0090-9556/06/3408-1288–1290$20.00
DRUG METABOLISM AND DISPOSITION
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DMD 34:1288–1290, 2006

Vol. 34, No. 8
10280/3128274
Printed in U.S.A.

Short Communication

KINETIC ISOTOPE EFFECTS IMPLICATE A SINGLE OXIDANT FOR CYTOCHROME P450-MEDIATED *O*-DEALKYLATION, *N*-OXYGENATION, AND AROMATIC HYDROXYLATION OF 6-METHOXYQUINOLINE

Received March 23, 2006; accepted May 12, 2006

ABSTRACT:

One major point of controversy in the area of cytochrome P450 (P450)-mediated oxidation reactions is the nature of the active-oxygen species. A number of hypotheses have been advanced which implicate a second oxidant besides the iron-oxo species designated as compound I (Cpd 1). This oxygen is thought to be either an iron-hydroperoxy species (Cpd 0) or a second spin-state of Cpd 1. Very little information is available on what fraction of P450 oxidations is mediated by the two different oxidants. Herein, we report results on three cytochrome P450-mediated reactions: *O*-dealkylation, *N*-oxygenation, and aromatic hydroxylation, which occur by three distinct chemical mechanisms. We have used kinetic isotope effects to test for branching from *O*-demethylation to *N*-oxygenation and aromatic hydroxylation, using 6-methoxyquinoline and $^2H_3$-6-methoxyquinoline as substrates for P4501A2. Identical large inverse isotope effects on V_{max}/K_m are obtained for the formation of both the *N*-oxide and the phenol. This indicates that all three reactions occur through the same enzyme-substrate complex and, thus, through a single iron-oxygen species. The nature of the iron-oxygen species is less certain but is more likely to be iron-oxo Cpd 1, given the energetics of these reactions.

The ubiquitous cytochrome P450 enzymes (P450s) are a superfamily of monooxygenases that function to metabolize a variety of endogenous and xenobiotic compounds (Ortiz de Montellano, 1995; Meunier et al., 2004). A great deal of effort has been expended on probing the oxygenation mechanisms and identifying the intermediate species formed in the catalytic cycle of the enzyme. Until recently, a consensus seemed to have been reached that the high-valent iron-oxo species (Cpd 1) is the sole active oxygenating species in P450 (Groves et al., 1978; Guengerich and MacDonald, 1990; Ortiz de Montellano, 1995). This mechanism was first elaborated by Groves et al. (1978) for aliphatic substrates as being an initial hydrogen atom abstraction followed by recombination to produce an alcohol. However, many recent publications implicate an iron-hydroperoxo species (Cpd 0) as a second electrophilic oxidant (Vaz et al., 1998; Hutzler et al., 2003; Chandrasena et al., 2004). The hypothesis that Cpd 0 is a second oxidant in the P450 catalytic cycle began with a series of site-directed mutagenesis experiments performed by Coon and coworkers (Vaz et al., 1998) in which an active-site threonine is replaced by an alanine. The mutation is thought to affect proton delivery that converts Cpd 0 to Cpd 1. The results indicate that some of the mutant enzymes will perform olefin epoxidation at enhanced rates while decreasing aliphatic C-H hydroxylation (Vaz et al., 1998). Radical probe and kinetic isotope effect experiments were used by Newcomb and coworkers (Chandrasena et al., 2004) to conclude that Cpd 0 is the predominant oxidant affecting aliphatic hydroxylation of substituted cyclopropanes but that Cpd 1 oxygenates higher barrier reactions such as hydroxylation of the methyl group of straight-chain aliphatic compounds. In separate studies, Hutzler et al. (2003) have suggested that two different oxidants may be involved in *O*-dealkylation and *N*-dealkylation. Jin et al. (2003) came to the conclusion that Cpd 0 could epoxidize alkenes but not hydroxylate the high-energy aliphatic C-H bond on camphor, and Volz et al. (2002) suggested that Cpds 0 and 1 may be the sole oxidants involved in *N*-dealkylation and sulfoxidation, respectively.

