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OXIDATIVE N-DEMETHYLATION OF *N,N*-DIMETHYLANILINE BY PURIFIED ISOZYMES OF CYTOCHROME P-450

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Abstract—The metabolism of *N,N*-dimethylaniline (DMA) by rabbit liver microsomes results in the formation of *N*-methylaniline (NMA) and formaldehyde. The *N*-oxide of DMA (DMA *N*-oxide) has been suggested as an intermediate in the cytochrome P-450-catalyzed demethylation reaction. The role of DMA *N*-oxide as an intermediate in demethylation has been investigated in a reconstituted system consisting of NADPH–cytochrome P-450 reductase, phospholipid, and several different purified isozymes of cytochrome P-450. The abilities of several cytochrome P-450 isozymes from rabbit liver (P-450 form 2 and P-450 form 4) and rat liver (P-450b and P-450c) to catalyze *N*-oxide formation and their abilities to catalyze demethylation of the *N*-oxide were determined and compared with their abilities to catalyze the demethylation of DMA. The metabolism of DMA by the purified isozymes of cytochrome P-450 in the reconstituted system did not result in the formation of measurable amounts of the *N*-oxide. The turnover numbers for the metabolism of DMA and DMA *N*-oxide to formaldehyde by the reconstituted system containing cytochrome P-450 form 2 were 25.6 and 3.4 nmol/min/nmol cytochrome P-450, respectively. The three other isozymes (P-450 form 4, P-450b, and P-450c) also exhibited significantly greater rates for the demethylation of DMA than for the *N*-oxide. If the *N*-oxide were an intermediate in the demethylation reaction, it should be metabolized at a rate greater than or at least equal to DMA. Therefore, these data, along with the inability to detect *N*-oxide formation during the cytochrome P-450-catalyzed demethylation of DMA, suggest that the *N*-oxide of DMA is not an intermediate in demethylation of DMA by these forms of cytochrome P-450 and that DMA *N*-oxidase activity is not associated with these isozymes.

The initial oxidative metabolism of many secondary and tertiary *N*-alkylamines by liver microsomes can be catalyzed by either of two mixed-function oxidase systems: the cytochrome P-450-dependent oxidase or the mixed-function amine oxidase [1–3]. The oxidative N-dealkylation of tertiary amines may involve the initial oxidative attack on the carbon alpha to the nitrogen to form a carbinolamine intermediate or oxidation at the nitrogen to form a tertiary amine *N*-oxide intermediate which would subsequently be demethylated. Due to the variable reactivity of tertiary amines and the complexity of the microsomal monooxygenases, which can catalyze both α-carbon and N-oxidation [4], it is not possible to generalize in terms of which enzymes or mechanism(s) will be operative for the microsomal dealkylation of a given amine substrate.

Studies by Brodie *et al.* [5] on the metabolism of *N,N*-dimethylaniline (DMA)¶ by liver microsomal preparations led to the conclusion that *N,N*-dimethylaniline *N*-oxide (DMA *N*-oxide) was not an intermediate in DMA metabolism. Subsequently, Ziegler and Pettit [6] suggested that DMA *N*-oxide was an intermediate in the demethylation of DMA to give *N*-methylaniline (NMA) and formaldehyde. They reported that DMA was first oxidized by the microsomal flavin containing mixed-function amine oxidase to give the *N*-oxide, which was then demethylated by cytochrome P-450. Studies from several laboratories have provided strong support for the involvement of the *N*-oxide as an intermediate in demethylation [7–10], whereas studies from other laboratories suggest that it is not involved in the reaction [11–14]. These studies were performed using microsomal preparations, and the conclusions were based on the effects on *N*-oxide formation or N-demethylation of enzyme inhibitors or inducers which were assumed to be specific for the microsomal monooxygenases. Since DMA may be metabolized

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¶ Abbreviations: DMA, *N,N*-dimethylaniline; DMA *N*-oxide, *N,N*-dimethylaniline *N*-oxide; NMA, *N*-methylaniline; $P\text{-}450_{LM}$, liver microsomal cytochrome P-450. The isozymes of rabbit liver microsomal cytochrome P-450 are numbered according to their electrophoretic mobilities in accord with the general recommentations of the Committee on Biochemical Nomenclature. For example, the phenobarbital and 3-methylcholanthrene-inducible isozymes are referred to as cytochrome P-450 form 2 and cytochrome P-450 form 4, respectively, or simply as isozymes 2 and 4. The isozymes of rat liver microsomal cytochrome P-450 are designated: cytochrome P-450b, major liver isozyme of cytochrome P-450 isolated from phenobarbital-induced rats; and cytochrome P-450c, major liver isozyme of cytochrome P-450 isolated from 3-methylcholanthrene-induced rats.

