An assessment of the relative contributions of redox and steric issues to laccase specificity towards putative substrates

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Laccases catalyze the one-electron oxidation of a broad range of substrates coupled to the 4 electron reduction of O_2 to H_2O. Phenols are typical substrates, because their redox potentials (ranging from 0.5 to 1.0 V *vs.* NHE) are low enough to allow electron abstraction by the T1 Cu(II) that, although a relatively modest oxidant (in the 0.4–0.8 V range), is the electron-acceptor in laccases. The present study comparatively investigated the oxidation performances of *Trametes villosa* and *Myceliophthora thermophila* laccases, two enzymes markedly differing in redox potential (0.79 and 0.46 V). The oxidation efficiency and kinetic constants of laccase-catalyzed conversion of putative substrates were determined. Hammett plots related to the oxidation of substituted phenols by the two laccases, in combination with the kinetic isotope effect determination, confirmed a rate-determining electron transfer from the substrate to the enzyme. The efficiency of oxidation was found to increase with the decrease in redox potential of the substrates, and the Marcus reorganisation energy for electron transfer to the T1 copper site was determined. Steric hindrance to substrate docking was inferred because some of the phenols and anilines investigated, despite possessing a redox potential compatible with one-electron abstraction, were scarcely oxidised. A threshold value of steric hindrance of the substrate, allowed for fitting into the active site of *T. villosa* laccase, was extrapolated from structural information provided by X-ray analysis of *T. versicolor* lac3B, sharing an identity of 99% at the protein level, thus enabling us to assess the relative contribution of steric and redox properties of a substrate in determining its susceptibility to laccase oxidation. The inferred structural threshold is compatible with the distance between two phenylalanine residues that mark the entrance to the active site. Interaction of the substrate with other residues of the active site is commented on.

Introduction

Laccases (EC 1.10.3.2, *para*-benzenediol:oxygen oxidoreductases) are a family of multi-copper ('blue copper') oxidases widely distributed in nature.[1–3] Although the predominant sources of laccases are fungi and higher plants, laccase activity has recently been documented in some insects and bacteria as well.[4,5] Laccases typically contain histidine-rich copper-binding sites, which coordinate one paramagnetic T1 Cu(II), the blue colour being provided by the charge-transfer band of a strong Cys·S → Cu(II) ligation, and a T2/T3 trinuclear copper cluster.[1,3,6,7] The enzyme catalyzes the one-electron oxidation of four substrate equivalents, coupled to the four-electron reduction of O_2 to water.[1,3,6,7] Abstraction of electron from the substrate takes place at the T1 copper, which is proximal to the substrate binding site, and progressively leads to the reduction of all four Cu(II) ions to Cu(I). Re-oxidation of the cuprous ions to Cu(II) occurs *via* electron donation to an O_2 molecule complexed at the trinuclear T2/T3 cluster, leading to the formation of water without release of toxic peroxide intermediates.[6–8]

What are typical roles of laccases in nature? White-rot fungi excrete laccases, in association with other extracellular oxidases, to degrade complex natural polymers, such as lignin in rotten wood or humic acids.[1,2,9] This is a difficult oxidation, owing to the high redox potential of the polymeric matrices and to the heterogeneity of inter-unit linkages;[10] radical fragments arising from the oxidative degradation are attacked by O_2 and further transformed.[11,12] Interestingly, laccases from plants bring about the opposite process, because they participate in early stages of lignin formation by means of the oxidative polymerisation of monolignols.[1,2,12b] These laccases are also involved in wound response for repairing structural damages in vascular tissue,[1,2] or in the biosynthesis of pigments such as melanin.[13] In all cases, cross-linking of phenolic (or aniline) monomeric precursors takes place by enzymatic aerobic oxidation, and yields polymers through the intermediate formation of radicals. Similarly, oxidative coupling of phenolic precursors with proteins is induced by laccases in insects for cuticle sclerotization.[14]

Phenols play a central role as natural substrates of laccases in view of their redox potential, which is conveniently low and in the 0.5–1.0 V *vs.* NHE (normal hydrogen electrode) range.[15] This enables electron abstraction by the T1 Cu(II) ion, a relatively modest oxidant (0.4–0.8 V, depending on the enzyme source),[16,17] and the ensuing formation of phenoxyl radicals (Scheme 1).

Less straightforward is to understand how the stereochemical problem inherent to the interaction of fungal laccases with a

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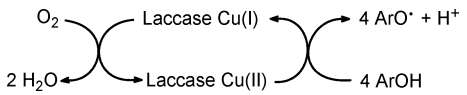

Scheme 1 The catalytic cycle of laccase.

structurally complex polymer such as lignin may be solved.[1] Despite the fact that lignin is a macromolecule where phenolic residues amount to *ca.* 20% of the total,[18] steric hindrance ought to prevent bulky lignin to access the active site of laccase in order to be oxidatively degraded. To cope with this steric problem, the concept of 'mediators' of the enzymatic activity has been suggested.[19] More specifically, easily oxidisable small-sized substances, once mono-electronically oxidised by laccase, are held to mediate the 'indirect' oxidation of lignin outside the enzymatic active site,[20,21] by resorting to either a monoelectronic or a radical hydrogen-abstraction pathway of oxidation.[21,22] In particular the radical route, which is not accessible to laccase, allows the degradation of non-phenolic benzyl alcohols, which comprise more than 75% of lignin residues[18] but that are not susceptible to monoelectronic oxidation by T1 Cu(II).[21,23] While the use of a few 'non-natural' redox mediators is fairly well established for this task,[19–22] a few phenolic compounds have recently shown proficiency in the mediation process, thereby emerging as likely 'natural' mediators of laccase.[21,24–26] Consequently, the steric hindrance of lignin, as well as the redox inadequacy of laccase towards non-phenolic compounds, can be solved by the use of mediators, thereby assigning an expanded role as delignifying agent to this enzyme in nature.

With regard to the enzymatic oxidation of monomeric putative substrates, such as simple phenols and anilines, the attention has been so far focussed mainly on the redox issue. Interpretation of reactivity data in a broader context has been constrained by the limited information on the enzyme structure. The currently increased availability of crystallographic data on fungal laccases might undoubtedly contribute to shedding light on many factors underlying the reactivity of these enzymes, with particular regard to steric issues with enzyme–substrate interactions.