An alternative explanation for these results, as proposed by Shaik and coworkers (Ogliaro et al., 2002), is a two-state reactivity model in which Cpd 1 has two accessible spin states that can behave like two different oxidants. Shaik used density functional theory to conclude ". . . that sulfoxidation and *N*-dealkylation proceed largely via different spin states of Cpd 1," consistent with the two oxidations occurring by different pathways (Sharma et al., 2003). Furthermore, Shaik and coworkers (Ogliaro et al., 2002) used density functional theory to conclude that Cpd 0 is a weak oxidant in comparison to Cpd 1. This has been confirmed with model systems (Park et al., 2006). In contrast, Bach (Bach and Dmitrenko, 2006) used theoretical calculations to conclude that the peroxy species was the active oxidant.

Given the controversy, and the conflicting data, a simple experiment is required to determine whether, and when, multiple oxidants are catalyzing a reaction. One such experiment involves the use of isotopically sensitive branching, which can be used to determine whether products come from rapidly interchanging enzyme-substrate complexes, or whether they arise from kinetically independent enzyme-substrate complexes (Jones et al., 1986; Atkins and Sligar, 1988; Wagschal et al., 1991). Three things led us to consider the possibility that *N*-oxygenation may occur through Cpd 0. 1) Sulfoxi-

This research was supported by National Institute of Environmental and Health Sciences Grant 09122.

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Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.
doi:10.1124/dmd.106.010280.

ABBREVIATIONS: P450, cytochrome P450; Cpd, compound; LC/MS, liquid chromatography/mass spectroscopy.

dation, a reaction closely related to *N*-oxygenation, has been shown by isotopically sensitive branching experiments to occur from a second oxidant (Volz et al., 2002). 2) *N*-Oxygenation possesses a relatively low energy barrier in comparison to aromatic hydroxylation (Dowers et al., 2004), and low barrier reactions have a higher probability of occurring through a reaction with Cpd 0, given its low reactivity. (Ogliaro et al., 2002). 3) The possibility that *N*-oxygenation occurs through Cpd 0 has been suggested in the past (Coon et al., 1998). Therefore, we aimed to test whether *N*-oxygenation occurs through the same active iron-oxygen species as *O*-dealkylation, a P450-mediated reaction that has a relatively high energy barrier in comparison to *N*-dealkylation (Jones et al., 2002) and is generally accepted to occur through Cpd 1 (Lindsay-Smith and Sleath, 1983; Harada et al., 1984; Vaz and Coon, 1994; Higgins et al., 2001; Meunier et al., 2004).

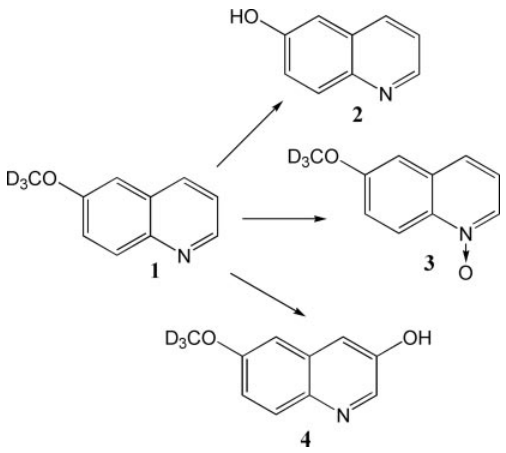
Materials and Methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. The aromatic hydroxylation product, 6-methoxy-3-quinolinol, was purchased from ChemBridge Corporation (San Diego, CA). $^2H_3$-6-Methoxyquinoline was synthesized from 6-hydroxyquinoline and $^2H_3$-methyl iodide, which was purchased from Cambridge Isotope Laboratories (Andover, MA). The product was purified on a gravity silica column and verified by liquid chromatography/mass spectroscopy (LC/MS). The deuterium content from the LC/MS was consistent with that in the $^2H_3$-methyl iodide (99.5%), and no attempt was made to correct for incomplete incorporation. Incubations were performed using RECO system CYP1A2, purchased from Invitrogen (Carlsbad, CA). Invitrogen's CYP1A2 enzyme mix consists of 0.5 μM CYP1A2, 0.2 μM NADPH P450 reductase, 0.5 μg/μl 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, 0.1 μg/μl liposomes [dilauroyl phosphatidylcholine, dioleoyl phosphatidylcholine, dilauroyl phosphatidylserine (1:1:1)], 3 mM reduced glutathione, and 50 mM HEPES/KOH, pH 7.4. Invitrogen's buffer mix is in 1 M potassium/sodium phosphate (pH 7.4). The incubations consisted of 200 μl enzyme mix, 270 μl buffer mix, 1 mM NADPH, and 0.04 mg/ml catalase. Varying concentrations of substrate in methanol were added to the incubations in 5-μl quantities. The samples were incubated in a shaker bath for 30 min at 37°C. The reactions were quenched with 3 ml of ethyl acetate, and 9.2 nmol of quinoline *N*-oxide was added as an internal standard. The samples were extracted with ethyl acetate, which was evaporated under nitrogen. The resulting residue was dissolved in approximately 200 μl of methanol for LC/MS analysis. LC/MS analysis was performed as described previously (Dowers et al., 2004).