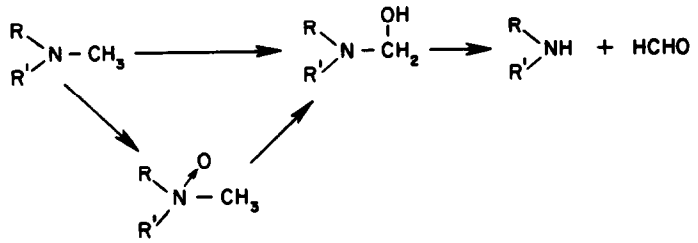

Fig. 1. Two possible routes for the metabolism of tertiary amines such as DMA by the cytochrome P-450-dependent mixed-function oxidases.

to give NMA and formaldehyde by two different pathways (Fig. 1) involving different monooxygenase enzyme systems, the interpretation of these experiments was complicated by the use of microsomal preparations containing several different enzyme systems. In addition, although DMA *N*-oxide is formed during the microsomal metabolism of DMA, it is not clear whether the *N*-oxide is an intermediate in the demethylation reaction or merely a by-product of oxidative metabolism. To investigate the possible role of DMA *N*-oxide as an intermediate in the cytochrome P-450-catalyzed N-demethylation to NMA and formaldehyde, we investigated the metabolism of DMA to DMA *N*-oxide in the reconstituted cytochrome P-450 system using four different purified isozymes of cytochrome P-450.

Although liver microsomal cytochrome P-450 has been shown to catalyze the N-oxidation of tertiary amines [15], the metabolism of DMA by rabbit liver cytochrome P-450 forms 2 or 4 or rat liver cytochrome P-450b or P-450c in the reconstituted system did not result in the formation of DMA *N*-oxide as a product. If the *N*-oxide were formed as an intermediate in the cytochrome P-450-catalyzed demethylation of DMA, then the *N*-oxide should be demethylated at a rate equal to or greater than the rate for the demethylation of DMA. However, the rates of formaldehyde formation by all four isozymes of cytochrome P-450 were significantly greater with DMA than DMA *N*-oxide. These results suggest that the *N*-oxide is not an intermediate in the cytochrome P-450-catalyzed demethylation of DMA and provide additional support for the initial formation of a carbinolamine as an intermediate in the demethylation reaction.

Houdi and Damani [16] and Ziegler [17] have provided evidence suggesting that the N-oxidation of DMA is not mediated by cytochrome P-450. Ziegler has also pointed out [17] that it is difficult to reconcile the mechanism of action of cytochrome P-450 and the N-oxygenation of a substrate such as DMA. The present studies are aimed at investigating the ability of purified isozymes of cytochrome P-450 to catalyze the N-oxygenation of DMA and to see if the *N*-oxide may be an intermediate in the metabolism of DMA to NMA.

MATERIALS AND METHODS

Chemicals. DMA and *m*-chloroperbenzoic acid were obtained from the Aldrich Chemical Co. (Milwaukee, WI). The DMA *N*-oxide was synthesized from DMA by oxidation with *m*-chloroperbenzoic acid using the method of Craig and Purushothaman [18]. The *N*-oxide was separated from the reactants by column chromatography on basic alumina and recrystallized from ethyl acetate. The *N*-oxide crystals had a melting point of 152–154° (literature 152–153°). Dilauroyl phosphatidylcholine was purchased from Serdary Research Laboratories (Ontario, Canada). All other chemicals used were analytical reagent grade from commercial suppliers.