For these reasons, the present study was aimed at investigating the relative contribution of both redox and steric properties of several putative substrates in determining their susceptibility to laccase oxidation, with specific reference to crystallographic information recently disclosed for a few laccases.[4a,27–29] The study was conducted with both *Trametes villosa* and *Myceliophtora thermophila* laccases, which markedly differ in redox potential properties (0.79 and 0.46 V, respectively).[16,17] The oxidation efficiency and kinetic constants for the laccase-catalyzed oxidation of substituted phenols and anilines were determined; the effect of the substituents and of the redox potential of the substrates upon the enzymatic reactivity was appraised within the frame of the Hammett and Marcus equations. Particular attention was devoted to specific substrates that are characterized by similar redox potentials but differ in steric hindrance, with the aim of determining a threshold of substrate size compatible with proper fitting into the enzymatic pocket. With this regard, the important role of some aminoacidic residues in the active site of the enzyme has been pinpointed. The results of this study allow a better understanding of structural features of putative substrates of laccase that are relevant for oxidation efficiency, but might also lead to devising phenolic mediators that are better tailored for the oxidative degradation of non-phenolic substrates, for synthetic purposes.

Results and discussion

Redox requirements of oxidation

A series of substituted phenols and anilines, which also includes two non-phenolic substrates, has been taken as a tool to appraise the redox requirements of enzymatic oxidation; the redox potential value across the series of substrates spans a sizeable range (from 0.5 to 1.9 V/NHE).[15] The oxidation has been performed in distinct experiments with two fungal laccases, namely, *Trametes villosa* (TviL) and *Myceliophthora thermophila* (MtL) laccases, markedly differing in redox potentials (0.79 and 0.46 V *vs.* NHE, respectively).[16,17] The same amount of activity units of the two enzymes, and the same concentration of the putative substrate have been used in each experiment, and the aerobic oxidation performed in 0.1 M sodium citrate at pH 5.0 (buffer A) at room temperature for 24 h (Table 1). In some cases a 2 : 1 buffer A : dioxane mixed solvent has been used to increase the solubility of hydrophobic substrates, at the expense of a moderate reduction of enzymatic activity.[30] Following the 24 h incubation, the extent of oxidation was inferred by determining the residual substrate concentration through gas-chromatography. This approach is, in fact, easier and less ambiguous than that based on determining the extent of end-products formation, because monoelectronic oxidation pathways with these substrates typically give rise to oligomers of uncertain structure.[11,12,24] The results are illustrated in Fig. 1, where the oxidation extent of the putative substrate by both MtL and TviL is shown as a function of the substrate redox potential.

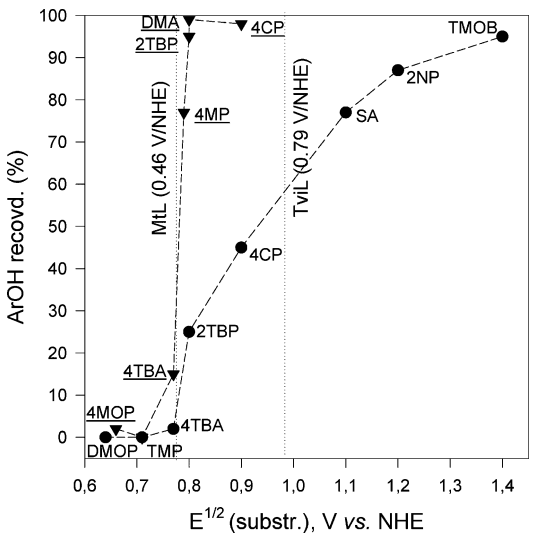

Fig. 1 Extent of recovery (%) of the substrate under aerobic oxidation by either *Trametes villosa* (TviL, ●) or *Myceliophthora thermophila* (MtL, ▼) laccase. Substrates code (underlined for MtL): DMOP, 2,6-diMeO-phenol; 4MOP, 4-MeO-phenol; TMP, 2,4,6-triMe-phenol; 4TBA, 4-Bu^t-aniline; 2TBP, 2-Bu^t-phenol; 4MP, 4-Me-phenol; DMA, 2,6-diMe-aniline; 4CP, 4-Cl-phenol; SA, 2-OH-benzaldehyde *viz.* salicyl aldehyde; 2NP, 2-NO_2-phenol; TMOB, 1,3,4,5-tetraMeO-benzene.

Table 1 Aerobic oxidation of a series of substrates with either *Trametes villosa* (TviL) or *Myceliophthora thermophila* (MtL) laccases, in buffered (0.1 M sodium citrate, pH 5; buffer A) water solution at room temperature for a 24 h reaction time. The extent of recovery of the substrate is given (%)

		Recovered substrate (%)[d]	
Substrate	$E^{\frac{1}{2}}$ (V *vs.* NHE)[a] in H_2O	with TviL	with MtL
4-OH-phenol (hydroquinone)	0.48	<1	<1
2,6-Dimethoxyphenol	0.57	<1	n.d.
4-MeO-phenol	0.66	<1	<1
2,4,6-Trimethylphenol	0.70	<1	<1
4-(Bu^t)-aniline	0.77	1	17
4-Me-aniline	0.79	<1	n.d.
4-Me-phenol	0.79	<1	75
2-(Bu^t)-phenol	0.81	25	95
4-(Bu^t)-phenol	0.82	<1	n.d.
2,6-Dimethylaniline	0.83	n.d.	99
β-Naphthol[b]	0.85	2	n.d.
4-Cl-phenol	0.90	45	100
2-OH-benzaldehyde	*ca.* 1.1	77	n.d.
2-NO_2-phenol	1.2	87	n.d.
2-NO_2-aniline	1.2	87	n.d.
1,3,4,5-Tetramethoxybenzene[b]	1.4	90	n.d.
N-Phenyl-acetamide[b]	1.84[c]	89	n.d.
2,4-Dinitroaniline[b]	>1.9	95	n.d.

[a] Redox potential of the substrate, from ref. 15. [b] In a 2 : 1 buffer A : dioxane mixed solvent. [c] Determined by cyclic voltammetry (E^p value) in MeCN; this work. [d] Abbreviation (n.d.) stands for 'not determined'. Average error in the determinations: ±4%.

Fig. 1 clearly shows that as the redox potential of the substrate rose, the oxidation efficiency of MtL markedly decreased up to a point where the substrate was quantitatively recovered. The inflection point in this sort of 'titration curve' of the oxidation efficiency (dotted line in Fig. 1) was located slightly above the redox potential value of MtL (0.46 V), indicating that the oxidation process may be endoergonic by no more than 0.2–0.3 V,[31,32] in contrast to previous claims where oxidation processes that are endoergonic by at least 0.7 V or more were considered to be feasible.[33] Analogously, the stronger oxidant TviL exhibited a sigmoid behaviour with an inflection point approximately located 0.2–0.3 V above the redox value of the enzyme (0.79 V). Clearly, substrates that were not oxidised by MtL, such as 4-Me-phenol, 2,6-diMe-aniline or 4-Cl-phenol ($E^{1/2}$ 0.79, 0.80, 0.90 V, respectively),[15] were fully or extensively oxidised by TviL, thereby stressing the need for a good match between the redox features of the enzyme and of the substrate.[31,32]

This qualitative information on the oxidation efficiency, inferred by the percentage recovery of the substrate, was integrated and confirmed by the determination of the kinetic constants of a set of significant phenols (Table 2).