Results and Discussion

The use of 6-methoxyquinoline as a substrate for P4501A2 allowed us to determine whether aromatic hydroxylation, *N*-oxidation, and *O*-dealkylation come from a common active-oxygen species (Scheme 1). Because isotopically sensitive branching from *O*-demethylation to aromatic hydroxylation has been shown previously (Lindsay-Smith and Sleath, 1983; Harada et al., 1984), we expected branching to occur to the aromatic hydroxylation metabolite of 6-methoxyquinoline (**4**), but not necessarily to the *N*-oxide (**3**). Obtaining an inverse isotope effect on the formation of the phenol **4** would ensure that the substrate's motion in the active site is fast relative to the oxidation reaction. In an elegant study by Regal et al. (2005) on the kinetics of caffeine metabolism by CYP1A2, fast interchange was observed for caffeine *N*-dealkylation; thus, fast interchange is likely for 6-methoxyquinoline as well.

Isotopically sensitive branching experiments test whether enzyme-substrate complexes for different products for a single substrate can rapidly interchange (Jones et al., 1986). If interchange is rapid, isotopic substitution will result in branching from the product with an isotope effect to a product that has no isotope effect associated with its formation. If the enzyme-substrate complexes are slow to interchange, no branching will be observed. In the case of 6-methoxyquinoline,



SCHEME 1. Metabolism of 6-methoxyquinoline (**1**) by P4501A2 to produce 6-hydroxyquinoline (**2**), 6-methoxyquinoline *N*-oxide (**3**), and 6-methoxy-3-quinolinol (**4**).

three products can be formed, as shown in Scheme 1. Substitution of deuterium on the methoxy group should lead to a significant normal isotope effect on the *O*-dealkylation pathway, leading to lower product formation. If aromatic oxidation and *N*-oxide formation occur from enzyme-substrate complexes that are rapidly interchangeable with the *O*-dealkylation enzyme-substrate complex, an inverse isotope effect will be observed on the aromatic oxidation and *N*-oxide products. The overall magnitude of the inverse isotope effect depends on the amount of each product formed and the magnitude of the isotope effect for *O*-dealkylation. If aromatic oxidation or *N*-oxide formation occur from enzyme-substrate complexes that *cannot* interchange with the *O*-dealkylation enzyme-substrate complex, no isotope effect will be observed on the aromatic oxidation or *N*-oxide products. The latter result was observed for *N*-dealkylation and sulfoxidation (Volz et al., 2002) of *N,N*-dimethyl-4-(methylthio)aniline, indicating that these two products do not come from interchangeable enzyme-substrate complexes and most likely come from two different oxidants. Given previous experiments that indicated that aromatic oxidation occurred from rapidly interchanging enzyme-substrate complexes, we anticipated two possible outcomes upon deuteration of the methoxy group: 1) a decrease in **2** as a result of a normal isotope effect, an increase in aromatic oxidation to form **4,** and the same amount of *N*-oxide formation, or 2) a decrease in product **2** and an increase in products **3** and **4** as a result of branching. Situation 1 would implicate a second oxidant for *N*-oxide formation, and situation 2 would indicate a common oxidant for all three products.

Large inverse isotope effects were observed on both *N*-oxygenation and aromatic hydroxylation of 6-methoxyquinoline (Table 1). The results are only consistent with all three products arising from a kinetically indistinguishable (rapidly interchanging) enzyme-substrate complex, i.e., the same oxidant. However, some fraction of the products may still come from a second noninterchangeable enzyme-substrate complex. If this is the case, only a fraction of the intrinsic isotope effect would be observed.

