Enzyme Catalysis

DOI: 10.1002/ange.200603411

# Is the Ferric Hydroperoxy Species Responsible for Sulfur Oxidation in Cytochrome P450s?**

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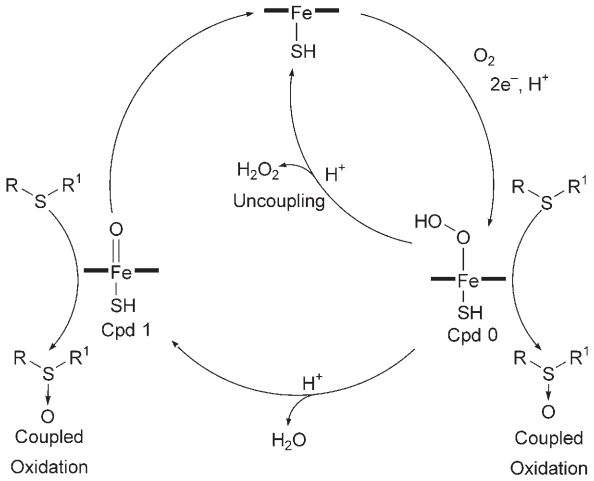
General research into cytochrome P450 (P450) mediated oxidation has led to a consensus view that the highly active, electrophilic Cpd I is responsible for the vast majority of such oxidations (Scheme 1).[1] The much less reactive Cpd 0 has

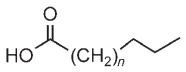



***Scheme 1.*** Abbreviated P450 oxidative cycle of thioethers, indicating possible sources of sulfoxidation and uncoupling.

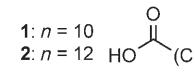
also been proposed as an oxidant in many P450-catalyzed transformations, including C-H hydroxylation, epoxidation, and sulfoxidation (Scheme 1). To date, however, no reaction has been unambiguously assigned to Cpd 0 and most results suggesting the presence of a second, electrophilic oxidant have been explained by Shaik's two-state reactivity paradigm, in which differing spin states of Cpd I react as different species.[2] However, the possibility remains that substrates containing centers that are much more susceptible to oxidation, such as sulfur, are oxidized by Cpd 0.[1] DFT calculations have suggested that Cpd I is a much more capable oxidant of sulfur than is Cpd 0,[3] and on this basis it was concluded that Cpd I is the major oxidant of thioethers. However, these results do not necessarily preclude Cpd 0 as an active oxidant in wildtype enzymes.

Decreasing the activity of Cpd I through the use of specific threonine-to-alanine active-site mutants has been demonstrated for $P450_{cam}$[4,5] and $P450_{BM3}$,[6,7], among others. These mutants have been used to investigate the oxidation of a wide variety of substrates, including thiothers.[8] Studies of the kinetics of wildtype and mutant $P450_{BM3}$ catalyzed oxidation of a subtrate with both amine or thioether moieties led to the postulate that two separate oxidants affect amine and thioether oxidation; the oxidation of sulfur was greater for the mutant in which Cpd 0 activity is potentiated.[8] An alternative explanation of these results that does not implicate Cpd 0 was proposed in which different spin states of Cpd I masquerade as two different oxidants.[3] Whether Cpd 0 does play a role in wildtype P450 catalyzed sulfoxidation was thus unclear.

Our interest in fatty acid oxidation by P450s[9–14] led us to investigate the use of thiafatty acids as potential mechanistic probes for P450s. Prior, elegant use of thiafatty acids by Buist and Marecak to probe the cryptostereochemistry of dehydrogenase enzymes indicated that these probes yield useful stereochemical data concerning reaction intermediates that are not readily isolatable.[15] We wished to use such probes to reveal the cryptostereochemistry of oxidative carbon–carbon-bond cleavage of fatty acids by $P450_{BioI}$.[11,13,16] Two isolated examples of P450-catalyzed oxidation of analogous thioether- or methylene-containing substrates appeared to indicate that these reactions proceed with the same stereochemical outcome.[17–19] However, we felt it prudent to validate the use of thiafatty acids as stereochemical probes with fatty acid metabolizing P450s for which the stereochemical outcomes were known. Thus, we chose to investigate thiafatty acid oxidation by the widely studied $P450_{BM3}$, a catalytically self-sufficient P450 isolated from *Bacillus megaterium*.[20] The regio- and stereoselectivity of fatty acid oxidation by $P450_{BM3}$ is well known for $C_{14}$ (**1**) and $C_{16}$ (**2**) fatty acids; thus, the thiafatty acids **3** and **4** were chosen as target substrate analogues.[20–23]




**1**: $n = 10$
**2**: $n = 12$




**3**: $n = 10$, R = SEt
**4**: $n = 12$, R = SEt
**5**: $n = 10$, R = S(O)Et
**6**: $n = 12$, R = S(O)Et

The synthesis of **3** and **4** was trivial and utilized the method of Yin and Pidgeon.[24] The synthesis of nonracemic sulfoxide standards (**5** and **6**) was also clearly important and was accomplished by using two distinct methods to incorporate a sulfoxide of known stereochemistry. Two independent methodologies were used to confirm the stereochemistry of **5** and **6** because of the results of enzymatic oxidation (see below). The first route utilized the displacement of the ethyl sulfinate ester of diacetone glucose, which is available in high diastereomeric ratio with a known diastereomeric preference.[25,26] The second route applied the Kagan oxidation to an alkyl/aryl thioether,[27] followed by Grignard displacement of

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[**] The authors are grateful to Prof. S. Chapman (University of Edinburgh) for the $P450_{BM3}$ clones used in this work.

