Metabolism of Nitrosamines by Purified Rabbit Liver Cytochrome P-450 Isozymes

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ABSTRACT

The metabolism of nitrosamines by microsomal cytochrome P-450 (P-450) isozymes was studied in a reconstituted monooxygenase system. P-450 LM2, LM3a, LM3b, and LM4a, and LM5 were purified, respectively, from the livers of phenobarbital-treated, ethanol-treated, untreated, isoasafrole-treated, and imidazole-treated rabbits. Of these isozymes, LM3a had the highest N-nitrosodimethylamine demethylase (NDMAA) activity with a K_m of 2.9 mM and V_max of 9.3 nmol/min/mg. LM2, LM4a, and LM5 exhibited NDMAA activity only at high N-nitrosodimethylamine concentrations, and isozymes LM3b and LM5a had poor activity even at the highest substrate concentrations examined. LM2, however, was more active than LM3a in the metabolism of N-nitrosomethyline. With each isozyme (LM3a or LM2), only one K_m for NDMAA was observed, whereas with rabbit liver microsomes, multiple K_m of 0.07, 0.27, and 36.8 mM were obtained. P-450 isozymes also catalyzed the denitrosation of nitrosamines at rates comparable to or lower than the demethylation, and the ratio of these two reactions was different with different nitrosamines. 2-Phenylethylamine and 3-amino-1,2,4-triazole, which were believed previously to affect NDMAA by mechanisms independent of P-450, were shown to be potent inhibitors of P-450-dependent NDMAA. These results further establish the role of P-450 isozymes in the demethylation of nitrosamines and indicate that LM3a is apparently responsible for the increased N-nitrosodimethylamine metabolism associated with ethanol treatment.

INTRODUCTION

The metabolism of nitrosamines has been a subject of extensive investigation because of its importance in understanding the carcinogenicity and toxicity of this group of compounds. Oxidation at the α-carbon (α-hydroxylation) is believed to be a key step in the metabolic activation of many nitrosamines (6, 17, 24, 28). There is evidence indicating that NDMAA is metabolized by a P-450-dependent monooxygenase system (5, 9, 10). The efficiency and the importance of this system in the metabolism of nitrosamines, however, have been questioned, and alternative mechanisms of metabolism have been postulated (18-21, 31-34). The following observations concerning NDMA metabolism by rat liver microsomes have previously puzzled many investigators. (a) NDMAA activity was not induced by classical inducers, such as phenobarbital, 3-methylcholanthrene, β-naphthoflavone, Aroclor 1254, and pregnenolone-16α-carbonitriile, especially when the activity was assayed with 1 to 4 mM NDMA (2, 3, 7, 17, 18, 20, 26, 32). (b) Studies with inducers led to the proposal of the existence of NDMAA I and II (17, 18). In fact, more than 2 K_m values can be observed for NDMAA even in hepatic microsomes of untreated rats (17-19, 38). Lake et al. (19) reported the existence of 3 K_m values: 0.32; 1.5; and 35 mM. In addition, Tu and Yang (38) reported recently the existence of a lower K_m (0.07 mM) NDMAA in control rat liver microsomes. (c) NDMAA was not significantly inhibited by classical monooxygenase inhibitors, such as SKF-525A and metyrapone, but was inhibited by compounds which are not known to be strong inhibitors of the commonly studied monooxygenase reactions, such as 3-amino-1,2,4-triazole and 2-phenylethylamine (20, 21, 31, 33, 34).

More recent work, however, is beginning to elucidate the role of P-450 isozymes in the metabolism of nitrosamines. The hepatic NDMAA activity in the rat liver was shown to exist predominately in the microsomes and to be P-450 dependent (Ref. 39; Footnote 4). Furthermore, the activity is inducible by pretreatment with acetone, isopropanol, ethanol, or pyrazole and by fasting and diabetes (29, 30, 36-39). A unique P-450 isozyme, LM3a, was isolated recently from ethanol-treated rabbits (14, 25), and the identical isozyme was shown to be inducible in rabbits by imidazole (13), a known inducer of hepatic microsomal NDMAA in rabbits (11). In the present work, we have studied the metabolism of NDMA by P-450 LM3a and by 5 other P-450 isozymes purified from rabbit liver microsomes. In addition, the specificity of the 6 isozymes in the demethylation and denitrosation of several other nitrosamines was examined.

