Oxidative *N*-demethylation of *N*,*N*-dimethylanilines catalysed by lignin peroxidase: a mechanistic insight by a kinetic deuterium isotope effect study

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Lignin peroxidase can catalyse the *N*-demethylation of *N*,*N*-dimethylanilines by an electron transfer mechanism, where the deprotonation of the intermediate radical cation is also an enzymatic process.

Lignin peroxidase (LiP), a heme-containing glycoprotein isolated from the ligninolytic cultures of the white-rot fungus *Phanerochaete chrysosporium*, is a very important enzyme capable of performing the oxidative depolymerization of lignin with hydrogen peroxide.[1] For this reason, studies of this enzyme have been focused so far on the oxidation of non-phenolic electron rich aromatic lignin model compounds,[1c,d,2] while less information is available about the catalytic activity of LiP in the H_2O_2-promoted oxidation of other substrates.[3]

Here we report on the ability of LiP to catalyse the oxidative *N*-demethylation of *N*,*N*-dimethylanilines, a process of great biological importance. We also provide some information about the mechanism of this reaction *via* a kinetic deuterium isotope effect (KDIE) study.

4-X-Substituted *N*,*N*-dimethylanilines were reacted with an equimolar amount of hydrogen peroxide in the presence of LiP (purified from the *Phanerochaete chrysosporium* culture medium according to the literature method[4]) in an argon-degassed 50 mM sodium tartrate buffered solution at pH 4. A clean *N*-demethylation reaction was observed, but only when X is an electron withdrawing group (X = Br, CF_3, CN and NO_2). The yields are reported in Table 1. *N*,*N*-Dimethylaniline and anilines with electron donating substituents exhibited no reactivity, probably due to the fact that they were almost completely protonated at the low pH value necessary for the enzyme catalysis. In all cases, beside the demethylated products, formaldehyde was also formed, which was detected by GC-MS after conversion into the dimedone adduct.

The capacity of LiP to catalyse the oxidation of electron rich aromatics ($E° \leqslant 1.25$ V *vs.* SCE in water) by an electron transfer (ET) mechanism is well documented.[2c,3d] Therefore, it is reasonable to suggest that an ET mechanism also occurs in the *N*-demethylation reaction, as a value as low as 1.1 V *vs.* SCE[5] has been estimated for the redox potential of *N*,*N*-dimethyl-4-nitroaniline, the least oxidizable aniline in Table 1. This mechanism is also consistent with the absence of reactivity observed when the aniline is in the protonated form. Moreover, an ET mechanism has been proposed for the *N*-demethylation of *N*,*N*-dimethylaniline by other peroxidases.[7]

According to this mechanism (Scheme 1), an ET occurs between the substrate and the porphyrinato iron–oxo complex (Compound I), which is the active oxidant formed by reaction of the native form of LiP with H_2O_2. An anilinium radical cation and the reduced form of Compound I (Compound II) are obtained. The anilinium radical cation then undergoes deprotonation to give an α-amino carbon radical and it has been suggested by Guengerich and his associates that this process can either involve the enzyme or take place by a non-enzymatic pathway, depending on the nature of the peroxidase.[7b] In the former case [path (b) in Scheme 1], Compound II is suggested to be the proton abstracting base. The α-amino carbon radical then undergoes oxygen rebound to form a carbinolamine that is eventually converted to the *N*-demethylated product. In the second case, the deprotonation is promoted [path (a)] by the medium (H_2O). The α-amino carbon radical (a very easily oxidizable species) is oxidized to a carbocation which is then converted into a carbinolamine by reaction with H_2O.

