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Evidence for Two Different Active Oxygen Species in Cytochrome P450 BM3 Mediated Sulfoxidation and N-Dealkylation Reactions

Trent J. Volz, Denise A. Rock, and Jeffrey P. Jones*

Department of Chemistry, P.O. Box 644630, Washington State University, Pullman, Washington 99164-4630

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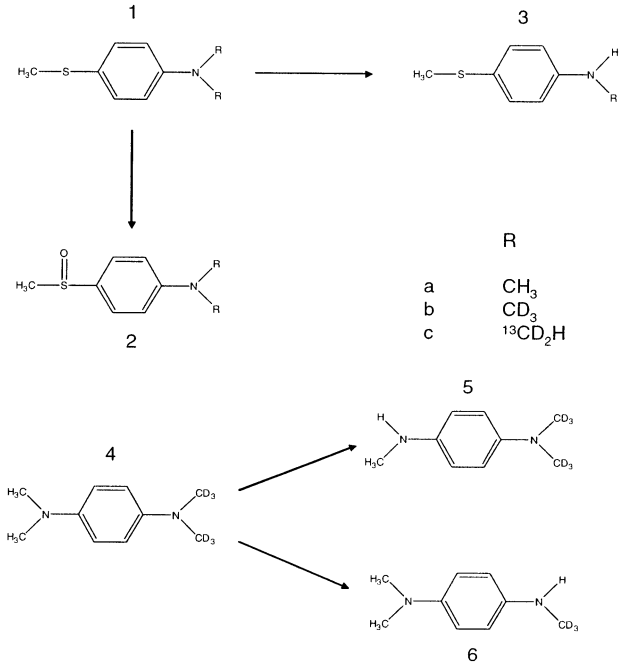
Cytochrome P450 is a superfamily of heme containing enzymes that is found in almost all organisms including bacteria, plants, and animals.[1] It can oxidize compounds of a broad chemical diversity and is perhaps the most important enzyme family involved in drug metabolism.[2] Herein, we report results on N-dealkylation and sulfoxidation, two of the most facile reactions mediated by Cytochrome P450. Results from two experiments are consistent with sulfoxidation and N-dealkylation arising from two different enzyme substrate complexes (ES) and thus two different active oxygen species that do not interchange. The first experiment involves the use of a mutant that has been postulated to increase the amount of the hydroperoxy-iron species ($Fe^{III}O_2H$). This mutant increases the amount of sulfoxidation relative to the amount of N-dealkylation by 4-fold. In a second experiment, deuterium substitution on the N-methyl groups of substrate **1** (see Scheme 1) does not result in an increase in sulfoxidation. This latter result is consistent with N-dealkylation and sulfoxidation being mediated by two different active oxygen species.

The consensus opinion has been that Cytochrome P450 employs an electrophilic oxenoid-iron species ($Fe^{IV}O$) as a catalytically active oxidant.[3−7] More recently, multiple active oxygen species have been postulated,[8] but no direct evidence has been obtained for their existence. On the basis of site-directed mutagenesis[9] and radical probe experiments,[10] Coon and co-workers have proposed hydroperoxy-iron as a second electrophilic active oxygen species.[8] Alternatively, Shaik and co-workers propose a two-state reactivity model that includes both a high spin ground state and a low spin excited state of oxenoid-iron as oxidizing species.[11,12]

Substrates (**1a**,[13] **1b**,[14] **1c**,[15] and **4**[14]) and chromatographic standards (**2a**[16] and **3a**[17]) were synthesized according to literature procedures. For enzymatic incubations, 10 mL scintillation vials were charged with substrate (1.6 μmol in 9 μL of methanol), purified P450 enzyme (200 pmol),[18] glucose-6-phosphate (9.2 μmol), glucose-6-phosphate dehydrogenase (0.40 nmol), catalase (0.34 nmol), and magnesium chloride (4.2 μmol) in phosphate buffer (0.1 M, pH 7.4)[19] to give a total volume of 990 μL. The incubations were initiated by adding NADP (1.2 μmol in 110 μL of the phosphate buffer) and placed in a shaker bath at 30° C for 25 min. The incubations were terminated by adding methanol (300 μL)[20] and filtered through a 0.45 μm filter. Enzymatic products, see Scheme 1, were analyzed by liquid chromatography mass spectrometry (LC-MS) using electrospray ionization monitoring positive ions (ESI+) for sulfoxidation/N-dealkylation, competitive intermolecular kinetic isotope effect (KIE), and intramolecular KIE experiments, while electrospray ionization monitoring negative ions (ESI−) was used for intrinsic KIE experiments.[21]

Table 1 displays the results of enzymatic incubations of substrates **1a**−**c** and **4** using both CYP BM3 wildtype and a CYP BM3 T268A mutant.[22] The T268A mutant, which may increase the steady-state levels of the hydroperoxy-iron,[8−10] increased sulfoxidation relative to N-dealkylation (one tailed P value from T test < 0.001). This altered reactivity of the T268A mutant is consistent with the existence of two different electrophilic oxidizing species:[8,9] one that

Scheme 1. Cytochrome P450 Reactions for Substrates **1a**−**c** and **4**[a]



[a] Substrates **1a**−**c** may undergo either sulfoxidation or N-dealkylation, while substrate **4** can be N-dealkylated at either end.

