Hydroxylation of acetone by ethanol- and acetone-inducible cytochrome P-450 in liver microsomes and reconstituted membranes

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Acetone oxidation in rat liver microsomes was induced 5- or 8-fold by the treatment of the animals with ethanol or acetone, respectively. The apparent K_m of the reaction was 0.9 mM, a value lower than the concentration reported for plasma acetone under starvation conditions. The major acetone metabolite was identified as acetol by GC-MS. Acetone oxidation in microsomes was inhibited by typical P-450 inhibitors as well as by compounds (e.g. imidazole) known to interact with the ethanol-inducible P-450 form. Antibodies against this P-450 isozyme were inhibitory for the reaction in rabbit liver microsomes and this isozyme was the only one that showed acetone hydroxylation activity in reconstituted membranes. Imidazole inhibited the conversion of [^{14}C]acetone into low-M_r compounds (e.g. glucose) in vivo. It is suggested that the ethanol- and acetone-inducible P-450 make use of acetone as an endogenous substrate in the utilization of the compound for, e.g. glucose production under conditions of starvation and diabetic ketoacidosis.

Acetone *Cytochrome P-450* *Starvation* *Ketoacidosis* *Gluconeogenesis* *Ethanol*

1. INTRODUCTION

Treatment of rabbits with ethanol or acetone causes the induction of a form of cytochrome P-450 in the liver microsomes called P-450 LMeb [1,2] or 3a [3–5]. It appears that this form of P-450 constitutes 20–40% of all cytochrome P-450 in liver microsomes from induced animals [4,5] and participates in the microsomal oxidation of ethanol and other aliphatic alcohols [2,4,6]. In the rat, similar forms of cytochrome P-450 are induced by ethanol, acetone and starvation (unpublished).

As shown by Reichard and collaborators [7,8], acetone appears to be a gluconeogenetic precursor in fasting and ketodiabetic humans. The plasma concentrations of acetone in such subjects are in the range 1–10 mM [7,8]. At the smaller acetone concentration, 75% of the acetone produced is metabolized in vivo, especially to glucose [8]. This glucose production could account for 10% of the gluconeogenetic demands in fasting humans [7]. The work by Casazza et al. [9,10] has suggested that the hydroxylation of acetone to acetol is the first step in the conversion of the ketone to glucose and involves either a glyoxalate pathway or a metabolic route with propanediol as an intermediary substance.

During the course of our studies concerning benzene metabolism by the ethanol-inducible form of P-450, it turned out that acetone was a competitive inhibitor of the P-450 LMeb-dependent hydroxylation of benzene. It was therefore hypothesized that this form of cytochrome P-450 would constitute an effective catalyst for acetone hydroxylation. Here, we present evidence that this indeed is the case and that the K_m of the enzyme for acetone is smaller than the plasma concentrations of this substance under fasting conditions.

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2. EXPERIMENTAL

Male Sprague-Dawley rats (175 g) were starved for 24 h and subsequently treated with acetone (5 ml/kg) as described [11]. Other groups of rats were treated with ethanol according to DeCarli and Lieber [12]. Control rats received food and water ad libitum until killing. The livers of the animals were homogenized in 3 vols of 10 mM sodium/potassium phosphate buffer, pH 7.4, containing 1.14% KCl. The microsomes were prepared by ultracentrifugation and washed once before suspension in 50 mM potassium phosphate buffer, pH 7.4. Acetone metabolism was studied using ^{14}C-labelled substrate. The incubations were performed in sealed tubes with liver microsomes corresponding to 1 mg protein in the presence of 1.36 mM acetone containing 0.4 μCi ^{14}C-labelled substrate in a total volume of 1 ml of 50 mM potassium phosphate buffer, pH 7.4. The incubations were started by the addition of 0.4 mg NADPH and stopped by the addition of 0.1 ml of 40% trichloroacetic acid. The tubes were subsequently treated with a stream of nitrogen at 56°C for 15 min and the residues were allowed to stand at room temperature for 48 h. Aliquots of 100 μl were counted in a scintillation counter after addition of water to the original volume. Acetone metabolites were also analyzed as dinitrophenylhydrazone derivatives [13] and subjected to analysis on HPLC. Metabolites eluted from the HPLC were further derivatized using *tert*-butyldimethylchlorosilane (tBDMClS) and analyzed by gas chromatography-mass spectrometry (GC-MS) [14]. Acetol was obtained from Aldrich, 2,4-dinitrophenylhydrazine from Riedel-De Haen, phenylhydrazine chloride from Merck and tBDMClS was purchased from Janssen, Beerse, Belgium.

Carbohydrates were analyzed in livers from rats receiving i.p. injections of [^{14}C]acetone. The livers were homogenized in 1 vol. redistilled water and ethanol was added to 80% (v/v). The samples were subsequently centrifuged and the supernatants, containing e.g. small carbohydrates, lipids and amino acids, were evaporated to dryness. The residues were dissolved in 5 ml water and an aliquot measured for radioactivity. Carrier glucose (3 mg), phenylhydrazine chloride (67 mg) and sodium acetate (100 mg) were added and the samples boiled for 30 min. The resulting osazones were collected by centrifugation, washed once in cold water and dissolved in 1 ml acetone:methanol (1:1, v/v) and subjected to TLC using the solvent system acetone:methanol (95:5, v/v). The glucose band was scraped off the plate and analyzed for radioactivity using a scintillation counter and Aqualuma plus as scintillator liquid.