Enzyme preparations. Rabbit liver cytochrome P-450 isozyme 2 was purified from liver microsomes of male New Zealand white rabbits that had been induced with 0.1% phenobarbital in their drinking water for 5–7 days prior to being killed [19]. The cytochrome P-450 isozyme 4 was prepared using microsomes from rabbits that had been pretreated with isosafrole (150 mg/kg) for 3 days prior to being killed. The two isozymes of rabbit liver cytochrome P-450 were purified to electrophoretic homogeneity by the procedure of Coon *et al.* [19]. The rat liver cytochrome P-450 was isolated from liver microsomes of male Sprague–Dawley rats that had been pretreated with 0.1% phenobarbital in the drinking water for 5–7 days using the method of Ryan *et al.* [20]. The cytochrome P-450c was purified from microsomes of 3-methylcholanthrene-induced rats using the method of Elshourbagy and Guzelian [21]. The isozymes of cytochrome P-450 used for these studies were electrophoretically homogeneous and had the specific contents (expressed as nanomoles of cytochrome P-450 per milligram of protein) indicated: isozyme 2, 14.5; isozyme 4, 19.0; P-450b, 8.1; and P-450c, 10.2.

NADPH–cytochrome P-450 reductase was purified from liver microsomes of phenobarbital—pretreated rabbits using the method of French and Coon [22]. The preparations were electrophoretically homogeneous and had specific contents of at least 9.5 nmol reductase/mg protein.

Demethylation assays. The N-demethylation of DMA and DMA *N*-oxide was measured using the reconstituted system consisting of 0.1 nmol cytochrome P-450 isozyme, 0.4 nmol NADPH–cytochrome P-450 reductase, 20 μg sonicated dialuroylphosphatidylcholine, 100 μmol potassium phosphate buffer, pH 7.4, 1 μmol DMA or DMA *N*-oxide (added in 10 μl of methanol), and 1 μmol NADPH in a final volume of 1.0 ml. The enzymes and substrate were preincubated at 30° for 5 min

Table 1. Formaldehyde formation from DMA and DMA *N*-oxide by cytochrome P-450 in the reconstituted system

Cytochrome P-450 isozyme	Turnover number (nmol formaldehyde formed/min/nmol cytochrome P-450)	
	DMA	DMA *N*-oxide
2	25.6 ± 1.6	3.4 ± 0.3
4	7.7 ± 1.7	3.2 ± 0.7
P-450b	29.4 ± 2.4	3.4 ± 0.4
P-450c	8.1 ± 1.1	2.3 ± 0.8

The incubations, sample treatments, and measurements of formaldehyde formation are described in Materials and Methods. The data are average values (±SE) taken from three separate experiments with each point determined in duplicate.

prior to the initiation of the reaction by the addition of NADPH. The reaction mixtures were incubated at 30° for 10 min in a shaking water bath, and the reactions were terminated by the addition of 0.5 ml of 60% trichloroacetic acid (TCA). The formaldehyde formed in the reaction was measured using a modification of the procedure of Nash [23] as described by Kedderis *et al.* [24] except that the absorbance was read at 412 nm on a Gilford 2400-S UV-visible spectrophotometer.

Assay for DMA N-oxide formation. The formation of the DMA *N*-oxide during the metabolism of DMA was investigated in a reconstituted system consisting of 0.5 nmol cytochrome P-450 isozyme, 2.0 nmol NADPH–cytochrome P-450 reductase, 20 μg sonicated dilauroylphosphatidylcholine, 100 μmol potassium phosphate buffer, pH 7.4, 1 μmol DMA (added in 10 μl of ethanol), and 1 μmol NADPH in a final volume of 1.0 ml. The enzymes and substrate were preincubated at 30° for 5 min, and the reaction was then initiated by the addition of the NADPH. The reaction mixtures were incubated at 30° for 60 min, and the reaction was then terminated by the addition of 0.1 ml of 3.3 M perchloric acid.

The amount of *N*-oxide formed was determined using the colorimetric assay of Ziegler and Pettit [6] for DMA *N*-oxide.

Other assays. Protein concentrations were determined by the method of Lowry *et al.* [25] as modified by Bensadoun and Weinstein [26].