Table 1. Experimental Data for CYP BM3 Wildtype and T268A Mutant[a]

enzyme	sulfox/ N-dealk[b]	comp. KIE[c]	intrinsic KIE[d]	intra KIE[e]
BM3	15 ± 1 (4)	1.1 ± 0.1 (9)	2.1 ± 0.1 (4)	1.9 ± 0.1 (4)
T268A	60 ± 9 (4)	1.1 + 0.1 (5)	2.2 ± 0.2 (4)	2.0 ± 0.2 (4)

[a] The first number in each entry is the average of several independent determinations, the number preceded by ± is the standard deviation, and the number in parentheses is the number of independent determinations. [b] This ratio is the amount of **2a** divided by the amount of **3a** produced when using **1a** as the substrate. [c] The competitive intermolecular KIE was obtained from the ratio of the KIE for N-dealkylation (**3a**/**3b**) divided by the KIE for sulfoxidation (**2a**/**2b**) when equal amounts of **1a** and **1b** were used as substrate. [d] The intrinsic KIE was determined by analyzing the dimedone adducts of formaldehyde-d_1 and formaldehyde-d_2 produced during the enzymatic incubation of **1c** (see ref 15). [e] The intramolecular KIE was measured using substrate **4** as a model compound and dividing the amount of product **5** by the amount of product **6**.

* To whom correspondence should be addressed. E-mail: jpj@wsu.edu.

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mediates sulfoxidation and is increased in the mutant and another that mediates N-dealkylation.

However, while the mutant has been postulated to increase the hydroperoxy-iron species, the changes in reactivity could be a direct result of changes in the active-site structure and not different oxygenating species. It is not unexpected that a mutant in the active site would change regioselectivity. For example, if another mutant of BM3, F87A, is incubated with **1a**, we see a decrease in the amount of sulfoxidation relative to N-dealkylation. Because this mutant is not presumed to be involved in proton transfer to the hydroperoxy-iron species, this altered regioselectivity is presumably due to a change in active-site structure, and not a change in the active oxygen species. Thus, care must be used in interpreting the results of active-site mutations.

To test if the two products come from the same or different oxygenating species, we looked for isotopically sensitive branching[23] when deuterium is placed on the N-methyl groups of substrate **1**. Isotopically sensitive branching of the reaction pathway is observed when the product of the second pathway is increased by isotopic substitution on the first pathway. If the two products come from the same active oxygen species, an increase in sulfoxidation relative to N-dealkylation, and thus an isotope effect, would result from incubation of compound **1b**.[2,23] No significant isotope effect is observed for this compound as shown in Table 1. The isotope effects for both N-dealkylation (1.06) and S-oxidation (0.97) are not significantly different than 1.0 when compound **1b** is used as a substrate**.** The combined isotope effect which takes into account any possible branching to sulfoxide and should give the largest possible isotope effect is reported in Table 1. This lack of isotopically sensitive branching could arise from (1) a very small intrinsic isotope effect for N-dealkylation, (2) slow interchange as a result of substrate enzyme interaction, or (3) the two products originating from different oxidizing species.

The intrinsic isotope effect was measured to be 2.2 using substrate **1c**, which should lead to significant branching and an isotope effect for compound **1b** if the two ES complexes are able to interchange. Thus, the lack of isotope effect indicates a lack of interchange, not a small intrinsic isotope effect. When substrate **4** is used, rapid interchange is seen between oxidation at both ends of the molecule as a result of isotopically sensitive branching. Thus, rotation between both ends of **1** appears to be possible. This, combined with a measured intrinsic isotope effect of 2.2, is consistent with the sulfoxidation and N-dealkylation products arising from different oxidizing species.

While the data indicate two active oxygen species, they do not distinguish between the different possibilities for the active oxygen species. It is tempting to assume that sulfoxidation is mediated by a hydroperoxy-iron active oxygen species, while N-dealkylation has been reported to be mediated by hydrogen atom abstraction by the oxenoid-iron species.[15] However, Shaik's two-state reactivity model can also explain the observed data. In this mechanism, the high spin and low spin states of oxenoid-iron form different ES complexes. If the two ES complexes are noninterchangeable, with one mediating sulfoxidation and the other mediating N-dealkylation, this would account for the KIE data. Watanabe has proposed that instead of increasing the amount of hydroperoxy-iron, the threonine to alanine mutation may alter the organized network of water molecules around the oxenoid-iron species and thus change the hydrogen-bonding interactions of the oxenoid-iron ES complex.[24] This may alter the ratio of high to low spin oxenoid-iron and explain the altered reactivity observed in the T268A mutant.

In closing, site-directed mutagenesis and KIE data provide evidence for two oxidizing species in Cytochrome P450: one that mediates sulfoxidation and another that mediates N-dealkylation.

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(18) Enzyme concentration was determined by the method of Karki et al (see ref 15).
(19) For intramolecular KIE experiments, the phosphate buffer was pH 8.2.
(20) Intrinsic KIE incubations were terminated and derivatized by the method of Karki et al. (see ref 15), except that the resulting residue was dissolved in methanol (200 μL) for LC-MS analysis.
(21) LC-MS analysis was done using a Finnigan AQA LC-MS coupled to a ThermoQuest Surveyor high performance liquid chromatography (HPLC) system equipped with an Alltech Alltima C18 column (150 mm × 3.2 mm). The solvent for ESI+ was 85:15 methanol/aqueous ammonium acetate solution (0.13 M), while 85:15 methanol/water was used for ESI−. Solvent flow rates were 400 μL/min. The probe temperature was 250 °C, and an ionizing voltage of 25 V was used.
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