Activation of H_2O_2 by P450: Evidence that the Hydroxylating Intermediate is Iron(III)-coordinated H_2O_2 and not the Ferryl FeO^{3+} Complex

John M. Pratt,*[a] T. Ian Ridd[a] and Laurence J. King[b]

[a] *Department of Chemistry, University of Surrey, Guildford, Surrey, UK GU2 5XH*
[b] *School of Biological Sciences, University of Surrey, Guildford, Surrey, UK GU2 5XH*

Purified P4502B4 (LM2) catalyses the hydroxylation of lauric acid by H_2O_2 against a background of degradation of the porphyrin ring while iodosylbenzene, which rapidly forms the ferryl FeO^{3+} derivative, supports neither hydroxylation nor degradation and further inhibits degradation by added H_2O_2, indicating that the key intermediate in both hydroxylation and heme degradation is an Fe^{III} peroxide complex.

There is continuing active interest in the diverse reactions catalysed by the P450 superfamily of hemoprotein enzymes where, in contrast to the His imidazole common to many other hemoproteins [*e.g.* Hb, Mb, classical peroxidases, heme oxygenase (HO)], the proximal ligand is the Cys thiolate RS^-.[1a–d] One of the most characteristic reactions of the P450 enzymes is the hydroxylation of alkanes and unactivated alkyl groups in, for example, long-chain fatty acids (all here denoted by RH) according to eqn. (1), where 2H represents two reducing equivalents supplied by NADPH *via* a specific P450 reductase enzyme; the reaction involves reduction of Fe^{III} to Fe^{II}, followed by the coordination and reduction of O_2 to the key (but unidentified) hydroxylating intermediate.[1a–d] Reaction (1) is probably always accompanied by some degree of 'uncoupling' to produce H_2O_2 according to eqn. (2) and this can even be made the predominant reaction through site-directed mutagenesis or the use of non-hydroxylatable perfluorinated substrate analogues.[1c] Some P450 enzymes have been shown to catalyse hydroxylation by H_2O_2 [eqn. (3)][2,3] as well as by the more commonly studied RO_2H and RCO_3H; these reactions require no second protein and involve no reduction to the Fe^{II} level.[1a–d] Reactions (3) and, to a lesser extent, (1) and (2) are accompanied by some degree of degradation of the porphyrin ring and bleaching of the spectrum.[1a] The evidence therefore suggests that the overall reaction (1) occurs as reaction (2) followed by reaction (3) and heme-degradation.

$$O_2 + 2H + RH \rightarrow ROH + H_2O \quad (1)$$

$$O_2 + 2H \rightarrow H_2O_2 \quad (2)$$

$$H_2O_2 + RH \rightarrow ROH + H_2O \quad (3)$$

$$RCHO + H_2O_2 \rightarrow (R\text{–}H) + HCO_2H + H_2O \quad (4)$$

The key hydroxylating intermediate of (1) and (3) has never been detected and there is no agreement as to whether it either retains the O–O bond intact as a peroxide ligand coordinated to Fe^{III} or possesses only a single O atom as in the formally pentavalent iron-oxene or ferryl FeO^{3+} complex.[1] The study of (3), which might provide the easiest access to the key intermediate, is hampered by the simultaneous occurrence of degradation. However, Blake and Coon recently provided good evidence that the main product of reaction of P4502B4 (formerly LM2) with iodosylbenzene (PhIO) is the elusive ferryl complex;[4] comparison of P450-catalysed reactions using H_2O_2 and PhIO therefore provides a possible means of testing between peroxide and ferryl complexes as the key intermediate. Coon and coworkers subsequently reported that the P4502B4-catalysed reaction [eqn. (4)], where R is cyclohexyl and (R–H) is cyclohexene, was supported by H_2O_2 but not by PhIO; they concluded that 'the generally accepted pentavalent iron oxene is not the oxidant' and proposed the initial addition of an Fe^{III}-coordinated peroxide to the aldehyde carbonyl group.[5] Akhtar and coworkers have likewise concluded that the products from certain reactions of steroids catalysed by P450 17Al could only be explained by the reaction of some Fe^{III} peroxide complex with a ketone carbonyl.[6] No such test has yet been reported for any P450-catalysed hydroxylation or heme degradation. Ortiz de Montellano and coworkers have, however, shown that heme degradation by (HO), which shows many parallels to P450, also involves some Fe^{III}-peroxide complex (which adds to the bridge C atom) and that formation of the ferryl complex actually inhibits heme destruction by H_2O_2.[7,8] We have now studied heme degradation and hydroxylation in parallel, using lauric acid (LA) as a solubilised alkane (known to be hydroxylated in the ω, ω − 1 and ω − 2 positions)[3–9] and P4502B4[10] **1** (as used by Coon and coworkers)[4,5] and report here the results of tests (comparing H_2O_2 and PhIO) for peroxide *vs.* ferryl complexes as the key intermediate in these two reactions.