In particular, the oxidation efficiency of both enzymes, expressed by the specificity constant parameter k_{cat}/K_m, was found to decrease with the increase in redox potential values of the substrates under study. Dramatic drops by about two and three orders of magnitude were evident for TviL and MtL, respectively, as redox potential values of substrates increased from 0.57 to 0.90 V. The lower oxidation efficiency of MtL with respect to TviL, due to the lower E° value of the former enzyme, was also confirmed by comparing the absolute values of k_{cat}/K_m ratios for each substrate, which were uniformly smaller for the former laccase. The trends of the two kinetic constants V_{max} and K_m were also revealing. In particular, the K_m parameter increased steadily for both TviL and MtL within the substrates series, thereby suggesting an increasingly lower affinity of the two enzymes for the less reducing substrates. It is interesting to note that a better affinity of both laccases for a phenol than for an aniline was evident by comparing the K_m values of 2,6-diMe-phenol and 2,6-diMe-aniline, despite a very similar redox potential, in agreement with a previous study.[25b] The V_{max} values of TviL did not markedly vary along the substrates series, until dropping by a factor of around 2 in the case of phenol ($E^{\frac{1}{2}}$ 0.88 V) and 4-Cl-phenol ($E^{\frac{1}{2}}$ 0.90 V), where abstraction of electron becomes endoergonic. With MtL, the drop of V_{max} values was more pronounced along the series (*i.e.* up to two orders of magnitude), being already perceptible for substrates having $E^{\frac{1}{2}}$ value ≥ 0.8 V. Our kinetic data compare favourably with those that were determined for the same substrates by Xu,[32] and comply with the general notion that the less reactive a reagent (an enzyme, in our case), the more selective it is.

Hammett-like correlation

For a subset of 4-X-substituted phenols (from Table 2), the specificity constants of oxidation by the two enzymes were plotted in logarithmic form *vs.* the σ^+ parameter of the X-substituent according to the relationship (eqn (1)).[34]

$$\log (k_{cat}/K_m)_X = \rho\sigma^+ \quad (1)$$

Linear correlations were found for both enzymes (Fig. 2), and the ρ parameters obtained from the slope of the plots were −2.6 for TviL and −3.0 for MtL. The finding that the σ^+ parameters correlated better than the Hammett σ parameters (data not shown), in addition to the negative sign of the ρ values, were clues indicating an oxidation process where a positive charge develops on the phenolic substrate in the rate-limiting step. The positively charged intermediate is the radical cation of the phenol, arising from rate-limiting one-electron abstraction. Electron-donor substituents, by stabilising the radical cation, lower the transition state energy and speed up the enzymatic oxidation, whereas electron-withdrawing substituents do retard it. The phenolic radical cation subsequently

Table 2 Kinetic constants of TviL and MtL as measured from determining the rate of oxygen uptake during the enzymatic oxidation of selected substrates in buffer A solution at 25 °C[a]

Substrate ($E^{\frac{1}{2}}$, V *vs.* NHE, in H_2O)[b]	TviL				MtL			
	K_m (mM)	V_{max} IU (mg protein × min)$^{-1}$	k_{cat} (min^{-1})	Specificity constant k_{cat}/K_m (min^{-1} M^{-1})	K_m (mM)	V_{max} IU (mg protein × min)$^{-1}$	k_{cat} (min^{-1})	Specificity constant k_{cat}/K_m (min^{-1} M^{-1})
Hydroquinone (0.48)	0.13	22	1513	1.16×10^7	0.75	11	932	1.24×10^6
2,6-DiMeO-phenol (0.57)	0.14	24	1690	1.21×10^7	0.38	13	1100	2.90×10^6
4-MeO-phenol (0.66)	0.19	15	1053	5.54×10^6	0.91	8.5	742	8.15×10^5
2-MeO-phenol (0.70) (guaiacol)	0.13	19	1324	1.02×10^7	0.45	12	1014	2.25×10^6
2,6-diMe-phenol (0.75)	0.72	28	1966	2.73×10^6	6.1	6.3	535	8.76×10^4
4-Me-phenol (0.79) (*p*-cresol)	1.8	18	1260	7.01×10^5	8.2	3.1	261	3.19×10^4
2-(Bu^t)-phenol (0.81)	5.9	13	940	1.58×10^5	3.9	0.30	26	6.62×10^3
Phenol (0.88)	12	10	720	5.90×10^4	8.4	0.28	24	2.83×10^3
2,6-DiMe-aniline (0.83)	10	21	1500	1.47×10^5	15	0.62	53	3.61×10^3
4-Cl-phenol (0.90)	9.2	9.4	660	7.16×10^4	7.8	0.12	10	1.35×10^3
4-Ph-phenol (0.78)	0.61	11	774	1.27×10^6	n.d.	n.d.	—	—
4-CHO-phenol (1.04)	45	0.16	114	2.53×10^3	n.d.	n.d.	—	—

[a] Maximum relative standard deviations were in the 4-to-8% range. [b] Redox data from ref. 15

undergoes fast deprotonation to yield the phenoxyl radical. To our knowledge, this is the first Hammett correlation obtained for the oxidation of phenols by laccases. Keeping in mind the reactivity–selectivity principle, the effect of substituents is expected to play a more noticeable role with the weaker oxidant MtL, thereby explaining the slightly larger value of its ρ parameter.

Driving force of the oxidation step

Electron-donor substituents lower the redox potential value of the phenols,[15] thereby making the enzymatic oxidation easier. It is therefore expected that a reactivity parameter, such as the (k_{cat}/K_m) ratio (data from Table 2), follows the trend of redox potential of the substrate if the rate-determining step is the electron-transfer from the phenolic substrate to the electron-acceptor T1 Cu(II) site of the enzyme. Fig. 3 shows that this expectation is fairly well satisfied with both TviL and MtL, confirming that electron-rich phenols are oxidised faster, while the electron-poorer ones react more slowly. A similar linear dependence of laccase oxidation efficiency *vs.* the ΔE in redox potential between the substrate and the T1 Cu center had already been found by Xu.[32]

Comparison of the oxidation efficiency of the enzymes with that of a polyoxometalate

