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Lignin peroxidase catalysed oxidation of 4-methoxymandelic acid. The role of mediator structure

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Abstract—A large number of substances have been tested as redox mediators in the LiP-catalysed oxidation of 4-methoxymandelic acid (4-MMA) to anisaldehyde. In some cases (i.e. thioanisole), the mediation efficiency is almost equal to the maximum value displayed by the natural mediator veratryl alcohol. The mediation efficiency is a function of the redox potential of the mediator and also appears to depend on the kinetic effectiveness with which the mediator is oxidised by the enzyme. In contrast, the lifetime of the mediator radical cation seems not to play any significant role, which would support the idea that the redox mediation is actually accomplished by a complex between the mediator radical cation and the enzyme.

1. Introduction

Lignin peroxidase (LiP, EC 1.11.1.7), an extracellular heme enzyme isolated from ligninolytic cultures of the white-rot basidiomycetous fungus *Phanerochaete chrysosporium*[1] has attracted great attention mainly because of its capacity to degrade lignin,[2,3] a process which is of fundamental importance both in the carbon earth cycle and in the paper and pulp industry.[4] Interestingly, LiP is also able to promote the H_2O_2-dependent oxidation of several compounds characterised by redox potential <1.4 V.[5–16]

The LiP catalytic cycle involves initial reaction of the ferric heme (the native state) with H_2O_2 to yield Compound I (LiP I), characterised as having an oxy-ferryl heme with porphyrin π-cation radical ($P^{+\cdot}$–Fe(IV)=O) (Eq. (1)). Thereafter, two steps of single electron oxidation of substrates restore the enzyme to the native state, via the intermediate Compound II (LiP II), which retains the oxy-ferryl heme (P–Fe(IV)=O) (Eqs. (2) and (3)). LiP II can also react with H_2O_2 to yield the catalytically inactive Compound III (LiP III) (P–Fe(III)O_2^-) (Eq. (4)).[17]

$$\underset{\text{LiP}}{\text{P–Fe(III)}} + H_2O_2 \rightarrow \underset{\text{Compound I (LiP I)}}{P^{+\cdot}\text{–Fe(IV)=O}} + H_2O \quad (1)$$

$$P^{+\cdot}\text{–Fe(IV)=O} + S \rightarrow \underset{\text{Compound II (Lip II)}}{\text{P–Fe(IV)=O}} + S^{+\cdot} \quad (2)$$

$$\text{P–Fe(IV)=O} + S + 2H^+ \rightarrow \text{P–Fe(III)} + S^{+\cdot} + H_2O \quad (3)$$

$$\text{P–Fe(IV)=O} + H_2O_2 \rightarrow \underset{\text{Compound III (Lip III)}}{\text{P–Fe(III)}O_2^-} + H_2O \quad (4)$$

It seems that LiP alone can catalyse lignin degradation; however, there is evidence that such a process is significantly accelerated by the presence of veratryl alcohol, 3,4-dimethoxybenzyl alcohol (VA), a secondary metabolite of the fungus *P. chrysosporium*, which acts as a cofactor.[18–20] This observation has led to a lively discussion about the actual role played by VA and three hypotheses have been presented in order to explain the beneficial effect of VA. The first hypothesis suggests that VA protects LiP against H_2O_2 inactivation preventing the formation of the inactive form LiP III.[21,22] The second is that VA reduces LiP II back to the native enzyme, thus completing the catalytic cycle.[23–25] The third hypothesis, which presently seems to receive the major credit, is that VA acts as a one-electron redox mediator transferring oxidising equivalents from the enzyme to the large, insoluble and hydrophobic lignin polymer, as illustrated in Scheme 1. The oxidation of VA would involve the transfer of one electron to LiP I, leading to the formation of $VA^{+\cdot}$ which would then exchange one electron with one of the aromatic rings of the macromolecule, thus initiating its degradation.[11,19,26,27]

Very interestingly, a beneficial effect of VA has also been observed in the oxidation of simple organic substrates which are 'recalcitrant' towards the oxidation by LiP. Accordingly, LiP alone is practically not able to oxidise 4-methoxybenzyl alcohol (anisyl alcohol) and 2-hydroxy-2-(4-methoxyphenyl)acetic acid (4-methoxymandelic acid, 4-MMA), but these oxidations take place with good efficiency in the presence of VA.[19] An additional observation is that other compounds (i.e. 1,4-dimethoxybenzene[19] and 2-chloro-1,4-dimethoxybenzene[28]) can replace VA in

Keywords: lignin peroxidase; mediation; redox potential; radical cation lifetime.