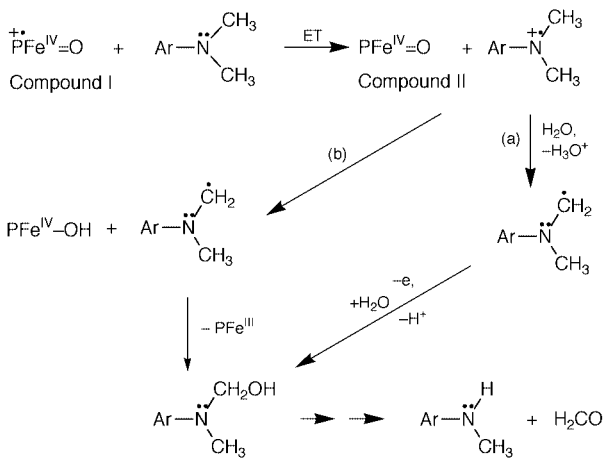
Guengerich also suggested, on the basis of intramolecular KDIE values, that chloroperoxidase followed the enzymatic path, while horseradish peroxidase followed the non-enzymatic one, probably due to the difficult accessibility of the ferryl oxygen in the latter enzyme.[7b]

In the case of LiP, the size of the substrate-access channel to the heme is still smaller than in HRP so that the substrate should have access only to the heme edge, where the electron transfer

Table 1 Products and yields in the reaction of 4-substituted *N*,*N*-dimethylanilines with hydrogen peroxide catalyzed by LiP[a]

Substrate (4-substituent)	Product yield ArNHMe (%)	Material balance[b] (%)
Br	16	> 99
CF_3	28[c]	75
CN	62	86
NO_2	20	87

[a] Experimental procedures are as follows: the oxidant (10 μmol) was added, over a period of 1 h by an infusion pump, to a stirred degassed solution of the substrate (10 μmol), LiP (0.96 units, 1.16 nmol) and MeCN (50 μl) as the co-solvent, in 3 ml of Na–tartrate buffer (50 mM, pH 4) at 25 °C. Products analysis were performed by GC and GC-MS. [b] The sum of the moles of unreacted substrate and reaction product relative to the initial moles of substrate. [c] In this case 4-trifluoromethylaniline (5%) was also observed.



Scheme 1

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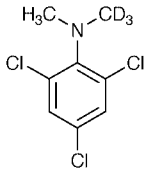
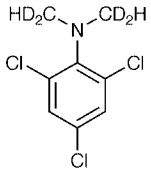
process can still take place, but not the deprotonation of the radical cation by the ferryl oxygen.[8] One would therefore predict a non-enzymatic pathway for the deprotonation of the radical cation in the LiP-catalysed *N*-demethylation reactions. However, strong evidence in favour of a deprotonation promoted by the enzyme has been provided by the complete masking [k_H/k_D = 1.04 (± 0.06)] of the intramolecular KDIE observed for the *N*-demethylation of *N*-methyl-*N*-trideuteromethyl-2,4,6-trichloroaniline **1** catalysed by LiP (25% yield).[9,10]



1



2

In this system, due to the steric effect of the two *ortho*-chlorine atoms, the two *N*-methyl groups are forced to stay one above and the other below the plane of aromatic ring as confirmed by theoretical calculations based on the Density Functional approach (DFT), carried out on the radical cation of *N*,*N*-dimethyl-2,6-dichloroaniline, a model compound for **1**.[11] These calculations moreover show that there is a quite large barrier (33.5 kJ mol^{-1}) to the rotation around the C(aromatic)–N bond.[12] Thus, the absence of a deuterium isotope effect for this substrate strongly suggests that deprotonation has to take place in the enzyme pocket, being significantly faster than rotation of CH_3 and CD_3 groups around the C(aromatic)–N bond.[13] Under these conditions, the loss of hydrogen or deuterium will only depend on which one of the two methyl groups in the radical cation is oriented towards the proton abstracting center and reasonably there is the same probability that this group is CH_3 or CD_3. Thus, the intramolecular KDIE with this substrate should be completely masked, as is actually observed. This interpretation is confirmed by the significant value of intramolecular KDIE (3.36 ± 0.07) found instead in the oxidation of *N*,*N*-bis(dideuteromethyl)-2,4,6-trichloroaniline **2** (27 % yield), where deuterium and hydrogen are bonded to the same carbon.[14] In **2**, the KDIE is no longer influenced by the hindered rotation mentioned before. Further support also comes from the high intramolecular KDIE value (7.0 ± 0.8) measured in the oxidation of *N*-methyl-*N*-trideuteromethyl-3,4,5-trichloroaniline, where the absence of *ortho*-substituents allows the two methyl groups to freely interchange within the enzyme pocket.