We have recently published kinetic data for the oxidation of a series of substituted phenols by vanadium(V) polyoxotungstate,[35] *i.e.*, [α-$SiV^VW_{11}O_{40}]^{5-}$. This polyoxometalate (POM) is endowed with a redox potential of 0.67 V/NHE,[35] which is almost intermediate between those of MtL and TviL. POMs are considered to provide unambiguous examples of outer-sphere oxidants, because the redox center in these anionic electron acceptors is deeply buried within the oxometalate Keggin cage.[36] A Hammett ρ value (*i.e.*, −3.1) comparable to those of the present enzymes was obtained for the vanadium(V) POM,[35] and the rate constants of oxidation analogously found to increase with the decrease in redox potential for the same series of substituted phenols, giving a slope (*i.e.*, −10) intermediate in value between those of MtL and TviL (*cf.* Fig. 3). On the basis of the comparable value of redox potential, the POM compound can be considered to provide a legitimate approximation of the reactivity of fungal laccases in the electron-transfer oxidation of phenols.[35] Compelling evidence in favour of a rate-determining outer-sphere electron-transfer oxidation is therefore granted for the enzymes as well, because the reduction potential difference (ΔE) between the donor phenolic substrate and the acceptor site dominates the reactivity trend: this holds both for an inorganic electron acceptor, such as the V(V) POM, and for the T1 Cu(II) site of the metalloenzymes of the present study. Additional and more insightful evidence that ΔE provides the driving force of the enzymatic reaction has been acquired by analysing the kinetic data of oxidation according to the Marcus theory framework and the related quadratic eqn (2).[37]

$$\Delta G^{\neq} = (\lambda/4)(1 + \Delta G^{\circ}/\lambda)^2 \quad (2)$$

The Marcus equation in fact provides a quantitative relationship between the electron transfer reactivity ($\Delta G^{\neq}$) and the thermodynamic driving force (ΔG°) to the transfer of electron between a donor and an acceptor species.[35,37] We have taken the specificity constant ratios k_{cat}/K_m of TviL, which are more numerous (from Table 2), and converted them into $\Delta G^{\neq}$ according

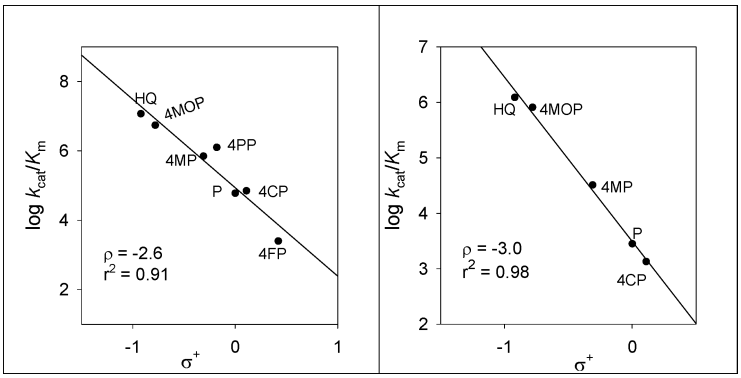

Fig. 2 Hammett plot for TviL (left) and MtL (right) for the aerobic oxidation of substituted phenols at 25 °C. Substrates code: HQ, hydroquinone; 4MOP, 4-MeO-phenol; 4MP, 4-Me-phenol; 4PP, 4-Ph-phenol; P, phenol; 4CP, 4-Cl-phenol; 4FP, 4-CHO-phenol.

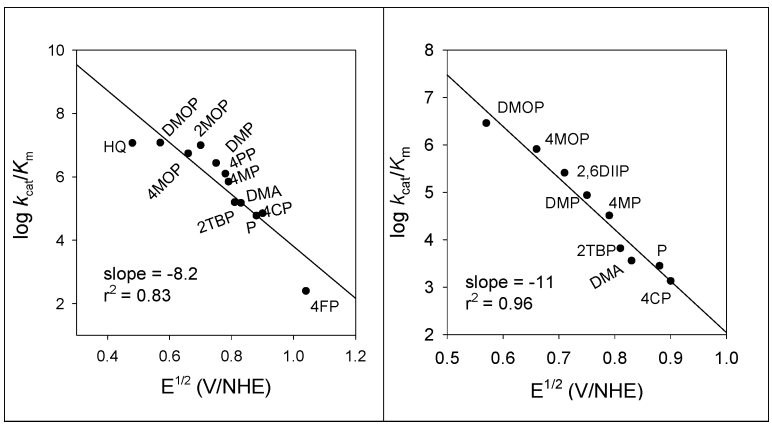

Fig. 3 Efficiency of oxidation by TviL (left) and by MtL (right) as a function of the redox potential of the substrate. Substrates code: HQ, hydroquinone; DMOP, 2,6-diMeO-phenol; 2MOP, 2-MeO-phenol; 4MOP, 4-MeO-phenol; DMP, 2,6-diMe-phenol; 4PP, 4-Ph-phenol; 4MP, 4-Me-phenol; 2TBP, 2-Bu^t-phenol; DMA, 2,6-diMe-aniline; P, phenol; 4CP, 4-Cl-phenol; 4FP, 4-CHO-phenol.

Table 3 Experimental specificity constants (from Table 2), ΔG° and $\Delta G^{\neq}$ data for the oxidation of substituted phenols by TviL ($E^\circ = 0.79$ V) in buffer A at 25 °C

ArOH	$E^{\frac{1}{2}}$ of ArOH V *vs.* NHE	ΔG°, in kcal mol^{-1} [a]	Specificity constant k_{cat}/K_m (min^{-1} M^{-1})	$\Delta G^{\neq}$, in kcal mol^{-1} [b]
4-Cl-phenol	0.90	2.54	7.16×10^4	10.83
Phenol	0.88	2.08	5.90×10^4	10.98
2,6-DiMe-aniline	0.83	0.92	1.47×10^5	10.44
2-(Bu^t)-phenol	0.81	0.46	1.58×10^5	10.40
4-Me-phenol	0.79	0	7.01×10^5	9.52
4-Ph-phenol	0.78	−0.23	1.27×10^6	9.16
2,6-DiMe-phenol	0.75	−0.92	2.73×10^6	8.71
2-MeO-phenol	0.70	−2.08	1.02×10^7	7.93
4-MeO-phenol	0.66	−3.00	5.54×10^6	8.29
2,6-DiMeO-phenol	0.57	−5.07	1.21×10^7	7.83
4-OH-phenol	0.48	−7.15	1.16×10^7	7.85

[a] From $\Delta E = E_{TviL} - E_{ArOH}$, and converted into kcal mol^{-1} ($\Delta G^\circ = -nF\Delta E$) (1 V = 23.06 kcal mol^{-1}). [b] From $k_{cat}/K_m = 6.7 \times 10^{12} \exp(-\Delta G^{\neq}/RT)$ at 25 °C.

to the relationship ln (k_{cat}/K_m) = ln $(k_B T/h) - \Delta G^{\neq}/RT$. The $\Delta E = E_{TviL} - E_{ArOH}$ for the phenolic substrates of the series have been converted into ΔG° data (given in Table 3; $\Delta G^\circ = -nF\Delta E$, where F is the Faraday constant) on taking 1 V = 23.06 kcal mol^{-1}.