MATERIALS AND METHODS

Chemicals. NDMAA was purchased from Aldrich Chemical Co., Milwaukee, WI. N-Nitroso-N-methylmethyamine, N-nitroso-N-methylbutyramine, N-nitroso-N-methylmethylamine, 2-phenylethylamine, 3-amino-1,2,4-triazole, N-1-naphthylethylenediamine, NADP, and NADPH generating reagents were obtained from Sigma Chemical Co., St. Louis, MO. N-Nitroso-N-methylbenzylamine was from Ash Stevens, Inc., Detroit, MI. Dilauroylphosphatidylcholine was purchased from Serdary Research Laboratories, London, Ontario, Canada.

Microsomes and Microsomal Enzymes. Hepatic microsomes were prepared from control or ethanol-treated adult male New Zealand rabbits (2.0 to 2.5 kg) according to previous procedures (14, 15). The control and ethanol-induced microsomes contained 2.16 and 2.30 nmol P-450 per mg protein, respectively. The P-450 isozymes were purified from rabbits which had been subjected to the following treatments: LM2, phenobarbital; LM3a, ethanol; LM5a, no treatment; LM4a, no treatment; LM5, isoasafrole; and LM5b, imidazole (13-15). These P-450 preparations were electrophoretically homogeneous, and the specific contents, expressed as nmol P-450 per mg protein, were 18.2, 19.0, 17.4, 13.5, 19.5, and 16.2 for LM2, LM3a, LM5a, LM4a, LM5, and LM5b, respectively. Electrophoretically pure NADPH-cytochrome P-450 reductase was prepared using the method of Andrew et al. (30). The activity of this preparation was 1.2 nmol NADPH oxidized/min/mg.

*J. Hong and C. S. Yang, manuscript in preparation.
pared from rabbit liver microsomes as described previously (8). The specific activity of the reductase was 54 units (which catalyzed the NADPH-dependent reduction of 54 μmol cytochrome c per min) per mg protein. The microsomes and enzymes were stored at −86° prior to use.

**Metabolism of Nitrosamines.** The demethylation and denitrosation reactions were assayed at 37° by previously described procedures (22, 38) with some modifications. To reconstitute the monoxygenase activity, 0.2 to 0.3 nmol of P-450, 1.2 to 2.0 units of NADPH-P-450 reductase, and 15 μg of dilauroylphosphatidylcholine were mixed in a glass test tube at room temperature. Buffer, water, and the NADPH-generating system were then added to obtain an incubation mixture containing 50 mM tris-HCl (pH 7.4), 10 mM MgCl₂, 150 mM KCl, 0.4 mM NADP, 10 mM isocitrate, and 0.15 unit of isocitrate dehydrogenase in 0.5 ml (38). Standards and 2 sets of blanks were run concomitantly; in one set, the nitrosamine substrate was omitted; and in the other, the NADPH-generating system was omitted. After preincubation at 37° for 2 min, the reaction was initiated by the addition of the nitrosamine substrate.

In some experiments, the nitrosamines were added before the preincubation, and the reaction was initiated by the addition of the NADPH-generating mixture. After a 20-min incubation, the reaction was terminated by the addition of 0.05 ml each of 25% ZnSO₄ and saturated Ba(OH)₂. The mixture was centrifuged, and 2 aliquots of the supernatant were transferred to glass test tubes: 0.2 ml for formaldehyde determination and 0.2 ml for nitrite determination. To the former, 0.1 ml of a concentrated Nash reagent (5 g ammonium acetate and 0.1 ml acetylacetone in 6 ml 3% acetic acid) was added, and the mixture was incubated at 55° for 10 min. The mixture was cooled to room temperature, and absorbance at 545 nm was measured (22). Results are expressed as nmol HCHO (or NO₂⁻) formed per min per nmol P-450 and per mg microsomal protein. The microsomes and enzymes were stored at −86° prior to use.

