Identification of Ethanol-inducible P-450 Isozyme 3a as the Acetone and Acetol Monoxygenase of Rabbit Microsomes*

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Recent reports of the presence of 1,2-propanediol and 2,3-butanediol in blood drawn from alcoholics while intoxicated (1) and in blood drawn from chronic acetone-treated rats after injection with acetone (2) suggest that similar metabolic pathways may be responsible for the production of these compounds in both circumstances. The chronic administration of acetone or ethanol to rats results in the induction of two microsomal activities, acetone monoxygenase, which converts acetone to acetol, and acetol monoxygenase, which converts acetol to methylglyoxal (2). These reactions require oxygen and NADPH, suggesting that both may be P-450 catalyzed. Acetone monoxygenase appears to play a key role in the production of 1,2-propanediol in acetone-treated rats and is the first step in a proposed gluconeogenic pathway for acetone involving hepatic and extrahepatic reactions (2).

Rabbit hepatic cytochrome P-450 isozyme 3a, which exhibits high activity in alcohol oxidation, has been isolated from ethanol-treated rabbits (3, 4). Experiments utilizing antibody to purified isozyme 3a indicated that in microsomes from ethanol-treated rabbits isozyme 3a represents about 20% of the total P-450 and catalyzes over 70% of the microsomal alcohol oxidation (5-7). Treatment of rabbits with acetone also induced microsomal alcohol oxidation, and the induced activity was inhibited by antibody to isozyme 3a (6). A protein immunochemically identical to isozyme 3a was increased about 5-fold by acetone (6).

In addition to alcohol oxidation, treatment of rabbits with ethanol results in the induction of hepatic aniline hydroxylation and a low $K_m$ N-nitrosodimethylamine demethylation; both activities are catalyzed primarily by isozyme 3a (7, 8). Similarly, the treatment of rats with ethanol or acetone results in an increase in aniline hydroxylation (9, 10) and N-nitrosodimethylamine demethylation (11, 12). It has also been reported that both activities are induced in the rat by fasting and diabetic ketoacidosis (13-16).

Both fasting and diabetic ketoacidosis are conditions in which serum acetone is elevated in both humans (17, 18) and rats (13). Consistent with a physiologic role for acetone monoxygenase, the activity is induced by starvation (2), and measurable acetol is present in serum from rats in diabetic ketoacidosis (19). The similarity between the enzyme activities induced by ethanol and acetone treatment and in vitro evidence showing that many of these activities are catalyzed primarily by the major ethanol-inducible P-450 in rabbit, isozyme 3a, suggest that the acetone and acetol monoxygenase activities may also be catalyzed by the same isozyme. In this report we demonstrate the identity of acetone monoxygenase and acetol monoxygenase and isozyme 3a in rabbit. An immunochemically similar isozyme is responsible for these activities in rats.

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EXPERIMENTAL PROCEDURES

Preparation of Microsomes and Purification of P-450 Isozymes—Adult male New Zealand rabbits (2-2.5 kg in weight) and male Wistar rats (250-350 g, weaners) were obtained from the Jackson Laboratory. Rabbits were housed in a room maintained at 15°C and given ad libitum access to Purina rabbit chow and water containing 1.5% aceto dine for 1 week with free access to Purina rabbit chow or a standard NIH rat ration. Pyrophosphate-washed liver microsomes were prepared (20) and stored at a final protein concentration from 25-40 mg/ml in 100 mM Tris acetate buffer, pH 7.4, containing 0.1 mM EDTA and 20% glycerol at -70°C. No significant loss of P-450, measured spectrally or catalytically, occurred over an 8-month period under these conditions. The specific contents of the preparations from untreated, acetone-, and ethanol-treated rabbits were 2.79 ± 0.21, 3.05 ± 0.38, and 2.36 ± 0.56 nmol of P-450/mg of protein, respectively.

P-450 cytochromes were purified to electrophoretic homogeneity by procedures already described (3, 20, 21), from rabbits treated with phenobarbital (for form 2, imidazole (for forms 3a and 6), isosafrole (for form 4), or untreated (for isozymes 3b and 3c). The specific contents of the preparations ranged from 16-20 nmol of P-450/mg of protein. Cytochrome P-450 reductase was purified from phenobarbital-treated rabbits (22) and catalysed the reduction of 45-55 pmol of P-450/mg of protein, respectively.

Antibodies to the purified or reconstituted cytochrome c were produced in sheep and purified as previously described (7). The IgG preparation inhibited greater than 95% of the activity of the purified enzyme. Weak cross-reactivity of the antibody with isoforms 3b was observed on Western blots but not with rabbit isoforms 3b in the reconstituted system. The antibody was passed over an isozyme 3b-Sepharose 4B column to remove the form 3b cross-reaction when used in immunological staining of Western blots (6).

