from hydrolysis, and possibly extend its lifetime long enough to enable the monoelectronic oxidation of electron-rich non-phenolic substrates, even though $ABTS^{++}$ would never accumulate in solution to extents spectrophotometrically detectable (*cf.* Experimental). A similar protection offered by the enzyme has been invoked by Aust *et al.*,[68] to explain the longer lifetime of the radical cation of mediator VA, whenever associated to LiP, *vs.* the shorter lifetime of *free* $VA^{\bullet+}$ in solution. We do confirm this sort of enzymatic shielding for $ABTS^{\bullet+}$, whose spontaneous decay in buffered water solution increases from $k_{(decay)} = 1.3 \times 10^{-4}$ s^{-1} (half-life of 90 min) when generated by Co(III)W, to 2×10^{-6} s^{-1} (half-life of 96 h) when generated by laccase (see Experimental). The relevance of this shielding, however, implies that the oxidised mediator remains associated with the enzyme and does not diffuse freely in solution, thereby disrupting the very idea that ABTS performs as a *diffusible* mediator of laccase.[20] In contrast to the measurable change in lifetime value of $ABTS^{\bullet+}$, no change in the redox potential values of ABTS is detected on running the cyclic voltammetry in the presence of laccase at saturation conditions (see Experimental). Therefore interaction with the enzyme binding site has no appreciable effect upon the propensity of ABTS to lose electrons.

When $ABTS^{\bullet+}$ is independently generated by means of Co(III)W, it is kinetically unable to oxidise substrates that both preformed $ABTS^{++}$ and the laccase–ABTS system do oxidise. For example, Table 1 shows that $PhCH_2OH$ is oxidised by laccase–ABTS, even though for a meagre 2%, while 4-methoxybenzyl alcohol reacts better (22%). There is no significant oxidation of these two substrates by preformed $ABTS^{\bullet+}$ (*cf.* Table 4). Furthermore, both $ABTS^{++}$ and laccase–ABTS do react with some benzylic probe substrates that are chemical models of lignin,[69] yielding oxidation products consistent with the operation of an ET route:[69a] no oxidation of these particular substrates occurs with preformed $ABTS^{\bullet+}$.[69b] Proposing $ABTS^{\bullet+}$ as the Med_{ox} species has therefore no consistent experimental support.

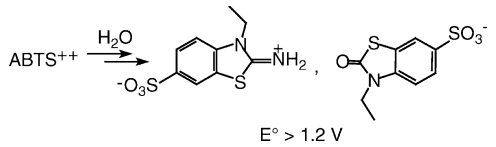
Another point can be stressed. The yields of oxidation in Table 1 were reckoned on the molar amount of *substrate*, whose initial concentration is three times that of the mediator. Scheme 1 points out that the Med_{ox} species is continuously regenerated by laccase–O_2. In fact, yields in excess of 100% are obtained if calculated *vs.* the molar amount of *mediator*, thereby implying an oxidation process with turnover.[24,40,69a] This is supported by the experiments reported in Table 5, where a 10 : 1 ratio of [Subst.] : [ABTS] was employed, and the amount of laccase was stoichiometric with respect to ABTS.