3. RESULTS

The oxidation of acetone in control microsomes or microsomes isolated from acetone-treated rats was linear for 20 min under the conditions used (not shown). The substrate curves obtained using the two types of microsomes (fig.1) revealed a K_m of slightly less than 1 mM (table 1). The V_{max} in acetone microsomes was at least 10-fold higher than in control microsomes (table 1). In microsomes from ethanol-treated rats, the rate of acetone oxidation was 5-fold higher, compared to control (table 1).

The microsomal oxidation of acetone was inhibited by compounds known to interact with the ethanol-inducible form of cytochrome P-450 (benzene, aniline, ethanol and imidazole, cf. [2,4]) as well as by typical cytochrome P-450 inhibitors (table 2). In contrast to the situation when analyz-

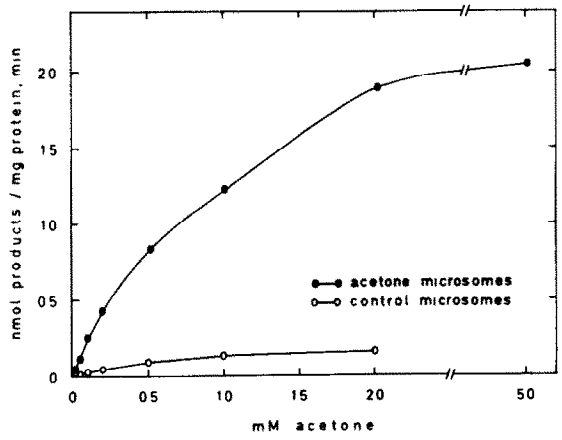

Fig.1. Effect of substrate concentration on the rate of acetone oxidation in liver microsomes isolated from acetone-treated (●—●) and control (○—○) rats. Incubations were performed as described in section 2 with liver microsomes corresponding to 1 mg protein for 20 min at 37°C.

Table 1

Oxidation of acetone by liver microsomes from control, ethanol- and acetone-treated rats

Type of liver microsomes	Acetone oxidation		K_m (mM)	V_{max} (nmol/mg per min)
	nmol/mg per min	nmol/nmol per min		
Control (4)	0.20 ± 0.03	0.35 ± 0.07	0.80	0.25
Ethanol (6)	1.11 ± 0.23[b]	0.81 ± 0.19[a]		
Acetone (5)	1.53 ± 0.17[b]	1.23 ± 0.20[b]	0.99	2.6

[a] $p < 0.005$; [b] $p < 0.001$, compared to control

The turnover values were obtained from incubations performed with 1.36 mM acetone and microsomes corresponding to 1 mg protein from the number of animals indicated within parentheses

ing the microsomal ethanol oxidation (cf. [2]), addition of Fe-EDTA was inhibitory for product formation and mannitol was a weak scavenger of the reaction (table 2). To evaluate further the participation of the ethanol-inducible form of cytochrome P-450, liver microsomes from acetone-treated rabbits were incubated with acetone in the presence of antibodies towards this form of P-450 (P-450 LMeb). As seen from fig.2, control IgG had a slight stimulatory effect on the reaction, whereas anti-P-450 LMeb-IgG was inhibitory. The acetone hydroxylation was also reconstituted in membranes containing various forms of rabbit liver microsomal cytochromes P-450 and NADPH-cytochrome P-450 reductase. The ethanol-inducible P-450 form was the only one that showed significant activity (table 3).

Table 2

Effect of Fe(III)-EDTA, mannitol, inhibitors of cytochrome P-450 and compounds known to interact with the ethanol-inducible P-450 form on the rate of acetone oxidation in liver microsomes from acetone-treated rats

Inhibitor added	Acetone oxidation (nmol/mg per min)	Inhibition (%)
None	1.44	–
Aniline, 1 mM	0.04	97
Ethanol, 200 mM	0.04	97
Mannitol, 500 mM	1.05	27
Imidazole, 0.05 mM	1.25	13
0.5 mM	0.29	80
5 mM	0	100
SKF-525A, 0.2 mM	0.78	46
Metyrapone, 1 mM	1.27	12
Fe-EDTA, 10 μM	0.48	67
Carbon monoxide	0.03	98
Benzene, 0.1 mM	0.36	75
1 mM	0.14	90

The substrate concentration was 1.36 mM and the conditions as outlined in section 2

To identify acetone metabolites produced in the microsomal system, the incubation residues (cf. section 2) were derivatized and analyzed by HPLC and GC-MS. As is evident from fig.3, two major acetone metabolites were eluted from the HPLC column. The first one (I) had identical retention time as did dinitrophenylhydrazine-derivatized