Determination of Acetol, 1,2-Propanediol, and 2,3-Butanediol in Serum—Rabbits that were untreated or given 1% (v/v) acetone for 7 days were bled from an ear vein on the morning of the eighth day. Blood was allowed to clot for 1 h, and serum was obtained by centrifugation. Both 1,2-propanediol and 2,3-butanediol were extracted as the phenylboronate esters from the serum, and the esters were measured by electron capture detection as described by Needham et al. (23).

Acetol was measured in perchloric acid-extracted serum (0.075 ml of 60% perchloric acid/ml of serum followed by neutralization of the supernatant with KOH) by HPLC separation of the dinitrophenylhydrazone derivative of acetol. Acetol dinitrophenylhydrazone was prepared by adding 0.6 ml of a solution containing 0.4% dinitrophenylhydrazine and 2 ml of 60% perchloric acid/ml of serum followed by neutralization of the perchlorate salt, and an aliquot was assayed by HPLC. The acetol dinitrophenylhydrazone was obtained from Boehringer Mannheim. Rabbit anti-sheep immunoglobulin and sheep peroxidase anti-peroxidase were obtained from Cooper Biomedical. The source of other chemicals and supplies has been described elsewhere (2, 3, 7).

RESULTS

Cassaza et al. (2) have demonstrated that rats given 1% (v/v) acetone exhibited significant serum concentrations of 1,2-propanediol, 2,3-butanediol, and acetol; these compounds were not detected in serum from untreated rats (2, 19). In order to determine if a similar pathway is present in the rabbit, rabbits were given 1% (v/v) acetone for 7 days. Serum from rabbits maintained on acetone contained 88 ± 14 nmol/ml of acetol, 70 ± 9 nmol/ml of 1,2-propanediol, and of the four treated rabbits only one had a detectable level of 2,3-butanediol, 7.9 nmol/ml, as shown in Table I. In contrast, serum from 4 untreated rabbits contained no detectable acetol, 1,2-propanediol, or 2,3-butanediol.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum concentration</th>
<th>nmol/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Acetol 1,2-Propanediol 2,3-Butanediol</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>2</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>3</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>4</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Acetone</td>
<td>100</td>
<td>66</td>
</tr>
<tr>
<td>2</td>
<td>47</td>
<td>52</td>
</tr>
<tr>
<td>3</td>
<td>111</td>
<td>84</td>
</tr>
<tr>
<td>4</td>
<td>53</td>
<td>67</td>
</tr>
</tbody>
</table>

*This value represents the limit of detection for the assays used.
Treatment of rabbits with 1% (v/v) acetone results in an increase in a protein immunologically identical with ethanol-inducible isozyme 3a (20). Microsomes from acetonetreated rabbits were submitted to the purification procedure developed for isozyme 3a (3), and an electrophoretically homogeneous preparation of P-450 form 3a was obtained; the preparation behaved identically to the ethanol form during all stages of purification. The preparation of isozyme 3a from acetonetreated rabbits could not be distinguished from the preparation from ethanol-treated rabbits by spectral, catalytic, or immunochemical methods (results not shown). The identity of the HPLC profiles of the tryptic digests of the two preparations (Fig. 1) supports the hypothesis that the two isozymes have identical primary structures, although absolute identity would require complete sequence determination. Using the same criteria it has been demonstrated that isozyme 3a induced by imidazole is identical with the ethanol form (21).

Acetone and acetal hydroxylase activity was localized in the microsomal fraction of rat liver (2). As shown in Fig. 2, hepatic microsomes from acetonetreated rabbits effectively catalyzed both hydroxylation reactions. The formation of acetal and d-lactate was linear with the length of incubation up to 30 min and was linear with microsomal protein up to 1 mg/ml.

Treatment of rabbits with either ethanol or acetone increased the rate of microsomal hydroxylation of acetone and acetol (Table II). Acetone hydroxylase activity was increased about 6-fold by ethanol and about 11-fold by acetone treatment. Acetal hydroxylation was enhanced slightly less than 3-fold by ethanol treatment and slightly more than 3-fold by acetone treatment. The relative increase in the two hydroxylation activities is very similar to the increase reported for acetone treatment of rats, where acetone hydroxylation was increased about 10-fold and acetal hydroxylation was increased about 4-fold (2).