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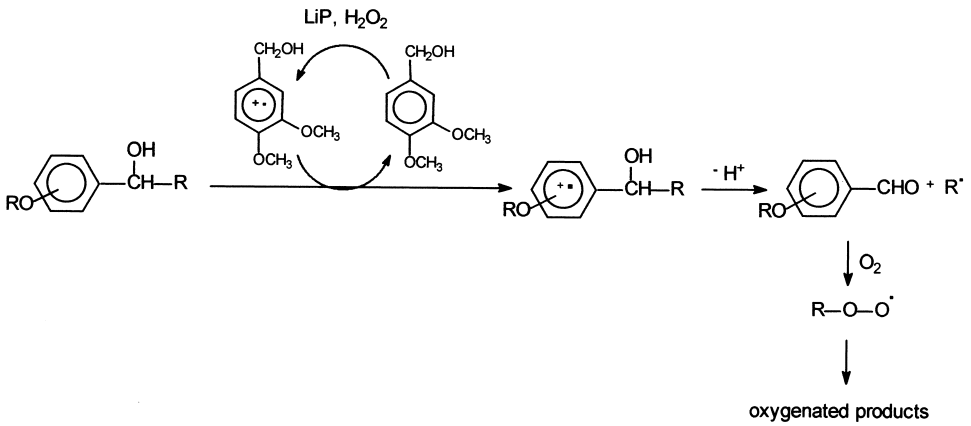
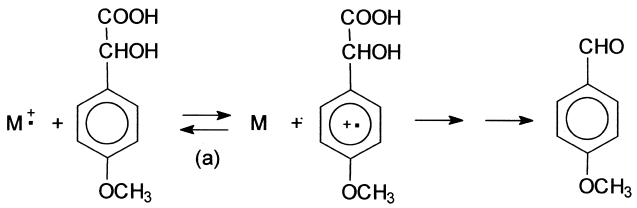


Scheme 1. VA as a redox mediator in LiP-catalysed oxidation of lignin.



Scheme 2. LiP-catalysed oxidation of 4-MMA.

increasing the efficiency of LiP-promoted oxidations. A mechanism similar to that in Scheme 2 has been proposed in the case of the oxidation of 4-MMA.[†]

Thus, it appears that the oxidation of these recalcitrant substrates represents a very simple system suitable to acquire information about the relative ability of different compounds to act as mediators in the oxidations catalysed by LiP and about the structural factors which play a role in this respect. Such information may also provide us further insight about the mechanism of mediation and the enzyme–mediator interaction.

In this paper, we report on the mediation efficiency of a large number of substances, measured in the LiP-catalysed oxidation of 4-MMA to 4-methoxybenzaldehyde (anisaldehyde) (Scheme 2).

We have tested a series of substituted *o*-, *m*-, *p*-dimethoxy, trimethoxy- and tetramethoxy-aromatic compounds, ring substituted (methylthio)benzenes (thioanisoles) and tryptophan. Whenever possible the redox potential and the lifetimes of the corresponding radical cations have also been determined. These parameters should play a role in a mechanism such as that shown in Scheme 1. Accordingly, the mediator has to be oxidised to radical cation and only a sufficiently long-lived radical cation (half-life of at least some milliseconds)[29] can escape from the interaction with the enzyme, diffuse into solution and induce the oxidation of other substrates (diffusible redox mediator). For some mediators, we have also measured the kinetic parameters k_{cat} and K_M for the enzymatic oxidation to acquire information on the enzyme–mediator interactions.