**RESULTS**

**Metabolism of NDMA by P-450 Isozymes.** Upon mixing P-450 LM₃a with purified NADPH-P-450 reductase and dilauroylphosphatidylcholine, the reconstituted monoxygenase system efficiently catalyzed the demethylation of NDMA (Table 1). No or very low activity was observed when any of the components, P-450, reductase, or the phospholipid, was omitted from the reconstituted system. The reaction was linear within at least 30 min, and the activity was proportional to the quantity of P-450 present, with a turnover number of 5.5/min. Other P-450 iso

zymes, however, were less effective in catalyzing the demethylation of NDMA (Table 2). When assayed with 4 mM NDMA, the demethylase activity of LM₄ was only 5% that of LM₃a, and the activity of other P-450 isozymes was even lower. At a substrate concentration of 100 mM, however, substantial NDMA activity was observed with LM₄ and LM₃a, which displayed 65, 33, and 30% of the activity of LM₃a, respectively. The 2 constitutive forms, LM₄ and LM₃a, had low activity even at high substrate concentrations.

**Substrate Specificities of P-450 Isozymes.** P-450 LM₃a, LM₄, and LM₄ were selected in this study to investigate whether other nitrosamines are also metabolized by P-450 isozymes (Table 3). For the convenience of the assay, only N-methyl-containing nitrosamines were used, and the demethylase activity was assayed at substrate concentrations of 4 and 40 mM. With P-450 LM₃a, the activity with nitrosothiophenylethylamine was about one half the rate with NDMA, and the activity was much lower when the other N-alkyl group was ethyl, butyl, or benzyl. With nitrosothiophenylcarbamoylamine and nitrosothiophenylurea, the demethylase activity was elevated 4- to 5-fold when the substrate concentration was increased from 4 to 40 mM. In contrast, a similar increase in substrate concentration produced only a 45 to 69% rise in activity with the other 3 substrates. The substrate specificity of LM₂ was quite different from that of LM₃a in its high activity toward nitrosothiophenylethylamine, showing a demethylase rate more than twice that of LM₃a. LM₄ also catalyzed the demethylation of nitrosothiophenylbutylamine, and the activity decreased when the N-butyl moiety was substituted by benzyl, ethyl, or methyl. When the substrate concentration was increased from 4 to 40 mM, a large increase in LM₄-dependent demethylase activity was observed with NDMA and nitrosothiophenylethylamine but not with the other 3 substrates. P-450 LM₄ was not effective in catalyzing the metabolism of most of the nitrosamines tested, and appreciable activity was seen only with nitrosothiophenylethylamine.

In addition to the demethylase reaction, the rate of nitrosamine denitrosation was measured concomitantly (Table 4). Because of the high rate of the nonenzymatic denitrosation of nitrosamine...
methylalanine, the enzymatic rate was not accurately measured and not included in Table 4. As was shown previously with rat liver microsomes (22), the ratio of the demethylation and denitrosation rates (HCHO:NO₂⁻ ratio) was different with different substrates. With LM₃a, the ratio was 6 to 7 for NDMA and ranged from 0.6 to 1.6 for the other substrates. Thus, even though LM₃a had higher NDMAd than nitrosomethyltryptophane demethylase activity, it catalyzed the denitrosation of these 2 nitrosamines at about equal rates, which were higher than the denitrosation rates of substrates with an N-butyl or N-benzyl group. Denitrosation of nitrosamines was also catalyzed by LM₂ and LM₄, but the possible error in measuring low quantities of NO₂⁻ in some of the data points prevented a more thorough analysis of the results. Nevertheless, parallelism between the denitrosation and demethylation reactions was observed. In comparison to LM₃a, LM₂ was less effective in catalyzing the denitrosation of NDMA and nitrosomethyltryptophane but more effective with nitrosomethylbutyramine and nitrosomethylbenzylamine. With LM₂, the denitrosation rates for NDMA and nitrosomethyltryptophane were also increased greatly upon changing the substrate concentration from 4 to 40 mm, analogous to the demethylase reaction. In reactions catalyzed by LM₂, the HCHO:NO₂⁻ ratio for NDMA was higher than that for other nitrosamines. The data, however, cannot distinguish with certainty whether the ratios produced by LM₂ were different from those produced by LM₃a. LM₄ which had low demethylase activities also was ineffective in catalyzing the denitrosation of the nitrosamines studied herein.