Conclusions

The phenoloxidase enzyme laccase becomes able to oxidise non-phenolic substrates, such as benzylic alcohols, in the presence of ABTS. Oxidation products (aldehydes or ketones) are accordingly obtained. What is the mediating role of ABTS in these oxidations? Is it initially oxidised by laccase to $ABTS^{\bullet+}$, or else to $ABTS^{++}$, and subsequently either one of these reactive intermediates carries out the non-enzymatic oxidation of the non-phenolic substrate? In this study we have (i) underlined the experimental difficulties associated with an unambiguous generation of $ABTS^{++}$, (ii) generated it independently and unambiguously by a chemical oxidant (*Ce*(IV)), and (iii) shown it to be able to monoelectronically oxidise substrates endowed with redox potential <1.4 V. Among these substrates is VA, which is indeed oxidised by the laccase–ABTS system. However, either $ABTS^{++}$ is too short-lived a species to serve as a diffusible mediator, or the experimental conditions under which it survives longer are too harsh (2 M H_2SO_4 as the solvent) and incompatible with the normal operation of the enzyme. The intermediacy of $ABTS^{++}$ appears unlikely therefore in laccase–ABTS oxidations. In contrast, $ABTS^{\bullet+}$ survives easily at the natural pH of laccase (*i.e.*, 4–5). However, our kinetic study with $ABTS^{\bullet+}$ (generated by Co(III)W) shows that this weaker ET oxidant is unable to react with substrates that laccase–ABTS does oxidise. Binding in the active site of laccase does not affect the redox properties of ABTS, as we establish from an electrochemical experiment. If neither $ABTS^{++}$ nor $ABTS^{\bullet+}$ can be regarded as reliable responsibles for the results obtained with laccase–ABTS, then there would be no apparent role for mediation! A shielding effect by the enzyme has been suggested as being capable of protecting either one of the oxidised derivatives of ABTS from hydrolysis, thereby extending their lifetime and making their intervention more likely. However, this implies that the oxidised mediator remains associated with the enzyme, and does not operate freely in solution, thereby disrupting the concept of a *diffusible* mediator.[20]

An explanation for this paradox could be that degradation by-products of either $ABTS^{++}$ or $ABTS^{\bullet+}$ are formed *in situ*, and then react with non-phenolics. For example, $ABTS^{++}$ is reported

to give products of hydrolysis endowed with a redox potential > 1.2 V (Scheme 6),[44] *i.e.*, even *higher* than that of $ABTS^{++}$ (1.1 V). Consequently, 'moderately reluctant' non-phenolics could be oxidised *via* an ET mechanism through the intervention of hydrolytic by-products of $ABTS^{++}$.[40]



Scheme 6 Products of hydrolysis of $ABTS^{++}$, from ref. 44.

Additionally, other degradation fragments of the Med_{ox} species could be radicals, and one cannot exclude the contribution of radical oxidation routes towards very recalcitrant non-phenolics;[40,69a,c] the efficiency of a radical route is in fact not strongly affected by redox features of the substrate.[24,69a]

In conclusion, ABTS-mediated oxidations with laccase, despite being known and quoted as examples of the mediation phenomenon, represent a very complex and still ambiguous case of reactivity. No clear-cut evidence about the nature or structure of the reactive intermediate(s) generated from interaction of the enzyme with ABTS could be unambiguously assessed. It is likely that, depending on the electron richness of the non-phenolic substrate, more that one reactive intermediate and/or more than one oxidation mechanism operate. Further studies are necessary and in progress.

Experimental

Instrumentation

The HI-TECH SFA-12 stopped flow instrument was interfaced to a HP 8453 diode array spectrophotometer; a conventional UV–vis spectrophotometer (Perkin Elmer Lambda 18) was alternatively used. Quartz cells of 1 cm optical path were employed. A VARIAN 3400 Star gaschromatograph, fitted with a 25 m × 0.25 mm methyl silicone (OV5) capillary column, was employed in GC analyses. The identity of the reaction products was confirmed by GC-MS analyses, run on a HP 5892 GC equipped with a 15 m × 0.2 mm methyl silicone gum capillary column, and coupled to a HP 5972 MSD instrument, operating in electron impact at 70 eV.

General

Substrates and products were high purity commercial samples (Aldrich) and were used without further purification. Other precursors and products were available in the laboratory from previous work.[40,69] ABTS was recrystallised from ethanol–water. Samples of $Ce(SO_4)_2$ (*viz.* *Ce(IV)*) and of 12-tungstocobaltate(III) (*viz.*, Co(III)W) were employed as in previous cases.[24,69] MeCN was HPLC grade from Carlo Erba. Buffers were prepared using ultrapure water obtained with a MilliQ apparatus.