[†] The reaction (a) described in Scheme 2 is generally an endoergonic process which is driven to the right by the fast fragmentation of 4-MMA$^{+\cdot}$.[19]

2. Results

2.1. Measurement of mediation efficiency

The mediation efficiency was determined as the yield of anisaldehyde formed in the LiP-catalysed oxidation of 4-MMA in the presence of the mediator (4% based on 4-MMA). The enzymatic oxidation was carried out at 37°C, for 10 min, under an argon atmosphere, in 50 mM sodium tartrate buffer solution, pH 3.0, containing LiP (0.51 nmol, 0.3 U), the substrate (0.25 μmol) and the mediator (0.01 μmol).

The reaction was initiated by adding the oxidant, H_2O_2 (0.06 μmol). The reaction mixture was subsequently analysed by HPLC. It was first checked that in the absence of the mediator (blank reaction) only a small amount of anisaldehyde (2% based on the substrate) was formed and this amount has been subtracted from the reaction yields in the presence of the mediator.

2.2. Measurement of the redox potential

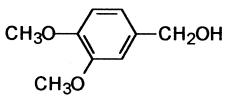
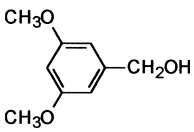
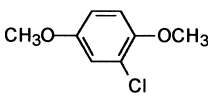
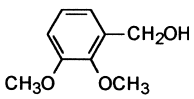
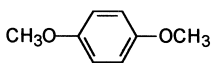
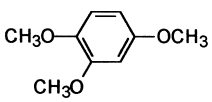
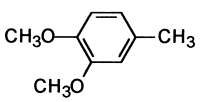
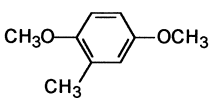
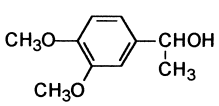
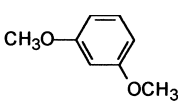
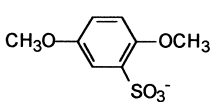
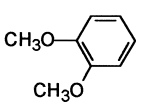
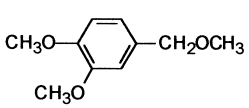
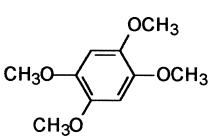
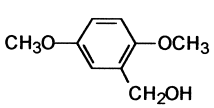
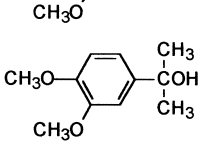
The redox potentials of the mediators have also been determined in aqueous solution by cyclic voltammetry. The measurements were carried out at 25°C, in argon degassed 10 mM sodium acetate buffer solution, pH 4.0, with 3% CH_3CN as cosolvent, containing $LiClO_4$ (0.12 M) as the supporting electrolyte and the substrate (0.10 mmol). Using a sweep scan up to 500 mV s^{-1} only irreversible oxidation waves were obtained.

Results of mediation efficiency together with the mediator's E_p values, in V vs NHE, are listed in Table 1 for di-, tri-, tetramethoxyaromatics and in Table 2 for ring substituted thioanisoles. For tryptophan, a mediation efficiency of 4.8 and E_p=1.09 (V vs NHE) have been determined.

2.3. Measurement of the kinetic constant k_{cat} and K_M

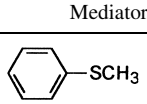
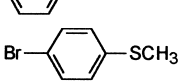
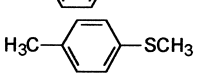
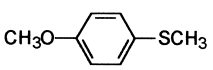
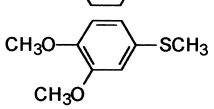
For some of the tested mediators, the enzymatic oxidation led to a single product (see Section 5) and it was thus possible to measure their enzymatic kinetic constants. These compounds are VA, its corresponding methyl ether and 1-(3,4-dimethoxyphenyl)ethanol.