Kinetics of NDMAd in Microsomes and Reconstituted Systems. Analogous to rat liver microsomes (38), rabbit liver microsomal NDMAd also displayed a complex set of kinetic parameters (Chart 1); the data were analyzed similarly (38). With control rats, the microsomal demethylase activity increased with increasing substrate concentrations, and saturation was not observed even at 200 nm NDMA (Chart 1A). With ethanol-pretreated rabbits, the activity was high in the low substrate concentration range (<1 mm), decreased in the range of 4 to 10 mm, and increased further in the range of 30 to 200 mm NDMA. In a double-reciprocal plot, at least 3 apparent Km values were observed for the NDMAd of control rabbit hepatic microsomes. They were 0.07, 0.27, and 36.8 mm with corresponding Vmax values of 0.65, 0.96, and 3.24 nmol/min/mg protein (Chart 1B). With ethanol-induced microsomes, the predominant NDMAd had an apparent Km of 0.11 mm and a Vmax of 2.66 nmol/min/mg (Chart 1B). In addition, an estimated Km of 33.9 mm and Vmax of 4.74 nmol/min/mg were also observed. In a second experiment with ethanol-induced microsomes, Km values of 0.08 and 42.3 mm with corresponding Vmax values of 1.73 and 3.84 nmol/min/mg were obtained.

In the reconstituted system with LM₃a, a single Km of 2.91 mm and a Vmax of 9.33 nmol/min/mmol were observed (Chart 2). In a second experiment, the corresponding values were 3.06 mm and 9.42 nmol/min/mmol, respectively. With LM₄, an estimated Km of 456 mm and Vmax of 7.60 nmol/min/mmol were obtained (Chart 3), and values of 602 mm and 9.35 nmol/min/mmol, respectively, were observed in a second experiment.

Inhibition of P-450-dependent NDMAd. The effects of several inhibitors on the NDMAd of microsomes and the reconstituted system were examined (Table 5). 2-Phenylethylamine, a compound known to be a substrate of monoamine oxidase and to inhibit NDMA metabolism by rat tissues (31, 34), was a potent inhibitor of NDMAd of the microsomes and of the LM₃a and LM₄ reconstituted systems. Since the reconstituted system had essentially no monoamine oxidase activity, the results were clearly due to inhibition of the P-450-dependent monoxygenase activity. The P-450-dependent NDMAd was also inhibited by 3-amino-1,2,4-triazole and pyrazole at concentrations similar to those used in previous studies (20, 31, 33). 3-Amino-1,2,4-triazole appeared to be more inhibitory toward the LM₃a-dependent system than microsomes, whereas pyrazole was less inhibitory.

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**Table 4**

| Substrate specificity of P-450 isozymes in the denitrosation of nitrosamines |
|---|---|---|
| Substrate | P-450 isozymes (nmol NO₂⁻/min/nmol P-450) | |
| | LM₂ | LM₃a | LM₄ |
| Nitrosodimethylamine | 0.05 | 0.40 | 0.81 |
| Nitrosomethyltryptophane | 0.04 | 1.03 | 1.09 |
| Nitrosomethylbutyramine | 0.64 | 1.02 | 0.38 |
| Nitrosomethylbenzylamine | 0.62 | 0.96 | 0.42 |

