Substrate Specificity and Alkyl Group Selectivity in the Metabolism of N-Nitrosodialkylamines

Maojung Lee, Hiroyuki Ishizaki, John F. Brady, and Chung S. Yang

ABSTRACT

Metabolic activation may be a key step in determining the tissue specificity of carcinogenic nitrosamines. In previous work, we characterized P450IIE1 (an acetone/ethanol-inducible form of cytochrome P-450) as the major enzyme for the metabolic activation of N-nitrosodimethylamine. In this work, we investigated the metabolism of other N-nitrosodialkylamines in rat liver microsomes and in reconstituted monooxygenase systems containing purified cytochrome P-450. The enzyme specificities in the metabolism of N-nitrosourethylmethylamine and N-nitrosodimethylamine were similar to those of N-nitrosodimethylamine; i.e., these substrates were more efficiently metabolized by acetone- or ethanol-induced microsomes than by other types of microsomes. However, substituting one methyl group with a benzyl or butyl group, as in N-nitrosobenzylmethylamine or N-nitrosobutylmethylamine (NBMA), substantially changed the enzyme specificity. P450IIE1 efficiently catalyzed the demethylation but not the debutylation of NBMA, whereas P450II1B1 (a phenobarbital-inducible form) efficiently catalyzed both the debutylation and demethylation reactions. In the demethylation of NBMA by P450IIE1, the addition of cytochrome b₅ markedly increased the activity at low but not at high substrate concentrations, suggesting a decrease in Kₘ value. This effect, however, was not observed in the debutylation of NBMA by P450IIE1 or P450II1B1, and in the demethylation of NBMA by P450II1B1. These studies demonstrate the substrate specificity and alkyl group selectivity in the metabolism of nitrosamines by cytochrome P-450 isozymes.

INTRODUCTION

Because of their wide occurrence and interesting carcinogenic properties, NDMA and other N-nitrosodialkylamines have received a great deal of attention in terms of their biological fates and activities (1-3). It is well recognized that metabolic activation of these compounds is required for the generation of electrophilic species which can elicit genotoxic or other damage in cells (3-7). This metabolic activation process is believed to be an important factor in determining the tissue and species specificities of some of these carcinogens. The enzymatic basis for activation and detoxication of many of these compounds, however, is not well understood. In previous work, we studied the enzymology of the metabolism of NDMA and characterized a specific type of cytochrome P-450 which displays low Kₘ and high turnover numbers in catalyzing the demethylation and denitrosation of NDMA (8-18). This P-450, with a recommended systematic name of P450II1E1 (19), is also known as P-450ac, P-450et, or P-450J in rats (13, 16, 20) and P-450LM3a in rabbits (14, 21). It exists in untreated rats, rabbits, and other animals and is inducible by a variety of inducers such as acetone, ethanol, pyrazole, and isoniazid as well as by physiological conditions such as fasting and diabetes (8-22). The P450II1E1 genes from rats and humans have been cloned, and the mechanisms of their regulation have been studied (23-27).

The Km value for NDMA demethylase, which was not accurately determined in the reconstituted system with purified P450II1E1 (16) because of the interference by glycerol and other competitive inhibitors, was recently determined in acetone-induced rat liver microsomes to be 20 to 50 µM. This value is slightly lower than our previously published value of 40 to 50 µM (17) and is much lower than the NDMA demethylase I and II studied by Arcos et al. (reviewed in Ref. 4). We also demonstrated that other P-450 species contribute to the metabolism of NDMA when this substrate is present at high concentrations (13, 14). Since animals are rarely exposed to such high concentrations of NDMA, we believe that the P450II1E1 species in animals and in humans (28, 29) are the enzymes responsible for the metabolism of carcinogenic levels of these compounds. Several lines of observations (10, 14, 17, 30) have lent support to this concept as well as to the role of this enzyme in the activation of NDMA to an alkylating, mutagenic, and cytotoxic agent (5-7). P450II1E1 is also capable of metabolizing other nitrosamines (13, 16). It was also observed from previous studies with microsomes and with reconstituted monooxygenase systems (10, 13, 14, 16) that many N-nitrosodialkylamines did not show the same enzyme preferences as NDMA in their dealkylation and denitrosation reactions. This difference was also observed in earlier studies by Farrelly et al. (31-33) and more recently by Kawanishi et al. (34) using rat liver microsomes. However, the enzymes involved in the metabolism and the kinetics of the reaction were not fully characterized.