Table 1. Mediation efficiency and redox potential of di-, tri- and tetramethoxy-aromatic compounds in the LiP-catalysed oxidation of 4-MMA

	Mediator	Efficiency (%)[a]	$E^{+\cdot}_M/_M$[b]		Mediator	Efficiency (%)[a]	$E^{+\cdot}_M/_M$[b]
1		25.5	1.36[c]	**9**		4.5	1.42[d]
2		24.7	1.44[e]	**10**		3.7	1.39[d]
3		15.5	1.39[e]	**11**		3.7	1.17[e]
4		11.2	1.46[e]	**12**		1.2	1.24[e]
5		8.0	1.46[e]	**13**		0	>1.60[e]
6		8.0	1.59[e]	**14**		0	>1.60[e]
7		7.5	1.36[c]	**15**		0	0.94[e]
8		6.0	1.33[d]	**16**		0	–

[a] Yield of anisaldehyde referred to the substrate subtracted of the amount (2%) formed in the absence of mediator. Average of at least two determinations, the error is in all cases $<\pm 0.3$.
[b] V vs NHE, measured in aqueous solution.
[c] Ref. 30.
[d] Ref. 31.
[e] E_p determined by cyclic voltammetry in aqueous solution pH 4.

Table 2. Mediation efficiency and redox potential of ring substituted thioanisoles in the LiP-catalysed oxidation of 4-MMA

	Mediator	Efficiency (%)[a]	$E^{+\cdot}_M/_M$[b]
17		25.0	1.49
18		17.6	1.55
19		18.9	1.46
20		9.0	1.36
21		5.4	1.30

[a] Yield of anisaldehyde referred to the substrate subtracted of the amount (2%) formed in the absence of mediator. Average of at least two determinations, the error is in all cases $<\pm 0.3$.
[b] E_p (V vs NHE) determined by cyclic voltammetry in aqueous solution pH 4.

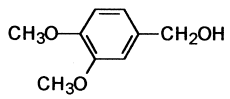
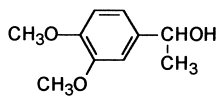
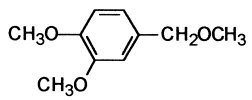
The k_{cat} and K_M values were obtained following the product formation spectrophotometrically at the λ value where the largest difference in absorbance between the substrate and the product is observed. The data obtained were then treated according to the Michaelis–Menten kinetic scheme.

The k_{cat} and K_M values are reported in Table 3 where again the mediation efficiencies are displayed.

2.4. Measurement of the half-life of the mediator radical cations

We have measured the half-life ($t_{1/2}$) of the radical cation of the dimethoxylated aromatic compounds that displayed the highest mediation efficiency (VA, 1,4-dimethoxybenzene, 2-chloro-1,4-dimethoxybenzene and 3,4-dimethoxytoluene) and, for comparison, the half-life of the radical cation of 2,5-dimethoxybenzylalcohol which displayed only a moderate mediation efficiency.

Table 3. Mediation efficiency in the LiP-catalysed oxidation of 4-MMA and k_{cat} and K_M values in the LiP-catalysed oxidation of aromatic mediators

	Mediator	Efficiency (%)[a]	k_{cat}	K_M	k_{cat}/K_M[b]
1		25.5	15.9	0.32	50
5		8.0	17.8	0.44	40
7		7.5	10.0	0.40	25

[a] Yield of anisaldehyde referred to the substrate subtracted of the amount (2%) formed in the absence of mediator. Average of at least two determinations, the error is in all cases $<\pm 0.3$.
[b] Average of three determinations, the error is in all cases $<\pm 2$.

The radical cation has been generated in acidic aqueous solution (6.5% $HClO_4$) by oxidation with cerium(IV) ammonium nitrate (CAN). Its decay has been followed in the region around 410–470 nm (where the studied radical cations exhibit a maximum of the absorbance) by a stopped-flow apparatus. For all the studied compounds, the half-life of the radical cations was determined as the time required to halve the initial absorbance. The radical cation half-lives are reported in Table 4.

3. Discussion

The results reported in Tables 1 and 2 indicate that most of the molecules tested can act as mediators in the oxidation of 4-MMA. Interestingly, it appears that the natural mediator, VA, is the species which displays the greatest mediation efficiency. However, an efficiency comparable to that of VA is also observed for some other mediators (i.e. thioanisole).