Antibody to isozyme 3a was used to determine the role of the isozyme in the microsomal activity. Fig. 3 shows the results obtained when hepatic microsomes from acetonetreated rabbits were assayed in the presence of increasing amounts of anti-3a IgG. The concentration of IgG was kept constant by the addition of IgG from preimmune sheep. The microsomal activity toward both substrates was inhibited by increasing concentrations of the antibody. At a final ratio of about 3 mg of IgG/nmol of microsomal P-450, acetal hydroxylation was inhibited about 70% while acetone hydroxylation was inhibited about 90%. When microsomes from untreated or ethanol-treated rabbits were assayed in the presence of anti-3a IgG the acetone hydroxylase activity was inhibited by 90% or more (Table III). In contrast, the acetal hydroxylation activity of microsomes from untreated rabbits was inhibited only 32% while the activity of microsomes from ethanol- or acetonetreated rabbits was inhibited 75 and 70%, respectively. The apparent disparity between the sensitivity of the two activities can be explained if isozyme 3a was the only isozyme active in acetal hydroxylation, while other isozymes...
Acetone and Acetol Hydroxylation

**FIG. 3. Inhibition of microsomal acetone and acetol hydroxylation by anti-3a IgG.** Reaction mixtures contained 1 mg of microsomal protein from acetone-treated rabbits and increasing amounts of anti-3a IgG. The concentration of IgG was kept constant by the addition of preimmune IgG. Other components were the same as described in Table II. Reactions were run for 30 min at 30 °C. Acetol and D-lactate were measured as described under "Experimental Procedures." Antibody-inhibited rates were obtained by subtracting the enzymatic rate obtained in the presence of anti-3a IgG from that obtained in the presence of control IgG.

**TABLE III**

Inhibition of microsomal acetone and acetol hydroxylation by anti-3a IgG

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Microsomal source</th>
<th>Acetone hydroxylation</th>
<th>Acetol hydroxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Antibody inhibited</td>
<td>Antibody inhibited</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>Acetone</td>
<td>Rabbit (untreated)</td>
<td>0.41</td>
<td>0.35</td>
</tr>
<tr>
<td>+Control IgG</td>
<td></td>
<td>0.04 (90%)</td>
<td>0.37 (32%)</td>
</tr>
<tr>
<td>+Anti-3a IgG</td>
<td></td>
<td>0.01 (80%)</td>
<td>0.26 (2%)</td>
</tr>
<tr>
<td>Rabbit (ethanol treated)</td>
<td></td>
<td>3.17</td>
<td>1.84</td>
</tr>
<tr>
<td>+Control IgG</td>
<td></td>
<td>0.15 (95%)</td>
<td>5.02 (75%)</td>
</tr>
<tr>
<td>+Anti-3a IgG</td>
<td></td>
<td>0.17 (98%)</td>
<td>2.14</td>
</tr>
<tr>
<td>+Anti-3a IgG</td>
<td></td>
<td>0.15 (97%)</td>
<td>5.02 (70%)</td>
</tr>
<tr>
<td>Acetol</td>
<td>Rabbit (acetone treated)</td>
<td>2.58</td>
<td>1.98</td>
</tr>
<tr>
<td>+Control IgG</td>
<td></td>
<td>0.07 (97%)</td>
<td>2.51 (73%)</td>
</tr>
<tr>
<td>+Anti-3a IgG</td>
<td></td>
<td>0.15 (97%)</td>
<td>5.02 (70%)</td>
</tr>
</tbody>
</table>

**TABLE IV**

Acetone and acetol hydroxylation by purified isozymes of rabbit hepatic cytochrome P-450

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity of isozyme</th>
<th>nmol/min/nmol P-450</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>2</td>
<td>&lt;0.5&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acetol</td>
<td>3</td>
<td>2.1&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*This value represents the lower limits of detection of the product by the methods used.

The activity of microsomes from acetone-treated rats was also inhibited by anti-3a IgG. The addition of the antibody resulted in 97 and 70% inhibition of acetone and acetol hydroxylation, respectively (Table III). Western blots were run with purified isozyme 3a and with microsomes from acetone-treated rabbits, acetone-treated rats, and untreated rats. The results obtained after immunohistochemical staining with anti-3a IgG are shown in Fig. 4. As previously reported (6), a single band corresponding to purified isozyme 3a was immunohistochemically stained with microsomes from acetone-treated rabbits. Two bands were stained with microsomes from untreated and acetone-treated rats. The lower band, having an apparent molecular weight of about 51,000, was not increased by acetone treatment. The more intense band, having a molecular weight of about 53,000, was increased after acetone treatment as seen qualitatively in Fig. 4.