In order to study the substrate specificity and alkyl group selectivity in the metabolism of N-nitrosodialkylamines, the different aldehydes produced from α-oxidation were measured in incubations containing different types of microsomes and reconstituted monooxygenase systems with purified P450II1E1 and P450II1B1. A key question in this investigation is whether two different alkyl groups on the same molecule display one or two Kₘ values when catalyzed by a P-450 species. This question was studied in detail using NBMA as a prototype substrate. Also included in this study is the effect of cytochrome b₅ which was found previously to enhance NDMA metabolism by decreasing the Kₘ value (16, 35).

MATERIALS AND METHODS

Chemicals. NEMA, NDEA, NBMA, NADP, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, MO). NDMA was from Aldrich Chemical Co. (Milwaukee, WI), and NBzMA was from Ash Stevens, Inc. (Detroit, MI). AKF 525A and benzphetamine HCI were gifts from Smith Kline & French (Philadelphia, PA) and Upjohn Company (Kalamazoo, MI), respectively.

Animals and Microsomes. Young male Sprague-Dawley rats (90 to 100 g of body weight) were obtained from Taconic Farms (Germantown, NY). They were kept in air-conditioned quarters in metabolism cages with a 12-h light-dark cycle and given a commercial laboratory chow (Ralston Purina Co., St. Louis, MO) and water ad libitum. In [CANCER RESEARCH 49, 1470-1474, March 15, 1989]
order to induce different P-450 enzymes, the rats were subject to the following treatments: (a) acetone administered i.g. as a 25% aqueous solution in a single dose of 2.5 ml/kg of body weight 18 h before sacrifice (11); (b) ethanol administered as a 15% solution in drinking water for 3 days (9); (c) phenobarbital administered i.p. in saline at a daily dose of 75 mg/kg for 3 days; (d) safrole administered i.p. in corn oil at a daily dose of 100 mg/kg for 3 days (36); and (e) 3-methylcholanganthrene administered i.p. in corn oil at a daily dose of 25 mg/kg for 3 days. Rats in the control group did not receive any treatment. Liver microsomes were prepared by differential centrifugation and washed once with a solution containing 154 mM KC1 and 10 mM EDTA as described previously (10). They were stored frozen in small portions at —80°C prior to use.

Reconstitution of P-450-dependent Monoxygenase Systems. P450IIE1, NADPH-P-450 reductase, and cytchrome b5 were purified as described (16). P450IIB1 was purified from phenobarbital-induced microsomes by the method of West et al. (37). For the reconstitution, P-450, NADPH:P-450 reductase, and dilaurylphosphatidylcholine were mixed in a test tube at room temperature before buffer and other reagents were added to give final concentrations of 0.1 nmol, 1500 units, and 22.5 μg, respectively, in 0.25 ml of incubation. The incubation period was 10 min. Cytochrome b5, when used, was present in the reconstituted system in equimolar concentration to P-450. Methods for other biochemical determinations have been described previously (10).