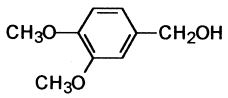
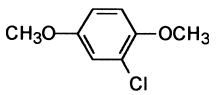
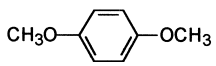
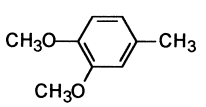
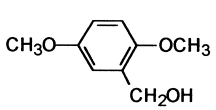
From the data reported in Table 1, we can observe that the redox potential of the couple $M^{+\cdot}/M$ (Table 1, M=mediator) is an important factor in determining the mediation efficiency. When a series of structurally related substrates is considered, we find that the mediation efficiency first increases and then decreases on increasing the $E_{p\ M^{+\cdot}/M}$ value. This trend can be easily seen in the series of 2-X-1, 4-dimethoxybenzenes (Fig. 1) and of ring substituted thioanisoles (Fig. 2).

A bell shaped curve is observed in both cases with a maximum efficiency at around 1.4–1.5 V. This result lends further support to the hypothesis of a redox mechanism for the mediation of LiP-induced oxidations (see above). Accordingly, the observed trend is exactly the one expected for such a mechanism (Scheme 1) when two contrasting factors must be at play: the mediator must have a redox potential sufficiently low to allow its oxidation by both LiP I and LiP II, but at the same time it should be high enough in order to promote the oxidative fragmentation of 4-MMA.

Even though the oxidation potential seems to play the major role, with respect to mediation, some influence is also exerted by other factors. Thus, the presence of an alcoholic group has a beneficial effect on the mediation efficiency as revealed by the fact that VA (**1**) is a much better mediator than its corresponding methyl ether (**7**), the two compounds exhibiting the same $E_{p\ M^{+\cdot}/M}$ value. Moreover, the mediation efficiency decreases regularly on going from primary to secondary and tertiary alcoholic group (compare VA with 1-(3,4-dimethoxyphenyl)ethanol, (**5**) and 2-(3,4-dimethoxyphenyl)-2-propanol) **16**; the absence of mediation with the latter compound is probably due to the steric hindrance of the side-chain.

In the search for other properties which, in addition to the oxidation potential, can influence the mediation efficiency, we have investigated the role of the mediator capacity to interact with the enzyme and therefore of the kinetic parameters k_{cat} and K_M. This investigation, however, was only possible for the series of the three mediators VA, VA

Table 4. Mediation efficiency in the LiP-catalysed oxidation of 4-MMA and lifetime of radical cations of dimethoxylated aromatic compounds

	Mediator	Efficiency (%)[a]	Half-life (s)[b]
1		25.5	0.04[c]
2		24.7	8.3
3		15.5	2.1
4		11.2	0.25
8		6.0	0.22

[a] Yield of anisaldehyde referred to the substrate subtracted of the amount (2%) formed in the absence of mediator. Average of at least two determinations, the error is in all cases $<\pm 0.3$.
[b] The error is ± 0.02 s for entries 1, 4, 8 and $\pm$ 0.1 s for entries 2 and 3.
[c] Ref. 32. The same value has been measured by Candeias and Harvey by pulse radiolysis.[29] The much shorter half-life (about 0.6 ms) measured by Khindaria et al. by ESR,[33,34] is probably wrong.