**DISCUSSION**

Two gluconeogenic pathways for acetone in acetone-treated rats have been proposed by Casazza et al. (2). The initial step of both pathways involves the hydroxylation of acetone to acetol. Acetol represents the branch point for the two pathways with one pathway involving the conversion of acetol to methyglyoxal and the other pathway involving the conversion of acetol to 1,2-propanediol (2). The production of acetol and 1,2-propanediol in rabbits treated with acetone suggests that a similar pathway to that proposed in rat is present in rabbit. In addition, both acetone and acetol hydroxylation are induced by acetone and ethanol treatment in the two species. The occurrence of 2,3-butanediol in the blood of only one of the four acetone-treated rabbits and at levels significantly lower than those observed in rat under similar conditions suggests that there is some species difference in the metabolism of 2,3-butanediol. Whether this difference is due to a difference in the rate of production or utilization of 2,3-butanediol is not clear.
Acetone and Acetol Hydroxylation

The nitrocellulose was immunochemically stained with antibody to...about 8- and 11-fold, respectively; acetone treatment increases acetone hydroxylation about 14-fold and acetol hydroxylation about 12-fold. Thus, while the microsomal activities suggest that the two activities are differentially induced by either of the two inducers, the increases in the antibody-inhibitable rates are in good agreement with each other.

The almost complete inhibition of microsomal acetone hydroxylation by anti-3a IgG indicates that isozyme 3a is most likely the sole catalyst of acetone hydroxylation in rabbit. The results of reconstitution experiments support this conclusion since isozyme 3a was the only form of six examined to exhibit detectable activity toward acetone. In contrast, while isozyme 3a is responsible for the majority of acetol hydroxylation in microsomes from treated rabbits, isozymes 2, 3b, and 4 are also able to catalyze this reaction. In results not presented, we found that acetone was a linear mixed type inhibitor of isozyme 3a-catalyzed aniline hydroxylation and acetol exhibited the same type of inhibition of butanol oxidation. At the same concentrations, acetone stimulated in vitro aniline hydroxylation by isozymes 2 and 4 about 2-fold, an indication that the inhibition by acetone was not due to denaturation of the protein.

A purified isozyme of P-450 from acetone- or ethanol-treated rats has not yet been characterized. Therefore, studies of this type could not be done in the rat. However, the inhibition of both rat acetone and acetol hydroxylase activities by anti-3a IgG revealed that these two activities are catalyzed by an immunochemically similar protein. The Western blot results in Fig. 4 suggest that the rat isozyme induced by acetone treatment has a molecular weight of about 53,000. This is in good agreement with previous reports of a microsomal protein induced in rats by ethanol which has a molecular weight ranging from 51,500–53,000 (10, 27–30). Proteins with similar molecular weights are induced in rats by fasting (14) and alloxan-induced diabetes (16). In addition, diabetic rats, fasted rats, and rats treated with acetone have increased rates of microsomal aniline hydroxylation (9) and N-nitrosodimethylamine demethylation (11, 14, 15), activities which are catalyzed primarily by isozyme 3a in the rabbit (5, 7, 8). Anti-3a IgG also effectively inhibits rat microsomal N-nitrosodimethylamine demethylation (31). The induction of rat microsomal aniline, acetone, acetol, and N-nitrosodimethylamine activity by acetone treatment and the susceptibility of the activities to inhibition by anti-3a IgG suggest that in the rat a single isozyme is catalyzing all of these reactions and the isozyme is immunochemically homologous to rabbit isozyme 3a. Whether this isozyme is the same as the protein identified on Western blots remains to be determined by a direct comparison of the purified isozymes.

The identity of P-450 isozyme 3a and the acetone monooxygenase and acetol monooxygenase systems suggests a unique physiological role for this isozyme in a gluconeogenic pathway. The report of the induction of acetone and acetol monooxygenase in the rat (2) and the data presented here suggest that isozyme 3a and homologous forms in other species can be induced in response to physiological conditions. The identification of acetol and 1,2-propanediol in fasting humans (2, 19), the incorporation of [14C]acetone into glucose, and the increase in serum acetone observed during fasting (17) suggest that a homologous form to isozyme 3a is induced in humans and catalyzes the initial hydroxylation of acetone to acetol enabling its conversion to glucose. Although it is possible that a similar situation occurs in alcoholics, it is not clear that this is the case. Despite the occasional occurrence of alcoholic ketoacidosis (32), alcoholics are not generally ketoacidotic. Although both acetone and acetol monooxygenase activities are induced by chronic ethanol consumption, the presence of
2,3-butanediol and 1,2-propanediol in alcoholics still remains unexplained.

REFERENCES