Dealkylation Assays. NDMA demethylase and benzphetamine demethylation activities were determined colorimetrically as described previously (10, 17) using glucose 6-phosphate and glucose-6-phosphate dehydrogenase for the NADPH-generating system. For the metabolism of other nitrosamines, a similar assay mixture was used. In brief, the assay mixture contained, in a total volume of 0.5 ml, 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 150 mM KC1, 0.4 mM NADP, 10 mM glucose 6-phosphate, glucose-6-phosphate dehydrogenase (0.2 units), microsomes (0.1 to 0.2 mg protein), and nitrosamine substrates as specified. For standards, different aldehydes were added at the midpoint of the incubation. Two different methods were used for carrying out the reaction. In Method I, nitrosamines were present at a concentration of 1 to 4 mM, and 5 mM semicarbazide was present to trap the aldehyde formed in uncapped test tubes (12 × 75 cm). In Method II, different concentrations of nitrosamines were used in the assay in the absence of semicarbazide. The test tubes were capped with rubber septa to minimize evaporation. The aldehydes were measured as 2,4-dinitrophenylhydrazone derivatives after separation by high-performance liquid chromatography as described by Brady et al. (38) based on the original procedure of Farrell et al. (31). All the assays were run in duplicate, and the differences between the duplicates were <15%. For kinetic analysis, apparent Michaelis-Menten parameters were estimated by a direct linear plot method (39).

RESULTS

Metabolism of Nitrosamines by Different Types of Microsomes. In order to study the substrate specificity in the metabolism of different nitrosamines, 6 different types of rat liver microsomes were used as the enzyme source, and the activity was expressed on the basis of per nmol of P-450 (Table 1). Treatment of rats with acetone or ethanol is known to induce P450IIE1 (16, 21, 23). In comparison to control microsomes, the acetone- and ethanol-induced microsomes were more active in the demethylation of NDMA (>2-fold), NEMA (170 to 175%), and NBMA (153 to 165%), but not for the metabolism of NBzMA. These microsomes were also more active in the deethylation of NEMA and NDEA (>2-fold), but not in the debutilylation of NBMA and debenzylation of NBzMA. Phenobarbital is known to induce P450IIB1 and other forms of P-450 (19). Pretreatment with this inducer produced microsomes that were much less active in catalyzing the demethylation of NDMA and NEMA, and the deethylation of NEMA. The phenobarbital-induced microsomes, however, were more active in catalyzing the debutilylation of NBMA (>2-fold) and the debenzylation of NBzMA (170%). Safrole treatment has been reported to enhance the metabolism of NBzMA by rat liver microsomes (35). This result was confirmed herein. The most noticeable differences between the duplicates were <15%. For kinetic analysis, apparent Michaelis-Menten parameters were estimated by a direct linear plot method (39).

Substrate Dependence in the Demethylation and Debutylation of NBMA. In order to characterize further the substrate specificity in the metabolism of nitrosamines, the rates of demethylation and debutilylation of different concentrations of NBMA were studied using control, acetone-induced, and phenobarbital-induced microsomes as the enzyme source (Fig. 1). With control microsomes, the rate of demethylation was higher than the rate of debutilylation at low concentrations (<3 mM) of NBMA, but the reverse was true at higher substrate concentrations. With acetone-induced microsomes, the pattern was similar except that the rate of the debutilylation reaction was lower than that with control microsomes at all substrate concentrations. With phenobarbital-induced microsomes, the rate of demethylation was similar to that with control microsomes, but the rate of debutilylation was much higher. In Eadie-Hofstee plots, the data generated from each set of experiments could not be fitted by a straight line (not shown). The data, however, could be approximately fitted by two straight lines: one for the 4 data points at low NBMA concentrations and the other for the 4 data points at higher substrate concentrations. The apparent kinetic param-