Fig. 4 shows that the experimental $\Delta G^{\neq}$ data points are interpolated by a theoretical $\Delta G^{\neq}$ curve calculated from eqn (2) according to the ΔG° data of Table 3 whenever a reorganisation barrier λ_{calc} = 39 kcal mol^{-1} is adopted for the electron-acceptor T1 Cu site. A much larger reorganisation energy (*i.e.*, λ = 79 kcal mol^{-1}) was instead required for fitting the oxidation data of the same substituted phenols by vanadium(V) POM in a Marcus plot.[35] Because λ_{calc} is given by eqn (3), as adapted to the present case,

$$\lambda_{calc} = (\lambda_{TviL} + \lambda_{ArOH})/2 \quad (3)$$

if one takes the λ_{ArOH} employed for the oxidation of substituted phenols with vanadium(V) POM (*i.e.*, 63 kcal mol^{-1}),[35]

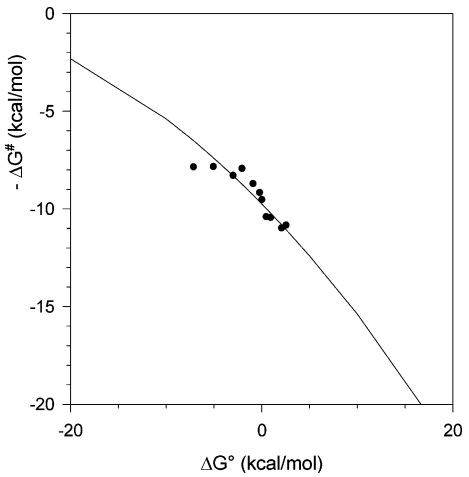

Fig. 4 Marcus plot for the oxidation of substituted phenols by TviL at 25 °C. The calculated curve (from eqn (2)) interpolates the experimental kinetic data for $\lambda = 39$ kcal mol^{-1}.

a $\lambda_{TviL} = 15$ kcal mol^{-1} comes out for the enzyme as opposed to the λ_{POM} of 85 kcal mol^{-1} determined in the previous study for POM.[35] This finding is in line with the expectation that the reorganisation energy (λ) of biological metal-containing electron-transfer sites has a lower value than that of synthetic metal-complexes[6a,8a,38,39] despite comparable redox features, because the T1 Cu site environment ensures minimal changes upon redox interconversion.[39d] This is the first Marcus treatment applied to the oxidation of phenols by the copper-enzyme laccase, and our experimentally determined λ_{TviL} value corroborates figures (in the 10–25 kcal mol^{-1} range) obtained with other enzymes.[38,39]

Kinetic isotope effect determination

The V_{max} for the oxidation of 2,6-diMeO-phenol by TviL has been determined spectrophotometrically (see Experimental) in buffered (0.1 M AcONa, pH 5) solution in H_2O at room temperature, and compared with the V_{max} value analogously measured in a separate experiment in D_2O solution buffered at pH 5 with AcONa–AcOD. Rapid exchange of D for H within the phenolic substrate in D_2O solution is ensured. The k_H/k_D ratio, obtained from the $V_{max}^{(H_2O)}/V_{max}^{(D_2O)}$ ratio, was found to be 1.4 ± 0.1. This 'solvent kinetic isotope effect' is sufficiently small in value and close to 1 to support a rate-determining electron-transfer mechanism (k_{ET}, in eqn (4)) from the reducing substrates to the T1 copper site of TviL, followed by fast deprotonation (or, de-deuteration) of the intervening radical cation of the substrate (2,6-diMeO-phenol in this case).

$$\mathrm{ArOH\,(D)} \xrightarrow{k_{ET}} \mathrm{ArOH\,(D)^{\bullet+}} \xrightarrow[\text{fast}]{} \mathrm{ArO\bullet + H\,(D)^{+}} \qquad (4)$$

This is the first kinetic isotope effect determination reported for a laccase, and complements the above assessments upon the mechanism of the enzymatic oxidation of phenols. The present result nicely compares with the 'solvent kinetic isotope effect' $k_H/k_D = 1.1$ analogously measured for the oxidation of 4-MeO-phenol by vanadium(v) POM, it being a 'redox functional model' of laccase.[35]

Steric problems with 'putative' substrates

For a successful enzymatic oxidation, the redox features of the substrate is not the only point that matters, because structural features are also important to enable a profitable interaction with the active site.[40] From this point of view, fundamental information deriving from the crystal structures of *Trametes versicolor* laccase,[27] as well as for two laccase-substrate complexes,[28,29a] has enabled us to tackle steric features of the laccase active site. The substrate-binding site of *Trametes versicolor* laccase is a negatively charged pocket near the T1 Cu, *i.e.*, the electron entry port.[28] Hydrophobic residues (Phe162, Leu164, Phe265) delineate one side of the binding pocket, and additional ones (Phe332 and 337, and Pro391) constitute the opposite wall (Fig. 5). The entrance path of the substrate is 'marked' by two phenylalanine residues (Phe332 and 265), which are 10.8 Å far apart[28] (not including the van der Waals radii of the H-atoms). Once the substrate fits in, it needs to approach His458, the electron-acceptor ligand of T1 Cu. The His458 is 6.5 Å recessed from the enzyme surface, and this is another structural restriction that a bulky substrate ought to overcome to be oxidised.[27,28]

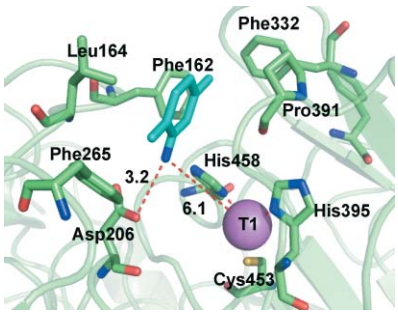

Fig. 5 View of the active site of *Trametes versicolor* laccase (distances in Åw), which binds substrate 2,5-dimethylaniline (in blue), elaborated with *PyMol* from the crystallographic structure, PDB code 1KYA (ref. 28).