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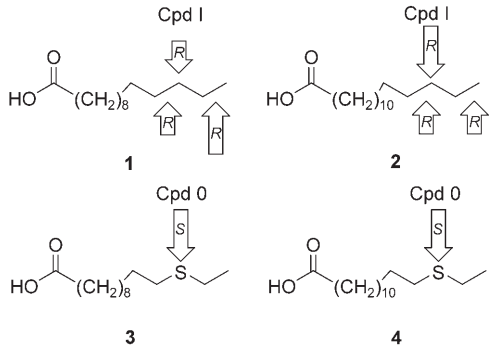
the *p*-chlorophenyl group.[28] Both routes afforded the same enantiomer, as predicted from previous work and determined by enantioselective HPLC (Chiralcel OB-H).[25–28]

With both product standards in hand, we investigated the $P450_{BM3}$-mediated oxidation of thiafatty acids **3** and **4** (see the Supporting Information for conditions). Analysis of the products of oxidation of **3** and **4** indicated that the presence of the sulfur dramatically alters the regiochemistry of the reaction. The metabolism of fatty acids **1** and **2** yields oxidation products across the three subterminal methylene groups; the highest regiochemical preference in both cases was approximately 50% (Table 1, Scheme 2).[10,20] With **3** and **4**, the relative oxidation at the sulfur atom ($\omega$-2) rose to 99% of the total oxidation, as might be expected after inclusion of a center susceptible to oxidation. Measurement of the stereochemistry of the resultant sulfoxide showed a decrease in enantiomeric purity (ratios change from 99:1 or 98:2 to 75:25). This drop in selectivity may be expected as the more easily oxidized sulfur atom may allow reaction from a greater range of reactive conformations than the more demanding C–H bond.[17–19] Intriguingly, however, the sulfoxides were produced with the opposite enantioselectivity to that observed for comparable fatty acid hydroxylation (*S* sulfoxides versus *R* alcohols).[21,22]

***Table 1:*** $P450_{BM3}$ wildtype (WT) and T268 mutant (TA) oxidation of probes **1–4**.

| Substr./ Enzyme | Oxidation Rate[a] | % Coupling[b] | % Oxidation at Site (% Stereochemistry)[c] | | |
|---|---|---|---|---|---|
| | | | $\omega$-3 | $\omega$-2 | $\omega$-1 |
| **1**/WT | 1.0±0.1 | 88±1 | 25±2 (74 *R*) | 27±2 (98 *R*) | 48±2 99 *R*) |
| **3**/WT | 0.4±0.1 | 81±9 | 1±1 | 99±1 (75 *S*) | 0 |
| **3**/TA | 0.4±0.1 | 88±5 | 1±1 | 99±1 (72 *S*) | 0 |
| **2**/WT | 1.6±0.5 | 93±6 | 24±2 (74 *R*) | 53±2 (99 *R*) | 23±2 (99 *R*) |
| **2**/TA | 0.3±0.1 | 21±5 | 25±2 (78±4 *R*)[23] | 52±2 (92±4 *R*)[23] | 23±2 (92±4 *R*)[23] |
| **4**/WT | 0.9±0.1 | 81±1 | 1±1 | 99±1 (75 *S*) | 0 |
| **4**/TA | 0.8±0.1 | 80±1 | 1±1 | 99±1 (75 *S*) | 0 |

[a] Quantified by monitoring the disappearance of NADPH at 340 nm (see the Supporting Information), μmol product formed $min^{-1}$ nmol $heme^{-1}$. [b] Expressed as moles of product formed (GC–MS determination with internal standard) divided by moles of NADPH consumed. [c] Stereochemistry determined by enantiomeric HPLC or GC[23] with isomer identification through comparison with enriched standards (see the Supporting Information).




***Scheme 2.*** Comparison of the products of oxidation of fatty acids **1** and **2** and thiafatty acids **3** and **4** by $P450_{BM3}$. The arrows indicate the position and stereochemistry of oxidation.

One explanation for this switch in enantiomeric preference for oxidation **3** and **4** relative to the analogous fatty acids is that they were accessing an unusual binding conformation that allowed the production of the *S* sulfoxide. This appears unlikely, as a range of modified fatty acids appear to undergo uniformly *R* oxidation.[9,22,29] Spectroscopic analysis of binding of **3** and **4** to $P450_{BM3}$ revealed no difference between their interactions and those of **1** and **2**: spectra of $P450_{BM3}$ bound to both fatty acids and thiafatty acids were the same in both the resting-state $Fe^{III}$ and in the reduced $Fe^{II}$ form. Fatty acids and thiafatty acids also afforded the same percentage spin-state change and spectral maximum of the $P450_{BM3}$ upon binding. This result is not suprising, as the replacement of a methylene group with a sulfur atom in the thiafatty acid does not result in a significant alteration in the hydrogen-bonding ability of the substrate and only slightly increases the overall chain length.