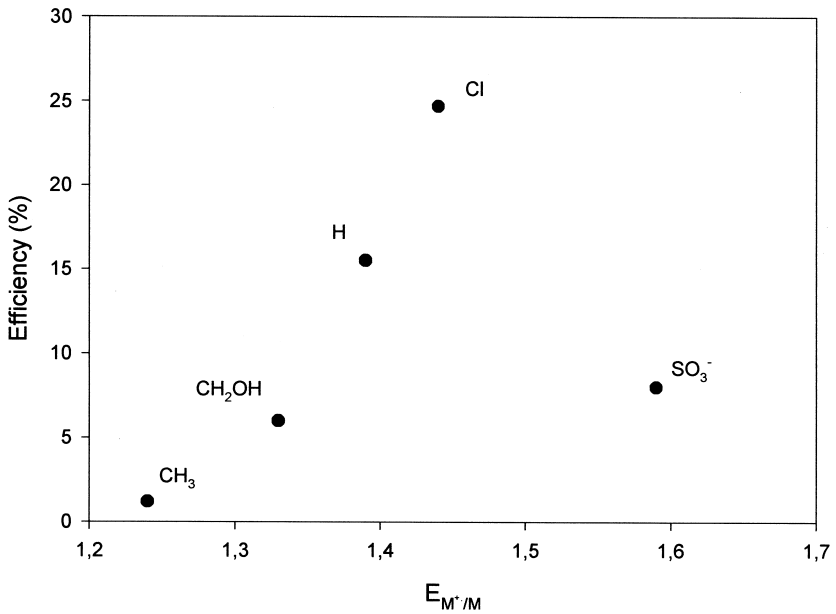


Figure 1. Mediation efficiency vs redox potential of 2-X-1,4-dimethoxybenzenes in the LiP-catalysed oxidation of 4-MMA.

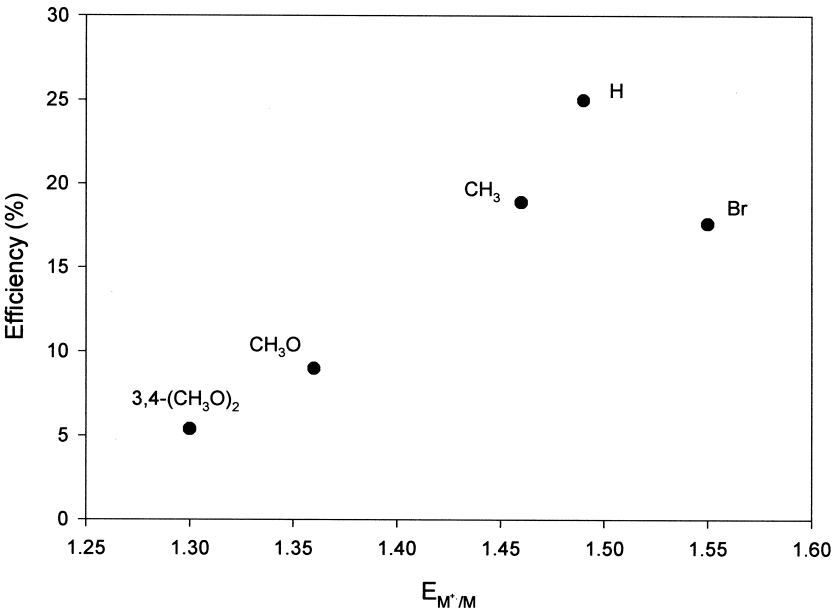


Figure 2. Mediation efficiency vs redox potential of ring substituted thioanisoles in the LiP-catalysed oxidation of 4-MMA.

methyl ether (**7**) and 1-(3,4-dimethoxyphenyl)ethanol (**5**), whose enzymatic oxidation led to the formation of a single product that could be monitored spectrophotometrically. Nevertheless, from the data reported in Table 3, it would seem that, whereas the oxidation potential remains almost constant, the mediation efficiency increases as the kinetic effectiveness (k_{cat}/K_M) becomes higher. Accordingly, VA, which is the best mediator among the substrates analysed has the highest kinetic effectiveness (k_{cat}/K_M= 50 s^{-1} mM^{-1}), while its methyl ether (**7**) (the worst mediator) has the lowest kinetic effectiveness (k_{cat}/K_M= 25 s^{-1} mM^{-1}). 1-(3,4-Dimethoxyphenyl)ethanol (**5**) shows intermediate values of both mediation efficiency and kinetic effectiveness (k_{cat}/K_M=40 s^{-1} mM^{-1}).