Table 1 Metabolism of nitrosamines by different types of microsomes

<table>
<thead>
<tr>
<th></th>
<th>NDMA</th>
<th>NEMA</th>
<th>NDEA</th>
<th>NEMA</th>
<th>NDEA</th>
<th>NDEA</th>
<th>NEMA</th>
<th>NBzMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment of rats</td>
<td>HCHO</td>
<td>HCHO</td>
<td>CH3CHO</td>
<td>HCHO</td>
<td>CH3CHO</td>
<td>HCHO</td>
<td>CH3CHO</td>
<td>HCHO</td>
</tr>
<tr>
<td>Untreated</td>
<td>2.25</td>
<td>0.44</td>
<td>1.55</td>
<td>1.14</td>
<td>1.23</td>
<td>1.52</td>
<td>0.69</td>
<td>1.59</td>
</tr>
<tr>
<td>Acetone</td>
<td>4.63</td>
<td>0.75</td>
<td>3.35</td>
<td>2.40</td>
<td>1.88</td>
<td>2.11</td>
<td>0.75</td>
<td>1.66</td>
</tr>
<tr>
<td>Ethanol</td>
<td>6.01</td>
<td>0.77</td>
<td>4.48</td>
<td>2.99</td>
<td>2.03</td>
<td>1.38</td>
<td>0.69</td>
<td>1.34</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>0.80</td>
<td>0.23</td>
<td>0.52</td>
<td>1.37</td>
<td>1.16</td>
<td>3.32</td>
<td>0.80</td>
<td>2.71</td>
</tr>
<tr>
<td>Safrole</td>
<td>1.86</td>
<td>0.28</td>
<td>1.13</td>
<td>1.31</td>
<td>1.24</td>
<td>2.93</td>
<td>1.33</td>
<td>3.22</td>
</tr>
<tr>
<td>3-Methylcholanganthrene</td>
<td>0.83</td>
<td>0.03</td>
<td>0.26</td>
<td>0.33</td>
<td>0.32</td>
<td>2.28</td>
<td>0.24</td>
<td>0.80</td>
</tr>
</tbody>
</table>

The reaction mixture was composed of microsomes corresponding to 0.5 nmol of P-450 and substrates at concentrations of 4 mM (NDMA), 1 mM (NEMA and NDEA), or 3 mM (NBMA and NBzMA) in 0.5 ml of reaction mixture containing 5 mM semicarbazide. The incubation time was 10 min. The activity, expressed as nmol of product formed per min per nmol of P-450, was the average of duplicated assays.
crosomes, respectively. Similar trends were obtained in 2 other sets of experiments using acetone-induced and phenobarbital-induced microsomes; II and C, reactions with acetone-induced and phenobarbital-induced microsomes. The assay was carried out by Method II. The reaction mixture (0.5 ml) contained 0.4, 0.8, and 1.6 mM NBMA concentrations, and each data point from the 4 lower (0.2, 0.4, 0.8, and 1.6 mM) NBMA concentrations, and each data point from the 4 higher (3, 6, 12, and 30 mM) NBMA concentrations. The V_{max} was obtained by subtracting V_{max} from the "observed V_{max}". The values for K_{m} are expressed as mM and for V_{max} as nmol of product/min/nmol of P-450.

<table>
<thead>
<tr>
<th>Substrate [mM]</th>
<th>Control</th>
<th>Acetone-induced</th>
<th>Phenobarbital-induced</th>
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<tbody>
<tr>
<td></td>
<td>K_{m}</td>
<td>V_{max}</td>
<td>K_{m}</td>
</tr>
<tr>
<td>I</td>
<td>0.07</td>
<td>1.16</td>
<td>0.54</td>
</tr>
<tr>
<td>II</td>
<td>2.47</td>
<td>1.25</td>
<td>12.88</td>
</tr>
<tr>
<td>I</td>
<td>0.18</td>
<td>1.39</td>
<td>0.32</td>
</tr>
<tr>
<td>II</td>
<td>1.62</td>
<td>0.62</td>
<td>13.29</td>
</tr>
<tr>
<td>I</td>
<td>0.34</td>
<td>1.48</td>
<td>0.58</td>
</tr>
<tr>
<td>II</td>
<td>1.80</td>
<td>1.27</td>
<td>9.89</td>
</tr>
</tbody>
</table>

The kinetic parameters were obtained from the data shown in Fig. 1 using the direct linear plot method (39). K_{m} refers to data points from the 4 lower (0.2, 0.4, 0.8, and 1.6 mM) NBMA concentrations, and K_{m} to data points from the 4 higher (3, 6, 12, and 30 mM) NBMA concentrations. The V_{max} was obtained by subtracting V_{max} from the "observed V_{max}". The values for K_{m} are expressed as mM and for V_{max} as nmol of product/min/nmol of P-450.