Enzyme preparation

Laccase from *Poliporus pinsitus* was kindly donated by Novo Nordisk Biotech; it was purified by ion-exchange chromatography on Q-Sepharose by elution with phosphate buffer; laccase fractions having an absorption ratio A_{280}/A_{610} of 20–30 were considered sufficiently pure.[70] The collected fractions were concentrated by dialysis in cellulose membrane tubing (Sigma) against poly(ethylene glycol) to a final activity of 9000 U mL^{-1}, as determined spectrophotometrically by the standard assay with ABTS, which requires the determination of the initial, linear increase of absorbance of $ABTS^{\bullet+}$ at 420 nm per unit time per mL of purified enzyme (AU s^{-1}).[38] In addition, this laccase-catalysed oxidation was carried out upon varying the concentration of ABTS, and the initial rate of appearance of $ABTS^{\bullet+}$ determined at 420 nm. A linear Lineweaver–Burke plot was thereby obtained (not shown), and the k_{cat} (510 s^{-1}) and K_M (1.1 × 10^{-5} M^{-1} s^{-1}) parameters reckoned from intercept and slope, under the assumption of simple Michaelis–Menten kinetics. Values of K_M clustering in a comparable range have been reported, as an index of the affinity of laccase for other mediators or substrates.[71] In contrast, it was verified that no absorption bands unambiguously pertaining to $ABTS^{++}$ can be detected on mixing suitable amounts of laccase and ABTS solutions, for observation times longer than 0.1 s.

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), containing 4% MeCN, and purged with O_2 for 30 min prior to the addition of the reagents.[24] The initial concentrations were: substrate (5 mM), ABTS (0.5 mM) and an amount of laccase (3 U ml^{-1}) almost stoichiometric with ABTS was used. After 24 h reaction time, GC analyses were performed with respect to an internal standard (acetophenone or *p*-methoxyacetophenone), suitable response factors being determined from authentic products. The yields of oxidation (Table 5) were reckoned *vs.* the molar amount of ABTS.

Kinetic study with $ABTS^{++}$

With reactive substrates the determinations were done with the stopped-flow device, interfaced to a diode-array spectrophotometer. The $ABTS^{++}$ species was generated by fast mixing a 3 × 10^{-4} M solution of $Ce(SO_4)_2$ (*viz.* *Ce(IV)*) and a 1.2 × 10^{-4} M solution of ABTS. Mixtures of H_2SO_4 and of MeCN in different proportions, or also mixtures of different solvents, had been preliminarily tested in order to single out the medium where the spontaneous decay of $ABTS^{++}$ was long enough to run the kinetic study conveniently. The 2 M H_2SO_4–MeCN 5 : 1 mixed solvent was the one selected for the study. In this medium, the k_{decay} of $ABTS^{++}$ was 8 × 10^{-3} s^{-1} ($t_{1/2}$ = 90 s) at 25 °C; it followed a kinetic first-order law, possible due to cleavage of the dication molecule on interaction with water.[44] In solvent mixtures of lower acidity the spontaneous decay of the dication was increasingly faster, and/or the absorption spectrum of $ABTS^{++}$ could not be attained; on the contrary, increasing the acidity above 2 M H_2SO_4 did not give additional advantages. In the kinetic study, pre-mixing of the ABTS and *Ce(IV)* (1 : 2.5 molar ratio) solutions generated $ABTS^{++}$ quantitatively (at the initial concentration of 1.2 × 10^{-4} M), and then this was added to the solution of the substrate by the stopped-flow instrument. The bleaching of $ABTS^{++}$ was time-recorded at 520 nm. The initial concentration of the substrate (1.2 × 10^{-3} M or higher) enabled a pseudo-first-order kinetic treatment of the data. With less reactive substrates, a conventional spectrophotometer was used, the mixed solvent and the initial concentration of the reactants being equal.