Moreover, for a series of mediators with an E_p value ranging from 1.33 to 1.46 V, we have tested the role with respect to the mediation efficiency of the half-life of the mediator radical cation.[‡] The results reported in Table 4 show that there is no apparent relation between the mediation efficiency and the radical cation half-life. Indeed VA and 2-chloro-1,4-dimethoxybenzene, the best mediators, have respectively the shortest (40 ms) and the longest (8.3 s) half-lives, the other radical cations exhibiting intermediate values and having lower mediation efficiencies. This finding is of interest as it might support the suggestion that the

[‡] Stopped-flow measurements have been carried out in a more acidic medium (where the radical cations are expected to be more stable) than the cyclic voltammetry measurements; however, we assume that the relative ordering of half-life and E_p values should be the same under these conditions.

mediation is carried out by a complex between the enzyme and the mediator radical cation, as suggested by several groups.[33–35] The half-life of such a complex might be different (and probably longer than that of the 'free' mediator radical cation).

An additional observation is that the radical cations in Table 4 display very different half-lives in spite of their similar thermodynamic stability as expressed by the $E_{p\ M^{+\cdot}/M}$ values. This is likely due to the different reaction paths which are available to these intermediates. Thus, the radical cations of VA, 3,4-dimethoxytoluene (**4**) and 2,5-dimethoxybenzyl alcohol (**8**) are short lived as they can undergo a relatively fast deprotonation from the benzylic position while longer lifetimes are observed for the radical cations of 2-chloro-1,4-dimethoxybenzene (**2**) and 1,4-dimethoxybenzene (**3**) which do not contain benzylic hydrogens and probably decay by different processes like dimerization, disproportionation or reaction with nucleophilic species.

Finally, in view of the fact that recent work has suggested that a tryptophan residue may be responsible for the electron exchange between the enzyme and VA,[36] we have also determined the mediation efficiency of tryptophan. However, this compound does not exhibit a significant mediation efficiency almost certainly because of its low redox potential (1.09 V vs NHE). In this respect, it would be of interest to test tryptophan derivatives with an oxidation potential of ca. 1.4 V. Work in this direction is under way in our laboratory.

4. Conclusions

On the basis of the results gathered in this work, several conclusions about the efficiency of a given substrate in the mediation of the LiP-catalysed oxidation of 4-MMA are possible. The redox potential of the mediator is probably the most important factor. Accordingly, in the structurally related series examined, the highest mediation efficiency has been reached when the $E_{p\ M^{+\cdot}/M}$ value is 1.4–1.5 V. The influence of oxidation potential on the mediation efficiency clearly supports the hypothesis of a redox mediation in LiP induced oxidations. Another factor which appears to play some role with respect to the mediation efficiency is the kinetic effectiveness as determined by the k_{cat}/K_M values, the best mediator displaying the highest kinetic effectiveness. In contrast, the mediation efficiency is not influenced by the lifetime of the free mediator radical cation. This observation might support the suggestion that the actual redox mediation is performed by a mediator–enzyme complex with a lifetime different (and probably longer) than that of the free mediator radical cations.

5. Experimental

5.1. Instrumentation

^{1}H NMR spectra were recorded on a Bruker AC300P spectrometer in $CDCl_3$. GC–MS analyses were performed on a HP5890 GC (OV1 capillary column, 12 m×0.2 mm) coupled with a HP5970 MSD. HPLC analyses were accomplished on a Hewlett–Packard 1050 liquid chromatograph fitted with a UV–vis detector HP79853A and a Supelcosil LC18 column (25 cm×4.6 mm). GC analyses were carried out on a Varian 3400 GC (OV1 capillary column, 25 m×0.2 mm). UV–vis measurement was performed on a Perkin–Elmer Lambda 18 spectrophotometer. For the cyclic voltammetry measurements, an Amel 5000 potentiostat was used, the cell was fitted with a glassy carbon working electrode (ϕ 3 mm) in combination with an aqueous SCE reference electrode.