The data points were utilized in the direct linear plot method (39) to obtain apparent K_{m} values of 1.04 mM and 0.63 mM for the debutylation and demethylation, respectively. The corresponding V_{max} values were 11.59 and 7.33 nmol/min/nmol. In the reactions catalyzed by P450IIE1, apparent K_{m} values for the debutylation and demethylation were 3.89 and 8.25 nmol/min/nmol. These results indicate that, in catalyzing the metabolism of NBMA, P450IIE1 has a slightly higher K_{m} value for debutylation than demethylation; both K_{m} values, however, are higher than those displayed by P450IIB1.

Effect of Cytochrome b_{5} on the Metabolism of NBMA. In previous studies, we have shown that cytochrome b_{5} enhances the P450IIE1-catalyzed metabolism of NDMA by decreasing the K_{m} value (16). In the present work, a similar result was observed with the demethylation of NBMA catalyzed by P450IIE1 (Fig. 3A). The addition of cytochrome b_{5} to the reconstituted system caused a 5-fold increase in the demethylase activity with 0.4 mM NBMA, a 60% increase with 1.6 mM NBMA, no increase with 6 mM NBMA, and a slight decrease with 30 mM NBMA. Such substrate concentration-dependent effects were not observed in the P450IIE1-catalyzed demethylation reaction which was increased an average of 36% by the addition of cytochrome b_{5}, as well as in the P450IIB1-catalyzed demethylation and debutylation, which were increased by an average of 30% (Fig. 3B). This conclusion is supported by two other sets of experiments using different concentrations of NDMA.

DISCUSSION

As was proposed and demonstrated in our previous work (10, 13, 14, 16), different cytochrome P-450 species are involved in the metabolism of NDMA in microsomes, and this is the basis for the multiplicity of the K_{m} values of NDMA demethylation. We believe that similar situations also exist in the metabolism of
of other nitrosamines. When the composition of the P-450 isozymes changes as a result of treatment with inducers, the rate of metabolism changes. This point is illustrated by the data in Table 1 concerning the metabolism of five nitrosamines. A previously unresolved problem is whether a certain inducer will affect the metabolism of the two alkyl groups of an N-nitrosodialkylamine differently. The present results clearly demonstrate that the acetone/ethanol-inducible P450IIE1 is more efficient in catalyzing the α-oxidation of the methyl and ethyl groups of NDMA, NEMA, NDEA, and NBMA than other constitutive forms. The phenobarbital-induced P-450(s), on the other hand, is less active in catalyzing the oxidation of these groups in NDMA and NEMA but more active in catalyzing the α-oxidation of the butyl group of NBMA. 3-Methylcholan-threne-induced P-450(s) also showed low activity toward methyl and ethyl groups but high activity toward the butyl group.

