Prochiral selectivity and deuterium kinetic isotope effect in the oxidation of benzyl alcohol catalyzed by chloroperoxidase

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The chloroperoxidase-catalyzed oxidation of benzyl alcohol exhibits a very high prochiral selectivity, involving only the cleavage of the *pro-S* C–H bond.

The mechanistic aspects of the reactions catalyzed by chloroperoxidase (CPO), a hemoprotein isolated from the fungus *Caldariomyces fumago*, are attracting continuous interest as this enzyme is able to catalyze the H_2O_2-promoted oxidation of a large variety of organic compounds.[1] There is general consensus that in all cases the active oxidant is an iron(IV) oxo complex porphyrin IX (P) radical cation [$P^{+\cdot}$–Fe^{IV}=O], also indicated as compound I, formed by reaction of the resting enzyme with H_2O_2 [eqn. (1)].[1d,2]

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

Here we report on a kinetic and products study of the oxidation of benzyl alcohol **1** and the enantiomeric and racemic forms of α-monodeuterated benzyl alcohol (**1**-*d*). The results obtained have shown that the process is characterized by an extremely high prochiral selectivity, providing us with useful information about the reaction mechanism.

The racemic [α-$^2H_1$]benzyl alcohol [(±)-**1**-*d*)] and the two enantiomers (*R*)- and (*S*)-[α-$^2H_1$]benzyl alcohol [(*R*)-**1**-*d* and (*S*)-**1**-*d*, respectively] were reacted with H_2O_2 in H_2O at pH 6 (0.1 M phosphate buffer), in the presence of CPO (2 nmol for 10 mmol of H_2O_2). In each case, the molar ratio between PhCDO and PhCHO produced in the reaction was measured by GC-MS and/or ^{1}H NMR, the two methods giving very similar results. The results are reported in Table 1. The kinetic parameters K_m and k_{cat} for the CPO-catalyzed oxidation of deuterated and undeuterated benzyl alcohols were obtained, as usual, by measuring the initial rates of formation of benzaldehyde at different concentrations of substrate (Lineweaver–Burk plots). These values are collected in Table 2.

Table 1 Oxidations of [α-$^2H_1$]benzyl alcohols with H_2O_2 catalyzed by CPO[a]

Substrate	Yields (%)[b]	PhCDO/PhCHO[c]
(*S*)-[α-^{2}H]benzyl alcohol	58	< 0.1
(*R*)-[α-^{2}H]benzyl alcohol	68	> 30
(±)-[α-^{2}H]benzyl alcohol	47	2.0

[a] The alcohol (0.1 ml of a solution 0.5 M in CD_3CN) and CPO (Sigma) (2 nmol) were magnetically stirred in 5 ml of 0.1 M phosphate buffer pH 6.0 at 25 °C, H_2O_2 (200 μl, 0.5 M) was then slowly added in 1 h *via* a syringe pump. After extraction with $CDCl_3$ reaction mixtures were analyzed by ^{1}H NMR, GC and GC-MS. Benzaldehyde and [α-$^2H_1$]benzaldehyde were the only products formed. [b] Yields are with respect to the starting material. Average of at least three determinations, the error is ±10%. [c] Molar ratio measured *via* GC-MS by the ratio between the corrected signal intensities of the two molecular ions at *m/z* 107 and 106. Average of at least three determinations, the error is ±10%. Similar values were obtained by ^{1}H NMR considering that the signal of the benzylic protons *ortho* to the carbonyl is due to the sum of the two products (PhCHO + PhCDO) and that of the aldehydic proton derives from PhCHO.

By looking at the data in Table 1, a very remarkable observation is that from (*S*)-**1**-*d* practically only PhCHO is produced, whereas the oxidation of its enantiomer (*R*)-**1**-*d* leads almost exclusively to PhCDO. Comparable amounts of deuterated and undeuterated benzaldehydes form instead from the racemic alcohol (±)-**1**-*d*.

These results clearly indicate that in the oxidation of (*S*)-**1**-*d* the bond broken is almost exclusively the C-D bond, whereas the oxidation of (*R*)-**1**-*d* involves almost exclusively the cleavage of the C–H bond. Thus, the ratio between the k_{cat} values for (*R*)-**1**-*d* (C–H bond cleavage) and (*S*)-**1**-*d* (C–D bond cleavage), reported in Table 2, should provide us with a lower limit for the *intrinsic* kinetic deuterium isotope effect of the reaction (k_H/k_D).[3] A value of 2.8 is obtained which is slightly smaller than those (3.3, 3.5) found in the benzylic hydroxylation of *p*-methylanisole.[1i] It therefore seems possible to suggest a similar mechanism for the two processes, *i.e.* the oxidation of benzyl alcohol catalyzed by CPO should involve the transfer of a hydrogen atom to the ferryl oxygen of the iron oxo complex (Scheme 1, path a).[5] An α-hydroxy carbon radical and the iron hydroxy complex P–Fe^{IV}–OH form. They may lead to the hydrated form of benzaldehyde directly (oxygen rebound, Scheme 1, path b) or stepwise with formation of the intermediate α-hydroxybenzyl cation (Scheme 1, path c and d). A similar sequence has also been proposed for the oxidation of benzyl alcohol catalyzed by cytochrome P-450.[6]

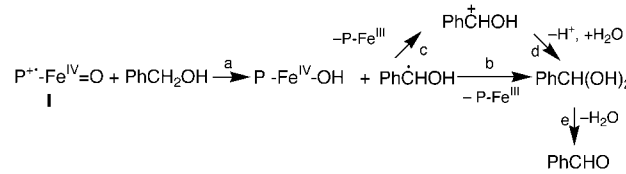
The possibility of an electron transfer mechanism facilitated by the aromatic ring seems unlikely since the value of k_H/k_D is significantly higher than that (1.8) determined in the oxidation of racemic **1**-*d* induced by the genuine one-electron oxidant $SO_4^{-\cdot}$.[7]