Because *N*-oxide formation and aromatic oxidation are minor pathways, the majority of the isotope effect is observed on these pathways, with only a small isotope effect on *O*-dealkylation. The lower limit of the intrinsic isotope effect can be estimated to be 6.5 by dividing the isotope effect on *O*-dealkylation (1.3) by the inverse isotope effect for either *N*-oxide formation (0.20) or the aromatic oxidation product (0.17). Some of the observed intramolecular and branched isotope effects for P450 *O*-dealkylation reactions that have been reported are

TABLE 1

*Isotope effects for metabolism of protio and deuterio 6-methoxyquinoline (**1**)*

	6-OH-Quinoline **2**	6-Methoxyquinoline *N*-Oxide **3**	6-Methoxy-3-Quinolinol **4**
$(V/K)_H$[a]	1994 ± 138	9.1 ± 1.1	24 ± 1.1
$(V/K)_D$	1552 ± 151	45 ± 15	140 ± 26
$(V/K)_H/(V/K)_D$	1.3 ± 0.22	0.20 ± 0.09	0.17 ± 0.04

[a] The V_{max} and K_m values were obtained by plotting the rate of product formation versus substrate concentration. The data were fit to Michaelis-Menton kinetics using the program EnzFitter. At higher substrate concentrations, a change in the ratio of product **3** to product **4** was observed, indicating two substrates binding in the active site. Therefore, the V_{max} and K_m values were obtained at low substrate concentrations (3 μM to 50 μM). Possible errors associated with two substrates binding should affect the V_{max} and K_m values of the protio and deuterio substrates equally and, therefore, would have no effect on $(V/K)_H/(V/K)_D$.

7.3 for anisole (Lindsay-Smith and Sleath, 1983), 5.1 (Watanabe et al., 1982) and 8.4 (Guengerich et al., 2004) for 4-methoxyanisole, 7.0 for methacetin (Guengerich et al., 2004), 8.4 for 4-cyanoanisole (Guengerich et al., 2004), 14.7 for 4-nitroanisole, and 5.5 for 7-ethoxycoumarin (Miwa et al., 1985). Thus, the magnitude of this isotope effect is consistent with other isotope effects that have been measured for P450-mediated *O*-dealkylation reactions indicating that the majority, or all, of the product comes from rapidly interchangeable enzyme-substrate complexes and, therefore, from the same oxidant. Again, however, this does not prove that 100% of the *N*-oxide arises from a single oxidant.

The strongest evidence that all the products come from the same oxidant is that the amount of branching to the *N*-oxide (**3**) and 6-methoxy-3-quinolinol (**4**) is identical. Isotopically sensitive branching requires that the relative amount of branching from a major pathway to alternate products will be the same for any two products; i.e., that the inverse isotope effects be the same for both products if the enzyme-substrate complexes are in rapid equilibrium (Korzekwa et al., 1995). However, if two oxidants catalyze the reactions that occur to form **3** and **4**, the fraction of **3** and **4** that comes from a given oxidant will most likely be different, reflecting the different activation energies for the two reactions. If this is the case, the observed branching isotope effects would be different, with a smaller inverse isotope effect for the pathway that favors the second oxidant. Clearly, this not the result obtained (see Table 1). Thus, for both the reactions to occur from both oxidants, it is required that the relative energy of formation of **3** and **4** be identical for both oxidizing species. In fact, mutants that purportedly enhance Cpd 0 have been shown to alter product ratios and rates of reaction in every case, indicating a difference in the energetics of the two oxidants (Vaz et al., 1998; Vatsis and Coon, 2002; Volz et al., 2002; Jin et al., 2003; Chandrasena et al., 2004) and consistent with computational results (Ogliaro et al., 2002).

From these results it is apparent that for the three reactions in Scheme 1 a single oxidant is responsible for most, if not all, of the products formed. The nature of the oxidant remains a matter of debate. However, the consensus oxidant for *O*-dealkylation reactions is Cpd 1. Since the results herein require a single oxidant for three energetically different reactions, it is most likely the iron-oxo species Cpd 1.

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