5.2. Substrates and reagents

All the reagents and solvents were of the highest purity available and used without further purification (unless otherwise specified). CAN (Aldrich) was dried at 85°C for 1 h. The concentration of H_2O_2 (Carlo Erba Reagents) was determined by titration with permanganate.[37] LiP was prepared and purified as described in the literature.[38] The concentration of the enzyme solution was determined spectrophotometrically ($\varepsilon_{409\ nm}$=169 $mM^{-1}\ cm^{-1}$).[39]

1-(3,4-Dimethoxy-phenyl)-ethanol (**5**),[40] 2,5-dimethoxybenzenesulfonic acid (**6**),[41] VA methyl ether (**7**),[42] 2-(3,4-dimethoxy-phenyl)-propan-2-ol[43] and 1,2-dimethoxy-4-methylthiobenzene[44] were synthesised according to the literature.

5.3. Mediation efficiency

The substrate, 4-MMA, (0.25 μmol), LiP (0.51 nmol, 0.3 U) and 4% of mediator (0.01 μmol) were magnetically stirred in 1 mL of 50 mM sodium tartrate buffer solution, pH 3, at 37°C, with 3% CH_3CN as cosolvent, under an Argon atmosphere. The oxidant, H_2O_2 (0.06 μmol) was added. After 10 min, the internal standard (4-methoxyacetophenone) was added and the products of the reaction were determined by HPLC on a reversed-phase column (Supelcosil LC18) using MeOH/H_2O (1/1 v/v) as the mobile phase. A good recovery of material was observed (>95%) in all the experiments.

5.4. Product analysis study

The mediator (10 μmol) and LiP (0.6 units, 1.02 nmol) were magnetically stirred in 5 mL of 50 mM sodium tartrate buffer solution, pH 3 containing 3% of acetonitrile at 37°C and under an Argon atmosphere. H_2O_2 (10 μmol) in 0.5 mL of buffer solution was added. After 10 min, the products of the reaction were extracted with CH_2Cl_2, dried over Na_2SO_4 and characterised by GC, GC–MS, ^{1}H NMR and UV–vis. The mediators that yielded a single product were VA and VA-methyl ether (veratraldehyde) and 1-(3,4-dimethoxyphenyl)ethanol (1-(3,4-dimethoxyphenyl)ethanone).

5.5. Steady-state kinetics

For VA, VA methyl ether and 1-(3,4-dimethoxyphenyl)-ethanol H_2O_2 (0.40 μmol) in 0.1 mL of buffer solution was added in a cuvette to 2.8 mL of 50 mM sodium tartrate buffer solution, pH 3.0, with 10% acetonitrile as cosolvent, containing the mediator (0.04–1.20 μmol) and LiP (0.42 units, 0.71 nmol), thermostated at 25°C. The formation of

the products was followed spectrophotometrically at a λ value where is the maximum difference in absorbance between the substrate and the corresponding product (310 nm for VA and its methyl ether, 302 nm for 1-(3,4-dimethoxyphenyl)ethanol). The kinetic parameters k_{cat} and K_M were obtained from Lineweaver–Burke plot of the data).

5.6. Measurement of the mediator redox potential

The oxidation potential was determined by cyclic voltammetry at 25°C in argon degassed 10 mM sodium acetate buffer solution, pH 4.0, with 3% CH_3CN as cosolvent, containing $LiClO_4$ (0.12 M) as the supporting electrolyte and the substrate (0.10 mmol). The cell was fitted with a glassy carbon working electrode (ϕ 3 mm) in combination with an aqueous SCE reference electrode. The waves were irreversible even at a 500 mV s^{-1} sweep rate, so that only E_p values are given.

5.7. Measurement of the mediator radical cation half-life

An aqueous solution of the mediator 1 mM in 10% CH_3CN as cosolvent and an aqueous solution of CAN 1 mM in 13% $HClO_4$ were introduced in two syringes of a stopped-flow apparatus. Radical cations were generated by mixing 50 μL of the solutions contained in the two syringes. The decay of each radical cation was followed in the region around 410–470 nm (where the studied radical cations exhibit a maximum of the absorbance). The filter time constant used varied in the range 1–100 ms. The apparatus was thermostated at 25°C.

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