We have taken a series of phenols and anilines progressively more hindered in the *ortho* positions, and subjected them to aerobic oxidation with the stronger oxidant TviL under the experimental conditions previously described. Table 4 reports the consumption of substrate upon laccase oxidation, where quantitative recovery means lack of oxidation after the 24 h reaction time. Reluctance by a few putative substrates to be oxidised by TviL clearly emerged. For example, the sterically encumbered 2,4,6-tri(Bu^t)phenol, that is 11.8 Å wide (*i.e.*, the distance between the farthest methyls of the two *ortho*-Bu^t groups, including the vdW radii) and 10.5 Å long, was not oxidised, whereas 4-(Bu^t)phenol and even 2,4,6-trimethylphenol, which is bulky but less wide (9.1 Å) than 2,4,6-tri(Bu^t)phenol, were fully oxidised. The redox potentials of 2,4,6-trimethylphenol and 2,4,6-tri(Bu^t)-phenol are the same (*i.e.*, 0.7 V),[15] so that monoelectronic oxidation by TviL ($E°$ 0.79 V) had to be feasible in both cases (*cf.* previous sections), and solubility problems can be rebuffed due to the mixed solvent (water–dioxane) employed.[24b,30] Similarly, 2-(Bu^t)phenol and 2,6-di(Pr^i)phenol, being 10.3 and 10.9 Å wide, respectively, and wider than 2,4,6-trimethylphenol but less than 2,4,6-tri(Bu^t)phenol, were oxidised although not quantitatively. Likewise, the encumbered 3,5-di(Bu^t)phenol, 2,4,6-tri(Bu^t)aniline

Table 4 Aerobic oxidations with TviL in 2 : 1 buffer A : dioxane, for a 24 h reaction time at room temperature. The extent of recovery of the substrate is given

Substrate	$E^{\frac{1}{2}}$ (V *vs.* NHE)[a]	Substrate width/Å[b]	Recovered substrate (%)[c]
2,4,6-Tri(Bu^t)phenol	0.70 (in H_2O)	11.8	99 ± 2
2,4,6-Tri(Bu^t)phenol			≤1[d]
4-(Bu^t)phenol	0.82 (in H_2O)	6.7	≤1
4-(Bu^t)phenol			50 ± 2 (pH 3)
2,4,6-Tri(Me)phenol	0.71 (in H_2O)	9.1	≤ 1
2-(Bu^t)phenol	0.81 (in H_2O)	10.3	27 ± 2
2,6-Di(Pr^i)phenol	0.71 (in H_2O)	10.9	5 ± 2
2,6-Di(Pr^i)phenol			23 ± 2 (pH 3)
3,5-Di(Bu^t)phenol	0.71 (in H_2O)	11.7	92 ± 2
2,4,6-Tri(Bu^t)aniline	0.82 (in MeCN)	11.8	98 ± 2
3,5-Di(Bu^t)aniline	0.80 (in MeCN)	11.7	95 ± 2
4-(Bu^t)aniline	0.78 (in MeCN)	6.7	5 ± 1
2,6-Di(Me)aniline	0.83 (in MeCN)	9.1	2 ± 1

[a] From ref. 15. [b] Including the vdW radii of the H-atoms; obtained by semiempirical calculations. [c] Average error in the determinations: ±4%. [d] In the presence of mediator ABTS.

and 3,5-di(Bu^t)aniline were not oxidised, whereas 4-(Bu^t)aniline and 2,6-di(Me)aniline were extensively oxidised.

The widths of phenols and anilines, all having a redox potential suitable for one-electron removal by TviL,[15] are reported in Table 4. Substrates too wide to fit in the binding pocket were found to be unreactive, and Fig. 6 points out graphically how the maximum width (*i.e.*, <11–12 Å) tolerated in a substrate can be inferred, beyond which no oxidation takes place.

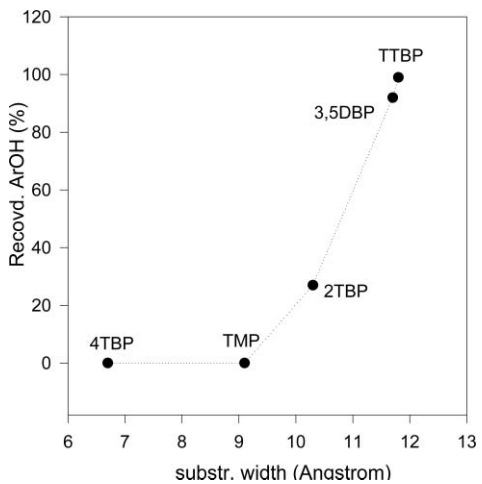

Fig. 6 Extent of recovery of encumbered phenols as a function of their width (in Å) in the aerobic oxidation by TviL. Substrates code: 4TBP, 4-Bu^t-phenol; TMP, 2,4,6-triMe-phenol; 2TBP, 2-Bu^t-phenol; 3,5DBP, 3,5-diBu^t-phenol; TTBP, 2,4,6-triBu^t-phenol.

This steric threshold is fairly compatible with the 11 Å distance between the two phenylalanines Phe332 and 265 that mark the entrance to the active site,[28] as we deduce it from the 3D structure of *Trametes versicolor* laccase. Interestingly, the reluctant 2,4,6-tri(Bu^t)phenol becomes fully consumed by TviL, and is converted into quinone and oligomeric products, whenever the oxidation is mediated by ABTS. In fact, TviL oxidises the redox mediator to ABTS$^{\bullet+}$ (E° 0.69 V),[23] which in turn oxidises the bulky substrate by electron abstraction outside the binding pocket,[24b] thus bypassing the steric restrictions of the phenylalanines 'gate', and fulfilling the requirement of being a mediator of the enzymatic activity.[40]

The reactivity problems observed for bulky substrates, and illustrated in Table 4 and Fig. 6, were further confirmed by measuring the kinetic constants of oxidation of four sterically hindered phenols by both TviL and MtL. The kinetic results shown in Table 5 are compared with those of 4-Me-phenol, taken as a non-encumbered reference compound. On going from 2,6-di(Pr^i)phenol to 2,4,6-tri(Bu^t)phenol the specificity constant of oxidation by TviL drops by three powers of ten, despite the equal redox potential value of the two substrates. The limiting factor is therefore the steric hindrance in 2,4,6-tri(Bu^t)phenol, that is *ca.* 1 Å wider than 2,6-di(Pr^i)phenol. As a consequence of the similar hindrance of 2-(Bu^t)phenol and 2,6-di(Pr^i)phenol, their k_{cat}/K_m values did not markedly differ from one another. The efficiency of oxidation of both 2-(Bu^t)phenol and 2,6-di(Pr^i)phenol was lower than that of 4-Me-phenol: this underlines the leading factor represented by steric hindrance in *ortho*, which hampers entrance into the active site. Consistently, a *t*-Bu substituent in *para* to the substrate ought to play a negligible role upon binding, as the similar k_{cat}/K_m ratios of 2,4,6-tri(Bu^t)phenol *vs.* 2,6-di(Bu^t)phenol do confirm. The results obtained with MtL are instead more difficult to disentangle, as this enzyme is a weaker oxidant. In fact the k_{cat}/K_m ratio for the unhindered 4-Me-phenol gave a lower value than that of the hindered 2,6-di(Pr^i)-phenol. Here the oxidation of the former substrate by MtL is more endoergonic than that of the latter, and the difference in redox potential between the two phenols offsets their difference in steric hindrance. Consistently, 2-(Bu^t)phenol appeared to be far less reactive than 2,6-di(Pr^i)phenol with MtL, as opposed to the outcome with TviL, because the redox potential of 2-(Bu^t)phenol is hardly matched by MtL. Finally, problems of very low reactivity prevented the experimental determination of the kinetic parameters for oxidation of 2,6-di(Bu^t)phenol and 2,4,6-tri(Bu^t)phenol with MtL.