The data presented in Table 1 also allow some speculation concerning the orientation of the substrate in the enzyme active site. In particular, it can be suggested that the substrate binds to the enzyme orienting the C–D bond of the *S* enantiomer, and the

Table 2. Steady-state kinetic constants for the chloroperoxidase catalyzed oxidation of benzyl alcohols by H_2O_2

Substrate	K_m/mM[a]	k_{cat}/s^{-1}[a]
1	1.3 ± 0.1	17.2 ± 0.7
(*S*)-**1**-**d**	1.1 ± 0.1	6.6 ± 0.4
(*R*)-**1**-**d**	1.6 ± 0.1	18.7 ± 0.9

[a] Obtained from a Lineweaver–Burk plot. The initial rates for the formation of benzaldehyde were determined spectrophotometrically at 25 °C and pH 6 following the increase of absorbance at 280 nm.



Scheme 1

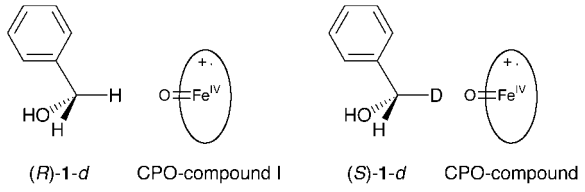


Fig. 1 Proposed orientation of (*R*)-**1**-*d* and (*S*)-**1**-*d* with respect to the iron oxo complex in the CPO active site.

C–H bond of the *R* enantiomer, towards the oxygen of the active oxidant, as illustrated in Fig. 1.

It follows that the oxidation of benzyl alcohol catalyzed by CPO should be characterized by a very high prochiral selectivity with *only* the *pro-S* hydrogen [Fig. 2(*a*)] involved in the process. An additional very interesting notation is that the same spatial orientation as that observed for the oxidation of benzyl alcohol appears to be also required in the CPO-induced oxidation of ethylbenzene, which accordingly has been reported to produce (*R*)-1-phenylethanol,[1j,8] exclusively [Fig. 2(*b*).

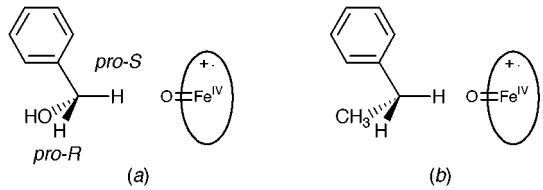


Fig. 2 Proposed orientation of (*a*) benzyl alcohol and (*b*) ethylbenzene with respect to the iron oxo complex in the CPO active site.

Thus, it would seem that in the oxidation of benzyl alcohol the OH group does not play any specific role with respect to the spatial orientation assumed by the substrate in the enzyme reacting pocket. Accordingly, the orientation appears not to change when OH is replaced by a Me group. Probably, the interaction of the aromatic ring with the side chains of Phe 103 and/or Phe 186 plays the major role in this respect. Both Phe 103 and Phe 186 are situated at the bottom of the opening of the CPO substrate-binding pocket and have been suggested to be of fundamental importance in establishing hydrophobic interactions with organic substrates.[9] Theoretical calculations to test this hypothesis are under way.

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Notes and references

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2 J. H. Dawson, *Science*, 1988, **240**, 433; M. C. R. Franssen and H. C. Van der Plas, *Adv. Appl. Microbiol*., 1992, **37**, 41.

3 Actually, k^R_{cat}/k^S_{cat} is a measure of the intrinsic deuterium isotope effect only if the rate of release of the product from the enzyme–product complex is not kinetically significant. If this condition does not hold, the intrinsic deuterium isotope effect is expected to be larger than k^R_{cat}/k^S_{cat} (ref. 4).

4 *Reaction Rates of Isotopic Molecules*, ed. L. Melander and W. H. Saunders, Wiley, New York, 1980, ch. 10, pp. 297–305.

5 However, a concerted oxygen insertion, as suggested by the use of radical probe substrates [ref. 1(*j*), (*k*)], might also be compatible with the observed intrinsic k_H/k_D values.

6 A. D. N. Vaz and M. J. Coon, *Biochemistry*, 1994, **33**, 6442. Interestingly, in this paper an intramolecular kinetic isotope effect of 2.6 was determined in the oxidation of [α-$^2H_1$]benzyl alcohol induced by purified rabbit liver cytochrome P450 2B4, using only the racemic monodeuterated benzyl alcohol. In preliminary experiments we have found significantly different values of the observed intramolecular kinetic deuterium isotope effect in the phenobarbital induced rat liver microsomal oxidation of (*R*)-**1**-*d* and (*S*)-**1**-*d* (1 and 4, respectively).

7 (±)-**1**-*d* was reacted with $SO_4^{\bullet-}$ (from $Ti^{III}/K_2S_2O_8$ or γ-radiolysis/$K_2S_2O_8$) in H_2O at pH 6 (0.1 M phosphate buffer). From the molar ratio between PhCDO and PhCHO produced in the reaction, measured by GC-MS, a k_H/k_D value of 1.8 was calculated.

8 The *pro-R* hydrogen in ethylbenzene corresponds to the *pro-S* hydrogen in benzyl alcohol.

9 M. Sundaramoorthy, J. Terner and T. L. Poulos, *Structure*, 1995, **3**, 1367.

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