We have compared the reactions of solutions† of **1** (in the absence of LA) at pH 7.5 and 25 °C with (*a*) 10 mmol dm^{-3} H_2O_2, (*b*) 0.1 mmol dm^{-3} PhIO and (*c*) 0.1 mmol dm^{-3} PhIO with 10 mmol dm^{-3} H_2O_2 injected after 20 min by following both the fall in A_{417} (Soret band of the starting Fe^{III} complex) and the changes in the 360–500 nm spectral region, with the following results: (*a*) H_2O_2 causes a relatively slow fall in A_{417} which follows first-order kinetics ($t_{\frac{1}{2}}$ *ca.* 30 min), with no significant formation of any new absorption bands, to leave a featureless tail of absorption extending from the UV with a low residual A_{417} (*ca.* 15% of the initial value after 200 min); degradation by H_2O_2 can be suppressed by the addition of ligands such as py or CN; (*b*) in agreement with Blake and Coon,[4] we find that PhIO causes a far more rapid ($t_{\frac{1}{2}} < 1$ min) but less extensive initial fall in A_{417} (to *ca.* 35% of its initial value after 10 min), accompanied by the rise of new bands in the 380–390 nm region (indicating the formation of FeO^{3+}),[4] which is then followed by a very slow further fall in A_{417} (still *ca.* 30% after 150 min); (*c*) injection of H_2O_2 after reaction with PhIO causes no significant change (other than dilution) in the spectrum (especially the bands at *ca.* 380–390 nm) or in the rate or extent of the very slow reaction observed with PhIO alone; this indicates that in P450, as in (HO),[7,8] the formation of FeO^{3+} inhibits degradation by H_2O_2 and, conversely, that degradation by H_2O_2 does not involve the FeO^{3+} intermediate.

Comparison of the yields of the hydroxylated products[11] (in triplicate experiments) from solutions‡ containing **1** and LA which were allowed to react for 10 min with (*a*) 15 mmol dm^{-3} H_2O_2, (*b*) 0.1 mmol dm^{-3} PhIO with LA added first and (*c*) 0.1 mmol dm^{-3} PhIO with LA added after 10 min gave a minimum ratio of ≥200 for products from (*a*) compared to those from either (*b*) or (*c*) where, in fact, none could be detected. Our results indicate that the P450-catalysed hydroxylation of LA by H_2O_2 does not involve the ferryl complex and that, at least in the case of P4502B4, neither PhIO nor the preformed FeO^{3+} are able to hydroxylate LA at any significant rate.

Simultaneous monitoring of the changes in spectrum ($t_{\frac{1}{2}}$ *ca.* 30 min for bleaching, with no new bands produced by the presence of LA) and the rate of increase in concentration of hydroxylated products (apparent $t_{\frac{1}{2}} \leq 1$ min, levelling off at *ca.* 6% of available LA converted after 20 min) showed that the rate of inactivation of the enzyme is faster than the rate of chromophore bleaching and that the ratio of products hydroxylated in the ω, ω − 1 and ω − 2 positions changed from *ca.* 0 : 1 : 1 at the start to *ca.* 0 : 3 : 1 at the end of the reaction. This smaller $t_{\frac{1}{2}}$ and changing ratio suggest that H_2O_2 reacts with one