In conclusion, our data clearly show that LiP can catalyse the oxidative *N*-demethylation of aromatic tertiary amines with fairly good efficiency. Moreover, the intramolecular KDIEs measured with **1** and **2** indicate that the aminium radical cation is deprotonated by the enzyme. Concerning the basic center, Compound II seems unlikely, in the light of the already mentioned current views about the accessibility of the heme in this enzyme.[8] Another hypothesis is that the deprotonation of the radical cation is promoted by some specific amino acid residue located in or very close to the active site. Histidine 82 might be a suitable candidate in this respect. An additional possibility might be a medium induced deprotonation of an enzyme complexed radical cation.[15]

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5 (*a*) V. D. Parker and M. Tilset, *J. Am. Chem. Soc.*, 1991, **113**, 8778. (*b*) This value has been measured in MeCN, the value in water should be significanly lower (of *ca*. 0.4 V) (ref. 6).

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9 All the KDIE values reported in this work were determined by GC-MS analysis of the formaldehyde–dimedone adduct, and they are an average of at least three independent determinations.

10 Compound **1** was prepared by reacting 2,4,6-trichloroaniline with MeI and then with CD_3I.

11 (*a*) The calculations were carried out, with the GAUSSIAN 94 package [ref. 11(*b*)], by using the DFT approach at the B3LYP/6-311+G(d,p) level of theory. Spin contamination due to states of multiplicity higher than the doublet state was negligible, in that the $< S^2 >$ parameter was, in all cases, well within 10% of the expectation value for a doublet (0.75). (*b*) M. J. Frisch, G. W. Trucks, H. B. Schlegel, P. M. W. Gill, B. G. Johnson, M. A. Robb, J. R. Cheeseman, T. Keith, G. A. Petersson, J. A. Montgomery, K. Raghavachari, M. A. Al-Laham, V. G. Zakrzewski, J. V. Ortiz, J. B. Foresman, J. Cioslowski, B. B. Stefanov, A. Nanayakkara, M. Challacombe, C. Y. Peng, P. Y. Ayala, W. Chen, M. W. Wong, J. L. Andres, E. S. Replogle, R. Gomperts, R. L. Martin, D. J. Fox, J. S. Binkley, D. J. Defrees, J. Baker, J. P. Stewart, M. Head-Gordon, C. Gonzalez and J. A. Pople, GAUSSIAN 94, Revision D.2, Gaussian, Inc., Pittsburgh PA, 1995.

12 This can also be argued by the oxidation peak potential (E_p) measured for **1** (1.34 V *vs.* SCE) [ref. 5(*b*)] which is significantly higher than that measured for its isomer *N*,*N*-dimethyl-3,4,5-trichloroaniline (E_p = 1.16 V *vs.* SCE) [ref. 5(*b*)].

13 Even assuming that the barrier to rotation does not increase in the enzyme pocket, a deprotonation faster than rotation around the C(aromatic)–N bond is plausible, as an activation enthalpy less than 33.5 kJ mol^{-1} is possible for the former reaction [ref. 5(*a*)].

14 Compound **2** was prepared by reacting 2,4,6-trichloroaniline with CD_2O and $NaBH_4$ (S. B. Karki, J. P. Dinnocenzo, J. P. Jones and K. R. Korzekwa, *J. Am. Chem. Soc.*, 1995, **117**, 3657).

15 We thank one of the referees for this suggestion.

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