Chart 1. Substrate dependence of microsomal NDMAd. The reaction mixture contained control microsomes (○) or ethanol-induced microsomes (△) corresponding to 0.95 and 0.30 mg protein, respectively, in an incubation mixture of 0.5 ml. The results are shown as velocity (v) (nmol HCHO per min per mg protein) versus substrate concentration (S) plots (A) and double reciprocal plots (B). In B, the data obtained with control microsomes are fitted by 3 linear regression lines with corresponding Km values of 0.07 mm (4 points with correlation coefficient, r = 0.983), 0.27 mm (3 points, r = 0.995), and 36.80 mm (5 points, r = 0.993). In B, the data obtained with ethanol-induced microsomes, the data are fitted by 2 lines with corresponding Km values of 0.10 mm (6 points, r = 0.994) and 33.90 mm (4 points, r = 0.973). The data points of the high Km are not shown due to space limitation.
DISCUSSION

When multiple forms of liver microsomal P-450 were first separated and characterized, the conclusion was reached that these cytochromes have the ability to bind many or all of the potential substrates but differ in their relative efficiency in the hydroxylation of such compounds (12, 23). The present work clearly provides another example of this principle, since all of the P-450 isozymes were found to be active with all of the nitrosamines examined and to differ only in the rates of catalysis. In the intact organism, however, such differences could determine the extent of toxicity and carcinogenicity when a particular animal species is exposed to varying levels of nitrosamines along with agents which induce the individual cytochromes. The results obtained in the present study show that LM₃₅, the ethanol-induced form from rabbits, is highly efficient in catalyzing the metabolism of NDMA, whereas the other forms are less efficient. The latter group of P-450 isozymes, however, can metabolize NDMA when assayed at high substrate concentrations (Table 2), suggesting that they function with a Km value higher than that exhibited by LM₃₅. In the case of LM₄, an apparent Km value of over 400 mM is seen (Chart 3).

The metabolism of nitrosomethyl-N,N-diethylamine follows the same pattern as NDMA, except that the demethylase rate is lower. LM₅, the phenobarbital-induced form, on the other hand, is more active than LM₃₅ in metabolizing nitrosomethylaniline and nitrosomethylbutylamine. With N-diethyl nitrosamines, both the N-methyl and the other N-alkyl (ethyl, butyl, or benzyl) groups are the targets of the oxygenation. Studies with microsomes have indicated that the other alkyl groups are usually more readily oxidized than the N-methyl group (1, 4). It is possible that the relative oxygenation rate of the 2 alkyl groups in a nitrosamine is also determined by the substrate specificity of P-450, but this point remains to be substantiated. In comparison to other P-450 isozymes, LM₃₅ is also more active in the p-hydroxylation of aniline and the oxidation of ethanol to acetaldehyde (14). The parallelism in the metabolism of NDMA and these 2 substrates was also observed in different types of rat liver microsomes.*

Denitrosation of nitrosamines has been studied previously with rat liver microsomes and P-450 isozymes purified from phenobarbital-treated rats (22). The present work demonstrates that other P-450 isozymes, particularly LM₃₅, can efficiently catalyze the oxidative denitrosation of nitrosamines. The relative rates of HCHO and NO₂⁻ formation are different with different nitrosamines. Concerning a specified nitrosamine substrate, however, the rates of these 2 reactions roughly parallel each other. This is consistent with the previous conclusion based on studies with rat liver microsomes (23). Although closely related to the demethylation, denitrosation is distinguished from the former reaction in its response to superoxide dismutase. In a reconstituted system consisting of P-450 isozyme purified from ethanol-treated rats, superoxide dismutase inhibited the denitrosation by 73%, but it inhibited demethylation only to a maximum of 20%.* Previous investigators have attempted to measure the quantity of N₂ formation upon oxidative metabolism of NDMA, and discrepancies exist between nitrosamine metabolized and N₂ recovered (18). The nitrite formation, as described herein, may account for some of the discrepancies. The mechanism of the denitrosation and the biological consequence of the reaction

*Y. Y. Tu and C. S. Yang, manuscript submitted for publication.

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Table 2. Substrate dependence of NDMA metabolism in the reconstituted system with P-450 LM₃₅. Data are shown as a double-reciprocal plot and, in the inset, a velocity (V) versus substrate concentration (S) plot. The regression line represents a Km of 2.91 mM (r = 0.999).