Kinetic study with $ABTS^{\bullet+}$

Due to the lower reactivity of $ABTS^{\bullet+}$ with non-phenolic substrates, a conventional spectrophotometer was used. The absorption spectrum of $ABTS^{\bullet+}$ was quantitatively generated in 0.1 M citrate buffer (pH 5) containing 4% MeCN (for solubility reasons) by mixing ABTS (7 × 10^{-5} M) and Co(III)W (9 × 10^{-5} M) solutions. The half-life of $ABTS^{\bullet+}$ is 90 min under these conditions. On rapid addition of the appropriate amount of the solution of substrate (in the 7 × 10^{-3} − 7 × 10^{-2} M range) to preformed $ABTS^{\bullet+}$, the drop of the absorption was time-monitored in the 690–730 nm range. No simple pseudo-first-order kinetic behaviour was obtained, at a 100 : 1 or even 1000 : 1 ratio of the [Subst.] : [$ABTS^{\bullet+}$] initial concentrations, and the kinetic treatment described in the text was followed, in order to extract the kinetic data reported in Table 4.

Determination of the spontaneous decay, and of the redox potential of ABTS$^{\bullet+}$, in the presence of laccase

On the basis of the activity value of the purified laccase, appropriate dilution with the 0.1 M citrate buffer enabled to add an amount of laccase stoichiometric with 83 μmol of ABTS in a cuvette containing 2.35 mL citrate buffer and 0.1 mL MeCN; the solvent mixture had been gently purged with O_2. The concentration of ABTS was 3.3×10^{-5} M, and spectrophotometric observation began. After 5 min, the A_{420} value of ABTS$^{\bullet+}$, expected for that concentration and ε value, had been fully developed. Decrease of that A_{420} value was time-recorded, and the k_{decay} value determined from curve fitting as 2×10^{-6} s^{-1}. This is almost 70 fold slower that the spontaneous decay determined in the absence of laccase.

Cyclic voltammetry was carried out in acetate buffer as previously described,[24] at a 2 mM initial concentration of ABTS. An amount of laccase was added to the ABTS solution that allowed full saturation: in fact, the solution turned blue. By cyclic voltammetry we then reduced the laccase-generated ABTS$^{\bullet+}$ to ABTS, and then re-oxidised it to ABTS$^{\bullet+}$ and further on to ABTS^{++}. No difference in the value of the redox potentials of ABTS could be determined in the presence of the enzyme, with respect to the values obtained without the enzyme. Consequently, binding in the active site of laccase does not affect the redox properties of ABTS.

Product analyses under kinetic conditions

Analysis of the oxidation products by ABTS^{++}, under experimental conditions strictly resembling the kinetic ones, was carried out as follows. In 5 mL of 2 M H_2SO_4 solution, suitable amounts of the *Ce*(IV) salt (4.6 mmol) and of ABTS (2 mmol) were mixed, and the red colour of ABTS^{++} developed immediately. A MeCN solution (1 mL; 20 mmol) of the substrate was quickly added by syringe, and the resulting mixture kept under stirring at room temperature for 3 min, or until the red colour of ABTS^{++} had turned blue–greenish. Addition of an internal standard (either biphenyl or *p*-MeO-acetophenone), conventional workup with ethyl acetate and GC analysis followed. The GC yields were calculated (Table 3) by means of the response factors. GC-MS analysis confirmed the nature of the products. Product analyses for the oxidations with ABTS$^{\bullet+}$ were run analogously, under the following conditions. In 10 mL of a 0.1 M citrate buffer solution (pH 5) containing 4% MeCN, 25 mmol of substrate, 2.5 mmol of ABTS and 3 mmol Co(III)W were dissolved, and kept at room temperature for 24 h. Workup, product analysis and yield determinations were as above.

Determination of the hν_{CT} data

Solutions (in MeCN) of the electron-donor substrate (5×10^{-2} M) and of the electron-acceptor TCNE (5×10^{-3} M) were introduced in the two separate compartments of a cuvette with a septum (1 cm optical path); the spectrum was registered in the 300–800 nm range and memorised. The cuvette was thoroughly shaken, to enable mixing of the two compartments, and the spectrum of the resulting mixture acquired. Electronic subtraction of the memorised spectrum of the separated partners allowed to appreciate the presence and position (λ_{max}) of the charge-transfer band of the donor-acceptor complex.[59] The hν_{CT} data (in eV) were obtained from the relationship $h\nu_{CT} = hc/\lambda_{max}$ (in Table 2).

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