or more amino acid residues, causing some conformational change(s) and inactivating the enzyme, more rapidly than it attacks the porphyrin ring and also that the product ratio is sensitive to such conformational changes. Useful comparisons can be made with the 2–3 fold increase in $\omega:\omega-1$ ratio from P4504A1 (using O_2 + NADPH + reductase) caused simply by the addition of cyt.b_5 and the greater than 100-fold difference between P4504A1 and P4502B1;[12] *cf.* also the *ca.* 20-fold difference in the $\omega:\omega-1$ ratio observed in the hydroxylation of hexane by the non-heme methane monoxygenase when using O_2 (+ NADH + reductase) or H_2O_2 (where other evidence indicated the same 'activated oxygen' intermediate), which was ascribed to a conformational change at the Fe^{II} level in the reaction involving O_2.[13] We find that the P4502B4-catalysed hydroxylation of LA using O_2 with NADPH and reductase§ gives a ratio of *ca.* 1 : 2 : 0 compared to 0 : 1–3 : 1 with H_2O_2. In view of the marked differences in product ratios observed in the cases mentioned, the variation observed here with P4502B4 (between O_2 and H_2O_2 and, in the latter case, with time) clearly does not exclude the formation of a common intermediate for reactions (1) and (3), which seems the simplest assumption to make.

Our results therefore indicate that in both heme degradation and LA hydroxylation by H_2O_2 (and hence, by extension, by O_2 + NADPH + reductase) the key intermediate is not the ferryl FeO^{3+} but must be structurally more closely related to the reagent H_2O_2 (pK 11.7).[14] Further experiments (details to be published) show that the rates of both reactions are H_2O_2-dependent but essentially pH-independent within the narrow range of pH available for study, *i.e.* they require H_2O_2 and not HO_2^-. The simplest scheme to explain our observations involves the reversible coordination of undissociated H_2O_2 (whether added or formed from O_2 + NADPH) according to eqn. (5), where only the distal ligand is given, followed by the hydroxylation of LA (denoted by RH) as in eqn. (6). This scheme closely resembles that proposed by White and Coon[15,16] [for H_2O_2 produced by eqn. (2) as well as for added ROOH] except that they separated (6*a*) into two successive steps, involving O–O fission to generate a free HO radical which only subsequently attacks RH; we suggest that O–O fission could also occur in step with H atom transfer. We agree that the role of Cys as proximal ligand (instead of His) is to stabilise the Fe^{IV} product of (6*a*), not the formally Fe^{V}, but they suggest this depends on its ability to form a thiyl radical (*i.e.* $RS\cdot + Fe^{III}$); we suggest that the greater electron donor power of RS^- compared to His^{17} serves both to stabilise the higher oxidation state Fe^{IV} and to suppress the formation of anionic HO_2^- as ligand, which would lead to the rapid formation of FeO^{3+} as in the peroxidases.[18]

$$H_2O_2 + [H_2O{\cdot}Fe^{III}] \rightleftarrows H_2O + [H_2O_2{\cdot}Fe^{III}] \quad (5)$$

$$RH + [HO-OH{\cdot}Fe^{III}] \xrightarrow{a} R\cdot + H_2O + [HO^-{\cdot}Fe^{IV}]$$

$$\xrightarrow{b} ROH + H_2O + [Fe^{III}] \rightarrow ROH + [H_2O{\cdot}Fe^{III}] \quad (6)$$

Our results serve to extend those of Coon[5] and Akhtar,[6] which have already established a key role for some Fe^{III}-peroxide intermediate in other reactions of P450, to the hydroxylation of unactivated alkyl groups. They also highlight a common denominator in the ability of H_2O_2 to hydroxylate alkanes when associated with a sufficiently strong acid, whether the proton (*cf.* $H_3O_2^+$ in liquid HF, *etc.* which can react well below 0 °C)[19] or certain Lewis acids such as Fe^{III} in P450. Coordination might, by analogy, activate other O-donors such as PhIO towards hydroxylation (*e.g.* by H abstraction from RH by a coordinated O atom, loss of PhI and return of HO to the radical R·) without prior conversion to a ferryl complex; this might explain the short-lived burst of hydroxylation of LA by PhIO catalysed by whole microsomes.[20] Alkanes may also be hydroxylated by oxo complexes as shown for a well-characterised complex of RuO^{3+} which is isoelectronic with FeO^{3+},[21] but this remains to be established for P450. There has been a natural tendency to assume one common intermediate for all combinations of O-donors, substrates and isoenzymes of P450; the situation may be more complex and more direct evidence is required.