Docking of the substrate

Crystallographic studies hint to a crucial role played by another aminoacidic residue upon enzymatic oxidation. This residue is Asp206, which is located at the rear wall of the binding

Table 5 Kinetic constants of TviL and MtL from determining the rate of oxygen uptake during the enzymatic oxidation of hindered substrates at 25 °C, in 2 : 1 buffer A : dioxane[a]

Substrate ($E^{\frac{1}{2}}$, V *vs.* NHE, in H_2O)[b]	TviL				MtL			
	K_m (mM)	V_{max} IU (mg protein × min)$^{-1}$	k_{cat} (min^{-1})	Specificity constant k_{cat}/K_m (min^{-1} M^{-1})	K_m (mM)	V_{max} IU (mg protein × min)$^{-1}$	k_{cat} (min^{-1})	Specificity constant k_{cat}/K_m (min^{-1} M^{-1})
2,6-Di(Pr^i)phenol (0.71)	1.4	5.3	370	2.70×10^5	2.7	8.2	694	2.56×10^5
2-(Bu^t)phenol (0.81)	5.9	13	939	1.59×10^5	3.9	0.30	26	6.62×10^3
2,6-Di(Bu^t)phenol (0.72)	33	0.24	17	0.52	n.d.	n.d.	n.d.	—
2,4,6-Tri(Bu^t)phenol (0.70)	50	0.16	11	0.22	n.d.	n.d.	n.d.	—
4-Me-phenol (0.79)	1.8	18	1260	7.08×10^5	8.2	3.1	260	3.19×10^4

[a] Maximum standard errors were in the 4-to-8% range. [b] Redox data from ref. 15.

pocket.[27,28,29a] Being deprotonated (pK_a 3.9)[28] at the pH of our experiments (*i.e.*, 5), it confers a negative charge to the active site. Substrates that bear the –OH or –NH_2 functionality, and that are not too wide for the 'gate' delimited by the two phenylalanines, are recognised and dragged inside by a specific hydrogen bonding interaction with Asp206. This interaction imposes a directionality in the docking of the substrate to the His458 ligand, where subsequent electron-transfer to T1 Cu will take place.[7] The successful oxidation of both 4-(Bu*t*)phenol and 4-(Bu*t*)aniline (*cf.* Table 4) confirms that a single substituent in the *para* position, even as bulky as the *t*-Bu group, does not impair access of the substrate because the directional docking to Asp206 causes the bulky substituent to point outside the binding cavity. In contrast, two *t*-Bu groups in *meta* positions obstruct the approach and prevent the satisfactory π-stacking interaction of both 3,5-di(Bu^t)aniline and 3,5-di(Bu^t)phenol with His458, no electron-abstraction taking place (Table 4). One *t*-Bu group in *ortho* is certainly more sterically demanding than in *para*, and this steric perturbation is comparable with, or even more pronounced than two i-Pr groups in *ortho* (*cf.* Table 5).

The efficiency of oxidation by laccase changes markedly when pH conditions other than the physiological ones (*i.e.*, pH 5) are adopted.[17b,41a] The role of Asp206 in this juncture is again important. At pH 3, Asp206 (pK_a = 3.9)[28] is not deprotonated and, as a neutral residue, drags a polar substrate inside less efficiently. The monoelectronic oxidation of phenols by laccase is thereby delayed,[41,42] as the lower conversion found at pH 3 for both 4-(Bu^t)phenol and 2,6-di(Pr^i)phenol confirmed (underlined results in Table 4). The recognition role of anionic Asp206 towards phenolic substrates is neatly supported by recent data acquired with mutants of *Trametes versicolor* laccase, where Asp206 was replaced by other aminoacidic residues.[42] In particular, when glutamate replaced aspartate in position 206, an efficiency of oxidation (expressed by k_{cat}/K_m) comparable to that of the Asp206-containing wild-type was determined towards 2,6-dimethoxyphenol.[42] Because glutamate is deprotonated at pH 5 as much as aspartate is, a comparable recognition proficiency of the active site is expected. In contrast, when the polar but not anionic asparagine replaced aspartate, the k_{cat}/K_m ratio for oxidation of 2,6-dimethoxyphenol decreased substantially, the same occurring on substituting Asp206 with the apolar alanine.[42]

Conclusions

The dependence of the oxidation proficiency of laccase from redox features of the substrate as well as from its steric hindrance has been investigated in the case of TviL and MtL. The extent of recovery of the substrate from the oxidation, and the determination of kinetic parameters by measuring the oxygen uptake rate, have been taken as an index of the enzymatic efficiency in the oxidation of significant phenolic substrates. The redox proficiency of the two enzymes has been comparatively assessed, and the stronger oxidant TviL came out as more reactive. Mechanistic insight, provided by a typical physical–organic approach based on Hammett-like correlations and kinetic isotope effect determination, which was acquired for the first time for laccases, confirms the electron-abstraction from the phenolic substrate to be rate-determining. The efficiency of the two laccases in the redox oxidation has been found comparable with

that of a vanadium(V) polyoxometalate, an outer-sphere electron acceptor endowed with a reduction potential value intermediate between those of TviL and MtL.[35] This finding further supports a rate-determining outer-sphere electron transfer mechanism in the enzymatic oxidation. The Marcus reorganisation barrier to electron-transfer has been determined for the T1 copper site of TviL, and found small in value as expected for biological metal-containing electron-transfer sites.[6a,8a,38,39] Our study additionally shows that some phenol and aniline derivatives, in spite of having a well suited redox potential, fail to be oxidised owing to the interplay of steric problems, thereby eluding the definition of being substrates of laccase. Information on the enzymatic active site provided by crystallographic data from the literature[4a,27–29] allows us to assess the optimal structural requirements for a putative substrate. The maximum width tolerated (<11–12 Å) in a substrate for proper fitting to the enzymatic pocket is evaluated, and found to match the distance between two phenylalanines that mark the entrance to the active site. Interaction of the putative substrate with an aspartate residue is another important structural feature of the active site, responsible for directional docking and efficient oxidation.