<table>
<thead>
<tr>
<th>Substrate Concentration (mM)</th>
<th>0</th>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>4</td>
<td>3.4</td>
<td>2.9</td>
<td>2.5</td>
<td>2.0</td>
</tr>
<tr>
<td>1/V</td>
<td>0.25</td>
<td>0.29</td>
<td>0.34</td>
<td>0.38</td>
<td>0.40</td>
</tr>
</tbody>
</table>

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Table 5. Inhibition of reconstituted and microsomal NDMA metabolism.

<table>
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<tr>
<th>Inhibitor</th>
<th>P-450 LM₃₅</th>
<th>P-450 LM₅</th>
<th>Control microsomes</th>
<th>Ethanol-induced microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0 (5.58)</td>
<td>0 (3.11)</td>
<td>0 (0.78)</td>
<td>0 (2.21)</td>
</tr>
<tr>
<td>2-Phenylethylamine (1 mM)</td>
<td>91</td>
<td>44</td>
<td>90</td>
<td>63</td>
</tr>
<tr>
<td>3-Amino-1,2,4-triazole (10 mM)</td>
<td>88</td>
<td>30</td>
<td>39</td>
<td>55</td>
</tr>
<tr>
<td>Pyrazole (1 mM)</td>
<td>40</td>
<td>20</td>
<td>70</td>
<td>80</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, the NDMA activity in the absence of inhibitor. The activity is expressed as nmol HCHO per min per nmol P-450 for the reconstituted systems and as nmol HCHO per min per mg protein for the microsomes.

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**Insert Table 2 and Table 5 here.**

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**Insert Chart 2 and Chart 3 here.**

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**Insert Figure 1 and Figure 2 here.**

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**Insert Figure 3 here.**
remain to be investigated.

Because different P-450 isozymes have different affinities for NDMA, it is understandable that multiple Km values are observed in microsomes. Depending on the number and type of P-450 isozymes present, many Km values with possible overlap may be expected. Our present approach of resolving them into 2 or 3 major Km values without correction for overlap is a treatment by approximation. The 3 apparent Km values for control rabbit liver microsomes are similar to those observed in rat liver microsomes (38). The kinetics of the ethanol-induced rabbit microsomes is also similar to that observed with rats in the concentration ranges studied (30). The increased NDMA activity is mainly due to the induction of a low Km form of this enzyme. The present data, however, do not distinguish whether or not the low Km NDMA activities in control and ethanol-induced microsomes are due to the same P-450 isoform. The observation that P-450 LMaa and LM each have a single and distinct Km value for NDMA is consistent with the concept that the multiple Km values in microsomes are due to the multiple P-450 isozymes present. It was unexpected, however, that the Km for LMaa, 2.95 mM, is much higher than the low Km, 0.07 to 0.10 mM, observed in microsomes. Antibodies prepared against LMaa have been shown to inhibit the low Km NDMA in microsomes. It appears that the kinetic constant is different when the cytochrome is in the membrane or in the reconstructed system.

Based on studies with inhibitors, some investigators (31, 33, 34) have previously doubted the involvement of P450 in the metabolism of NDMA and postulated that other enzymes, such as monoamine oxidase, might be involved in catalyzing the oxidation of NDMA. The present work with the reconstructed monooxygenase system demonstrates clearly that compounds, such as 2-phenylethylamine, 3-amino-1,2,4-triazole, and pyrazole, are potent inhibitors of P-450-dependent NDMA activity. Similar results were also obtained in studies with rat liver microsomal P-450 (39). These compounds were not known previously to be inhibitors of P-450-dependent reactions, because they do not effectively inhibit monooxygenase reactions with classical drug substrates (20, 39). The results of the present study suggest that many of the previous observations (20, 31, 33, 34) can be interpreted on the basis of inhibition of P-450-dependent NDMA and thus further establish the role of P-450 isozymes in the metabolism of nitrosamines.

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