RESULTS AND DISCUSSION

The abilities of the four isozymes of cytochrome P-450 to catalyze the formation of DMA *N*-oxide were investigated using the reconstituted system as described in Materials and Methods. However, DMA *N*-oxide formation could not be detected with any of the isozymes. The limit of detection for *N*-oxide determination by the method of Ziegler and Pettit [6] was approximately 10 nmol, suggesting that, if DMA *N*-oxide were formed as a product of the cytochrome P-450-catalyzed reaction, then the turnover number is less than 0.3 nmol/min/nmol cytochrome P-450.

As shown in Table 1, all four isozymes of cytochrome P-450 catalyzed the metabolism of DMA to formaldehyde at rates which were markedly greater than those for the dealkylation of DMA *N*-oxide. In fact, for isozymes 2 and P-450b, the two phenobarbital-inducible forms, the formation of formaldehyde from DMA was at least seven times as fast as from the *N*-oxide.

Hlavica and Hulsmann [27] have reported previously that a reconstituted system containing highly purified rabbit liver cytochrome P-448, NADPH-cytochrome *c* reductase, and lipid can catalyze *N*-oxide formation from *N*,*N*-dimethylaniline with an optimum turnover number of approximately 1.9. However, we could not detect any DMA *N*-oxidase activity associated with the four purified isozymes of cytochrome P-450 which we investigated. Guengerich [28] has suggested that the cytochrome P-450-mediated formation of *N*-oxides can be initiated by one electron oxidation of the amine, but that oxygen rebound to the aminium radical would only occur when: (a) the radical is rendered unusually stable (e.g. azo compounds) [29]; (b) no α-hydrogens are available; or (c) the α-hydrogens are inaccessible for abstraction (e.g. Bredt's rule) [30]. On the basis of these considerations, one would predict that cytochrome P-450 would not catalyze the oxidation of DMA to the *N*-oxide and also that the DMA *N*-oxide would not be an intermediate in the cytochrome P-450-catalyzed metabolism of DMA to NMA and formaldehyde.

Therefore, our results indicate that the *N*-oxide is not an intermediate in the demethylation of DMA catalyzed by these isozymes of cytochrome P-450 and support the suggestion that a critical step in the cytochrome P-450-catalyzed demethylation involves the initial oxygenation of the carbon atom to form a carbinolamine intermediate rather than the *N*-oxide, as shown in Fig. 1. Although the sequence of steps leading to the formation of the carbinolamine has not been fully elucidated, studies on the intramolecular isotope effects for the N-demethylation of *N*-methyl-*N*-trideuteromethylaniline suggest that the cytochrome P-450-catalyzed reactions proceed via a deprotonation step following the formation of an anilinium radical [31]. The carbon radical then recombines with the nascent iron-bound hydroxyl radical to give the carbinolamine. Based on studies on the rates of demethylation of five para-substituted *N*,*N*-dimethylanilines, Guengerich and co-workers [32] have suggested that the demethylation of dimethylanilines involves an initial electron abstraction from the nitrogen followed by the loss of a hydrogen atom rather than a proton. This results in the formation of an Fe^{III}-OH iminium ion pair which collapses to form the carbinolamine. The carbinolamine intermediate of DMA is too unstable in aqueous solution to isolate; however, in the case of *N*-methylcarbazole, a tertiary amine substrate of cytochrome P-450, the carbinolamine *N*-hydroxymethylcarbazole has been isolated [33, 34]. This compound slowly decomposes in aqueous solution to give carbazole and formaldehyde.

DMA *N*-oxide does not give a binding spectrum with either cytochrome P-450 isozyme 2 or P-450b (data not shown); however, studies with microsomes [35] and purified cytochrome P-450 form 2 [36] sug-

gest that DMA *N*-oxide is metabolized by the cytochrome P-450 enzyme system, though it is not a very good substrate.