Our reactivity results are mostly gathered with *Trametes villosa* laccase, whereas the crystallographic data refers to *Trametes versicolor* laccase,[27,28] and a similarity of the binding site between the two enzymes is assumed. This is confirmed by the fact that *Trametes versicolor* laccase 3B (GenBank Accession N° AF414109)[28] shares more than 63% identity at the protein level when compared with *Trametes villosa* laccases (GenBank Accession n° Q99044, Q99046, Q99049, Q99055 and Q99056), this identity being 99% with the deduced amino acid sequence of *Trametes villosa* lac1,[43] *i.e.* the enzyme employed by us. In addition, most of the aforementioned crucial residues and related surrounding motifs are highly conserved.[1,32,44] Finally, both the *Trametes* enzymes have a phenylalanine residue as the axial ligand to T1 Cu(II), and there is a consensus that this structural feature is associated with a high redox potential value in laccases.[8a,17,27,39] In contrast, MtL has an axial leucine ligand, as the laccase from *Melanocarpus albomyces* (PDB entry 1GM0),[29b] and accordingly shows a lower redox potential.[17b,41] Consistently, MtL behaved less efficiently in the rounds of oxidation with phenolic substrates.

In conclusion, our investigation on steric and redox features of putative substrates, inspired by available structural features of laccase enzymes, might even lead in the future to the design of mutants endowed with better oxidation proficiency towards a broader range of substrates, including encumbered ones.

Experimental

Reagents

All phenols employed (Aldrich) and ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) were used as received. Buffers were prepared using ultrapure water obtained with a MilliQ apparatus. *N*-Phenyl-acetamide was obtained from acetylation of aniline with Ac_2O and Zn dust in AcOH at 60 °C;[45] mp 115–117 °C.

Instrumentation

A VARIAN CP 3800 GC, fitted with a 30 m × 0.25 mm methyl silicone gum capillary column (CPSil5CB), was employed in the analyses. The identity of the compounds was confirmed by GC-MS run on a HP 5892 GC, equipped with a 30 m × 0.2 mm methyl silicone gum capillary column, and coupled to a HP 5972 MSD instrument, operating at 70 eV. A UV–Vis spectrophotometer (HP 8453 or PerkinElmer Lambda 18) was used. Quartz cells had a 1 cm optical path.

Enzyme purification

Crude laccases from *Trametes villosa* (*viz. Polyporus pinsitus*) and *Myceliophthora thermophila* were a kind gift from Novo Nordisk Biotech (presently, Novozymes). The enzymes were purified by anion-exchange chromatography on Q-Sepharose Fast Flow, as previously reported.[21] The activity was determined by the ABTS spectrophotometric assay,[46] and was found to be 6000 U ml^{-1} for TviL and 1450 U ml^{-1} for MtL. One unit (U) is defined as the amount of enzyme producing 1 μmol of product per min under the assay conditions. The method of Lowry was employed for the determination of the concentration of laccase in the purified samples, on using BSA.[47]

Enzymatic oxidations

Experimental details were given in previous papers.[21,24] Briefly, the oxidations are run in 3 mL of buffered water (0.1 M sodium citrate, pH 5; buffer A), or in a 2 : 1 buffer A : dioxane mixed solvent, purged with O_2 prior to the addition of the reagents. The substrate (60 μmol) is incubated with 15 Units of laccase for 24 h at room temperature under an oxygen atmosphere. Addition of an internal standard and work-up with ethyl acetate precede GC analysis for the determination of substrate consumption.

Determination of kinetic constants

The oxygen uptake rate has been determined with a SA 520 Clark oxygen electrode (Orion Instruments, Boston MA) connected with a LKB 481 single-channel potentiometric recorder. The reaction mixture (10 ml) containing variable concentrations (generally from 0.5 to 35 mM) of the tested substrate in 0.05 M citrate buffer (pH 5.0) was equilibrated at 25 °C in the electrode chamber, and the reaction initiated by adding appropriate amounts of the enzyme. Maximum reaction velocity (V_{max}), apparent K_m, k_{cat} values ($k_{cat} = V_{max}/[\text{enzyme}]$), and specificity constants (k_{cat}/K_m) were calculated by non-linear regression according to the Michaelis–Menten relationship. To this aim, the Enzfitter software (Elsevier Biosoft, Cambridge) was used. The V_{max} is expressed in International Units (IU), where the IU represents the amount of enzyme (in mg) that consumes 1 μmol of O_2 per min for the oxidation of the substrate.

Computational method

Evaluation of steric hindrance for significant substrates was carried out with the program HyperChem (a trademark of Autodesk, Inc., Sausalito, CA) at the semiempirical level PM3, full geometry optimisations being carried out using the Restricted Hartree–Fock method (RHF). For example, 2,4,6-trimethylphenol approximates a squared object 6.7 Å wide (the distance between the two *ortho*-methyls) and 6.5 Å long (from OH to the *para*-Me). On adding the van der Waals radii (*i.e.*, vdW) to the hydrogen atoms (1.2 Å per H),[48] the steric hindrance of the molecule steps up to

9.1 × 8.9 Å (Table 3). The rendering of the PDB file 1KYA of *Trametes versicolor* laccase[28] for graphic purposes has been done with PyMOL software.[49]

Kinetic isotope effect determination

The laccase-catalyzed oxidation of 2,6-dimethoxyphenol was monitored spectrophotometrically at 468 nm ($\varepsilon = 2.2 \times 10^4\ M^{-1}\ cm^{-1}$)[41] at 25 °C. Either a buffered (0.1 M AcONa–AcOH, pH 5) water solution or a D_2O solution buffered at pH 5 with AcONa–AcOD-d_4 were used. Rapid exchange of D for H within the phenolic substrate in D_2O solution is ensured, and the effect of this exchange upon oxidation of 2,6-$(MeO)_2C_6H_3OD$ by laccase was kinetically measured in comparison with the non-deuterated case. The initial absorbance changes were used to calculate the V_{max} of product formation (*i.e.*, the radical cation) in the reaction mixtures. From $V_{max} = 0.39$ IU in H_2O solution, and $V_{max} = 0.29$ IU in D_2O solution, a k_H/k_D ratio $= 1.4 \pm 0.1$ was determined.

Electrochemical determination

The redox potential of *N*-phenyl-acetamide was determined as an E^p value (1.84 V/NHE) by means of the cyclic voltammetry equipment previously described.[24a] Briefly, the experimental approach entails a three-electrode system consisting of a glassy-carbon disc ($\phi = 1.5$ mm) working electrode, an aqueous $Hg/HgCl_2$/saturated KCl reference electrode, and a Pt reference electrode (1 cm^2). The CV scans of 0.5 mM *N*-phenyl-acetamide were run at 0.5 V s^{-1} rate. All the $E^{\frac{1}{2}}$ values reported in this paper refer to NHE = SCE + 0.242 V.

Abbreviations

NHE, normal hydrogen electrode; ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate); HPI, *N*-hydroxyphthalimide; HBT, 1-hydroxybenzotriazole; TviL, *Trametes villosa* laccase; MtL, *Myceliophthora thermophila* laccase; POM, polyoxometalate; SCE, saturated calomel electrode.

Acknowledgements

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