We thank the SERC for a Research Studentship (to T. I. R.) and Professor Gordon Gibson for helpful discussions.

Received, 20th July 1995; Com. 5/04771D

Footnotes

† Reaction mixtures (total volume 1 ml) contained 1 μmol dm^{-3} **1**, 30 μg freshly sonicated phosphatidylcholine, 15 mmol dm^{-3} $MgCl_2$ and tris-HCl buffer pH 7.5; *cf.* ref. 11.

‡ Reaction mixtures as in footnote † together with 0.5 mmol dm^{-3} LA and 0.1 μCi [1-^{14}C]LA.

§ Reaction mixture as in footnote ‡ together with 1 mmol dm^{-3} NADPH and 0.9 units NADPH reductase.

References

1 See for example, (*a*) F. P. Guengerich, *Crit. Rev. Biochem. Mol. Biol.*, 1990, **25**, 97; (*b*) F. P. Guengerich, *J. Biol. Chem.*, 1991, **266**, 10019; (*c*) B. K. Hawkins and J. H. Dawson, in *Frontiers in Biotransformation*, ed. K. Ruckpaul and H. Rein, Academie Verlag, Berlin, 1992, vol. 7, p. 216; (*d*) M. J. Coon, X. Ding, S. J. Pernecky and A. D. N. Vaz, *FASEB J.*, 1992, **6**, 669.
2 E. G. Hrycay, J. Gustafsson, M. Ingelman-Sundberg and L. Ernster, *Biochem. Biophys. Res. Commun.*, 1975, **66**, 209.
3 M. C. Romano, K. M. Straub, L. A. P. Yodis, R. D. Eckardt and J. F. Newton, *Anal. Biochem.*, 1988, **170**, 83.
4 R. C. Blake and M. J. Coon, *J. Biol. Chem.*, 1989, **264**, 3694.
5 A. D. N. Vaz, E. S. Roberts and M. J. Coon, *J. Am. Chem. Soc.*, 1991, **113**, 5886.
6 P. Robichaud, J. N. Wright and M. Akhtar, *J. Chem. Soc., Chem. Commun.*, 1994, 1501.
7 A. Wilks and P. R. Ortiz de Montellano, *J. Biol. Chem.*, 1993, **268**, 22357.
8 A. Wilks, J. Torpey and P. R. Ortiz de Montellano, *J. Biol. Chem.*, 1994, **269**, 29553.
9 P. R. Ortiz de Montellano, W. K. Chan, S. F. Tuck, R. M. Kaikaus, N. M. Bass and J. A. Peterson, *FASEB J.*, 1992, **6**, 695.
10 M. J. Coon, T. A. van der Hoeven, S. B. Dahl and D. A. Haugen, *Methods Enzymol.*, 1978, **52**, 109.
11 G. Parker and T. C. Orton, in *Biochemistry, Biophysics and Regulation of Cytochrome P450*, ed. J. Gustafsson, J. Duke, A. Mode and J. Rafter, Elsevier/North Holland, Amsterdam, 1980, pp. 373–7.
12 C. A. Jacob, W. K. Chan, E. Shephard and P. R. Ortiz de Montellano, *J. Biol. Chem.*, 1988, **263**, 18640.
13 W. A. Froland, K. K. Andersson, S. Lee, Y. Liu and J. D. Lipscomb, *J. Biol. Chem.*, 1992, **267**, 17588.
14 P. M. Wood, *Biochem. J.*, 1988, **253**, 287.
15 R. E. White and M. J. Coon, *Ann. Rev. Biochem.*, 1980, **49**, 315.
16 M. McCarthy and M. J. Coon, *J. Biol. Chem.*, 1983, **258**, 9153.
17 M. Sono and J. H. Dawson, *J. Biol. Chem.*, 1982, **257**, 5496.
18 D. A. Baldwin, H. M. Marques and J. M. Pratt, *J. Inorg. Biochem.*, 1987, **30**, 203.
19 G. A. Olah, N. Yoneda and D. G. Parker, *J. Am. Chem. Soc.*, 1977, **99**, 483.
20 J. Gustafsson and J. Bergman, *FEBS Lett.*, 1976, **70**, 276.
21 C. Che, C. Ho and T. Lau, *J. Chem. Soc., Dalton Trans.*, 1991, 1259.