An alternative explanation involves sulfoxidation being catalyzed by a different oxidant. This might be either Cpd 0 or again different spin states of Cpd I as suggested by Shaik and co-workers.[3] Cpd 0 is rapidly converted into Cpd I in wildtype P450 enzymes through protonation of the distal oxygen atom of a heme-bound dioxygen intermediate. As this process is less favored than protonation of the proximal oxygen atom, a hydrogen-bonded water network is believed to control the regiochemistry of protonation through interactions with a conserved active-site threonine residue.[1] The TA mutants of both $P450_{cam}$ and $P450_{BM3}$ have reduced Cpd I activity and higher levels of uncoupling, as they lack this network to protonate the dioxgyen intermediate correctly. These mutants, therefore, should exhibit enhanced Cpd 0 activity and decreased Cpd I activity relative to wildtype enzymes.[4–6,22]

Thus, to examine the possibility that a Cpd 0 driven sulfoxidation reaction was occurring with $P450_{BM3}$, the activity of the T268A mutant of $P450_{BM3}$ on the thiafatty acid **4** and the corresponding fatty acid **2** was examined. The poor coupling (moles oxidized product formed/moles of NADPH consumed: see the Supporting Information for details) for oxidation of **2** (approximately 23% of wildtype coupling in our experiments) mirrors the results previously reported for lauric acid with this enzyme.[6,7] The unchanged regiochemistry and poor coupling also parallels the results reported for camphor oxidation by analogous $P450_{cam}$ mutants.[4,5] Importantly, the absolute configuration of the hydroxy fatty acids produced and the enantioselectivity of the $P450_{BM3}$T268A-catalyzed oxidation were unchanged.[23] The results thus support the hypothesis that the formation of Cpd I is slowed in this mutant, thus reducing the overall activity observed for oxidation by this mutant P450. Thus, applying such a mutant to the oxidation of **3** and **4** would

provide an indication of the oxidative contribution of Cpd I. Turnover of both **3** and **4** with $P450_{BM3}$T268A showed no difference in the coupling, rate, or regio- and enantioselectivity of oxidation relative to that seen with the wildtype enzyme. Thus, the oxidation reactions of **3** and **4** are unaffected by a decrease in the rate of Cpd I formation and represent the only examples, to our knowledge, of P450-catalyzed reactions that are unaffected by mutagenesis of the catalytically important, conserved threonine residue.[4–7,30] This result suggests that a different oxidant is responsible for the sulfur oxidation in both the $P450_{BM3}$T268A mutant and in the wildtype enzyme and implicates Cpd 0 in the oxidation of thioethers. We suggest that the sulfur atom of **3** and **4** reacts rapidly with Cpd 0, thus preventing "incorrect" protonation of Cpd 0 and resultant loss of hydrogen peroxide (uncoupling) (Scheme 1). The results also indicate that an explanation based on differential binding for the observed stereochemical differences between **1** and **2** and **3** and **4** is unlikely. Such an explanation would require that not only do **3** and **4** adopt the same binding orientation, distinct from **1** and **2**, but also that this conformation facilitates the conversion Cpd 0 to Cpd I in the mutant and thus maintains the observed rate and coupling of enzymic oxidation.

In conclusion, care must be exercized in utilizing thioethers to probe the stereochemical outcomes of more energetically demanding reactions catalyzed by P450s, such as C–H oxidation. The two prior reports of comparable P450-mediated sulfur or methylene oxidation suggest that they can proceed with comparable stereochemical outcomes,[17,18,21] but our results demonstrate that this is not universal. The postulate that Cpd 0 is responsible for thioether oxidation in P450s is in harmony with the work of Jones and co-workers that suggests that two different oxidants function in a $P450_{BM3}$-catalyzed oxidation of an amine or thioether.[8] Our proposed mechanism also agrees with earlier work that suggests that a direct oxygen-transfer step operates in P450-mediated thioether oxidation. Additionally, the intermediacy of Cpd 0 may provide a rationale for why amine oxidation, in contrast to thioether oxidation, appears to proceed through an initial electron-transfer step. Although other hypotheses can be advanced to account for the results presented herein, oxidation by Cpd 0 is the simplest one that explains both the observed stereochemical outcome and the efficiency of oxidation by the $P450_{BM3}$T268A mutant. It will be of interest to determine if this process is a general one for the metabolism of sulfur-containing substrates by wildtype P450s.

Received: August 21, 2006
Revised: September 28, 2006
Published online: November 17, 2006

**Keywords:** cytochromes · enzyme catalysis · oxidation · P450 · sulfur

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