The alkyl group selectivity in the metabolism of NBMA is clearly demonstrated in the kinetic studies shown in Figs. 1 and 2. Because of the existence of multiple P-450 forms in the microsomal samples, it is reasonable that the data points could not be represented by a single straight line in Eadie-Hofstee plots. Our simplified approach of fitting them into two Km values did not allow us to obtain the true Km and Vmax values; rather, it generated operational kinetic parameters. It allows us to compare our results with some previous studies. For exam-ple, with control microsomes, our KmII values for demethylation (2.47 mM) and for debutylation (12.88 mM) are close to the corresponding Km values of 3.4 and 14.2 mM observed by Farrelly et al. (33) with control microsomes from male Fisher rats. The corresponding Vmax values (VmaxI + VmaxII) in our study are much higher than those reported (33). These authors (33) apparently missed the KmI values because substrate concentra-tions <2.5 mM were not used. Since the carcinogenic doses of most nitrosamines are not very high (33), we believe that the low Km (KmI) enzymes are more important in the activation of these carcinogens in vivo. For the demethylation and debutylation of NBMA studied herein, these Km values ranged from 0.07 to 0.34 mM and from 0.32 to 0.58 mM, respectively. We may speculate that most of the observed activities in acetone-induced and phenobarbital-induced microsomes are due to P450IIIE1 and P450IIB1, respectively. However, additional work is needed to clarify this point. In control microsomes, P450IIE1 might contribute more in the demethyla-tion than the debutylation of NBMA. Further studies are needed to investigate the involvement of other constitutive P-450 forms in the metabolism of this carcinogen.

The kinetic data obtained in the reconstituted system (Fig. 2) were simpler than those observed with microsomes. However, some of the observed Km values may be higher than those displayed by the same P-450 species in microsomes. For example, the presence of cytochrome b5 is expected to decrease the Km value for the demethylation of NBMA by P450IIE1 in microsomes, a situation similar to the demethylation of NDMA by the same enzyme (16). By analogy, it may also be suggested that glycerol and other unknown factors may cause an overes-timation of the Km value for the demethylation of NBMA catalyzed by P450IIE1.

A key issue of the present investigation is whether the metabol-ism of the two alkyl groups of a nitrosamine displays one or two Km values. The results in Fig. 2 show that the demethylation of NBMA displayed a lower Km value than the debutylation in the reconstituted system with P450IIIE1. In the system with P450IIB1 the difference is not as clear. In addition, the Km for the demethylation by P450IIIE1 can be decreased by the presence of cytochrome b5. If we assume the Km is determined mainly by the binding of NBMA to the active site of the enzyme, we may suggest that the butyl group of NBMA binds to a proposed hydrophobic pocket in the active site of P450IIE1,4 leaving the methyl group more favorably positioned for oxida-tion. Especially in the presence of cytochrome b5, this mode of binding is preferred over the alternative mode, i.e., with the methyl group binding to the proposed hydrophobic pocket allowing the butyl group to be oxidized at the oxygenation site. On the other hand, P450IIB1 apparently does not possess such a clear alkyl group selectivity in substrate binding. It appears that cytochrome b5 may affect nitrosamine metabolism via two mechanisms: (a) by binding to P450IIE1 to elicit a confor-mational change at the active site of this enzyme which increases the affinity for the binding of NBMA in one mode (leading to the demethylation) but not in the other mode, and (b) by a previously proposed mechanism of donating a second electron to the heme-oxygen complex (40) leading to a substrate concentra-tion-independent enhancement of 30 to 36% in average magnitude.

The present work clearly demonstrates the substrate specificity and alkyl group selectivity in the metabolism of nitrosamines by P-450 isozymes. It is also clear that such selectivity is dependent on the concentration of the substrate used in the assay. Accordingly, in comparing metabolic activities, different conclusions may be reached if different substrate concentrations
are used. This point is important when we consider the effects of P-450 inducers on the metabolic activation of nitrosamines in vivo. We believe that the low Km forms of the enzymes are important in the activation of these carcinogens. With many N-nitrosodimethylamines, the formation of a methylating agent is believed to be the key activation step (1, 3, 41). Based on this concept, we may suggest that pretreatment of rats with acetone or ethanol, which decreases debutylation (and thus the formation of the methylating agent), may lower the activation of this carcinogen in the liver. However, this information is insufficient for predicting the carcinogenicity of NBMA which induces mainly esophageal cancer in rats (42). Whereas a decrease in the ratio of activation to detoxification pathways should decrease carcinogenesis, a net decrease in hepatic metabolism may increase the extent of exposure of the target tissue to this carcinogen.

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