Whether the DMA *N*-oxidase activity is associated only with mixed-function amine oxidase or whether a cytochrome P-450 enzyme system is also involved in the microsomal metabolism of DMA to form the *N*-oxide is controversial. It has been demonstrated [37, 38] that DMA *N*-oxidase activity is not affected by inhibitors of cytochrome P-450 such as carbon monoxide (CO) or SKF-525A. This suggests that cytochrome P-450 may not be responsible for the DMA *N*-oxidase activity in the microsomes. This view is further supported by the fact that inducers of cytochrome P-450 such as phenobarbital or 3-methylcholanthrene do not affect DMA *N*-oxidase activity [38]. However, based on inhibition studies with 2-bromo-4′-nitroacetophenone, steapsin, and menadione, Hlavica and Kohl [15] claim that 50–60% of the total DMA *N*-oxide formed by rabbit liver microsomes is mediated through cytochrome P-450. In addition, they observed that, during the microsomal metabolism of DMA, a peak appears in the difference spectrum at 448 nm which subsequently disappears when the sample is treated with sodium dithionite or potassium ferricyanide. They attributed this spectral perturbation at 448 nm to the formation of a ferrocytochrome P-450–DMA *N*-oxide complex. However, the spectral perturbation at 448 nm may also be due to the formation of an oxyferrocytochrome P-450 species which disappears either upon depletion of oxygen in the system by the addition of sodium dithionite or upon the oxidation of ferrous cytochrome P-450 to ferric cytochrome P-450 by the addition of potassium ferricyanide. If the spectral perturbation at 448 nm were due to formation of the DMA *N*-oxide–ferrocytochrome P-450 complex, the peak at 448 nm should disappear only upon conversion of the ferrous cytochrome P-450 to ferric cytochrome P-450 and should not be affected by the depletion of oxygen in the system by the addition of sodium dithionite. Hlavica and Hulsmann [27] have also reported formation of the DMA *N*-oxide by a reconstituted system containing 3-methylcholanthrene-induced cytochrome P-450 using a colorimetric assay. However, as reported here, we were unable to detect DMA *N*-oxide formation by the reconstituted system using cytochrome P-450 rabbit liver isozymes 2 or 4 or rat liver cytochrome P-450b or P-450c as the terminal oxidases, which suggests that for these isozymes the *N*-oxide is not an intermediate in the demethylation of DMA and these isozymes do not exhibit any DMA *N*-oxidase activity. The differences between our results and those of Hlavica and Hulsmann [27] may be due to differences in the identities of the isozymes studied. It is also possible that they may be detecting nonenzymatic conversion of DMA to DMA *N*-oxide as suggested by Ziegler [17]. Houdi and Damani [16] recently demonstrated that there is no DMA *N*-oxidase activity associated with two purified isozymes of rat liver cytochrome P-450.

Although DMA *N*-oxide could not be detected in the incubation mixture after 60 min, one can argue that there may be some DMA *N*-oxidase activity associated with cytochrome P-450. If a small amount of the *N*-oxide were produced and then further metabolized by cytochrome P-450 to give NMA and formaldehyde, the DMA *N*-oxide would not be detected even though it was formed. However, this possibility seems unlikely since the DMA *N*-oxide is highly hydrophilic and would be expected to leave the active site and go into solution upon its formation. Since the *N*-oxide is a poor substrate of cytochrome P-450, its further metabolism would occur only when its concentration in the incubation mixture reached a reasonably high level. Even if metabolism of the *N*-oxide were taking place, it would not be depleted completely from the system. The fact that DMA *N*-oxide could not be detected in the system indicates either that it was not formed at all or that its concentration was below the level of detection. In either case, the *N*-oxide was not formed as a major product during the cytochrome P-450-catalyzed demethylation of DMA nor was it an intermediate in demethylation.

The relatively poor catalytic activity of cytochrome P-450 for the metabolism of DMA *N*-oxide to give formaldehyde (Table 1) raises concerns about the appropriateness of mechanistic studies on the cytochrome P-450-catalyzed demethylation of DMA *N*-oxide [36].

In conclusion, the results of these studies demonstrate that DMA demethylation by four purified isozymes of cytochrome P-450 (form 2, form 4, P-450b and P-450c) does not proceed via the formation of DMA *N*-oxide as an intermediate in the overall reaction nor do these isozymes exhibit the ability to catalyze the metabolism of DMA to the *N*-oxide.

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