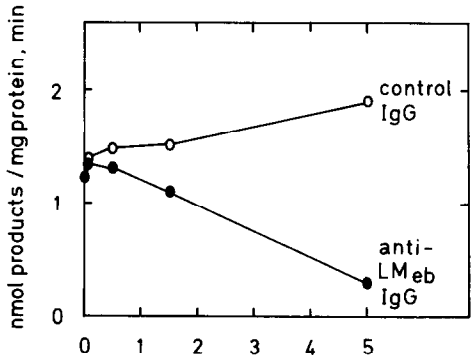

Fig.2. Inhibition of the liver microsomal NADPH-dependent oxidation of acetone by antibodies towards the ethanol-inducible form of cytochrome P-450 (P-450 LMeb). The anti-P-450 LMeb-IgG was prepared as in [4] and added 15 min prior to the initiation of the incubations.

Table 3

Oxidation of acetone in reconstituted membrane vesicles containing various forms of rabbit liver microsomal cytochrome P-450 and NADPH-cytochrome P-450 reductase

Cytochrome used	nmol/nmol P-450 per min
P-450 LM_2	<0.02
P-450 LMeb	0.15
P-450 LM_4	<0.02

The sources of enzymes [2] and procedures for preparation of membrane vesicles [15] were as described. The membranes contained P-450 reductase:P-450:phospholipid at a molar ratio of 1:4:1200. Incubations were performed with 1.36 mM acetone and vesicles corresponding to 0.2–0.45 nmol P-450 for 20 min

acetol and, furthermore, after derivatization with tBDMClS, exhibited an identical mass spectrum as authentic dinitrophenylhydrazine- and tBDMClS-derivatized acetol (fig.3). This metabolite was therefore identified as acetol. Approx. 30% of the total radioactivity in the incubation residues was recovered in the organic phase after dinitrophenylhydrazine derivatization.

Imidazole, a compound that has been previously found to interact with P-450 LMeb (cf. [4]), was a very effective inhibitor of acetone hydroxylation in vitro (table 2). It was therefore proposed that administration of this compound to rats prior to the injection of [^{14}C]acetone to the same animals should prevent the in vivo incorporation of radioactivity into glucose and other low-M_r compounds. As is evident from table 4, imidazole given

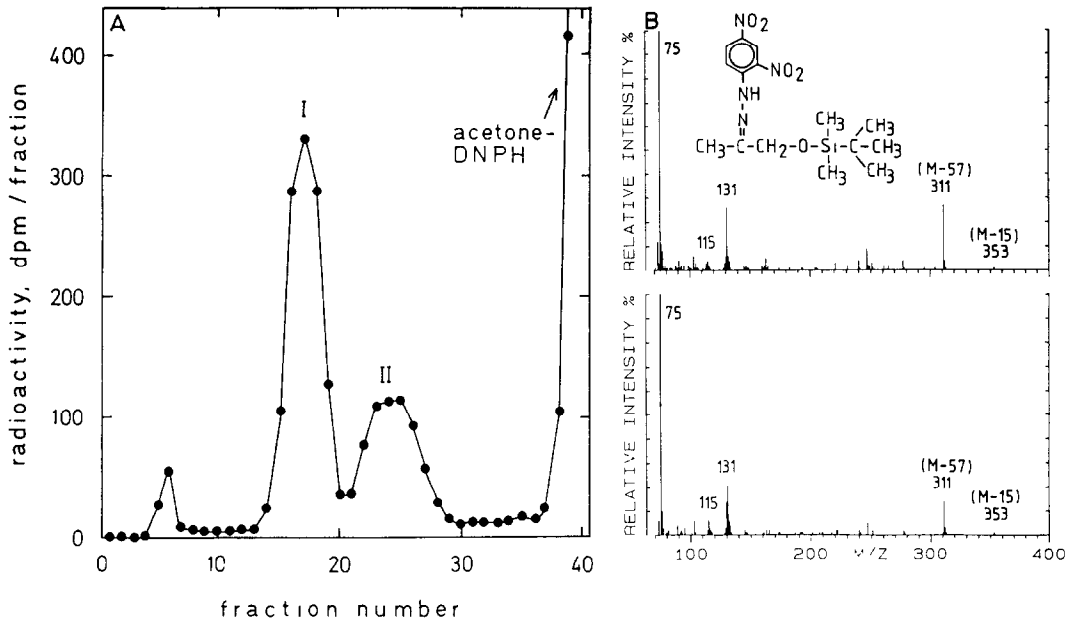

Fig.3. HPLC (A) and GC-MS (B) analysis of acetone metabolites produced from incubations with liver microsomes of acetone-treated rats. After termination, the incubations were derivatized with 2,4-dinitrophenylhydrazine and extracted with chloroform [13]. The organic phase was taken to dryness under nitrogen and dissolved in ethyl acetate before injection onto the HPLC column (μBondapack C_{18}). The column was eluted isocratically with methanol:water (1:1, v/v) at a rate of 1.5 ml/min. Fractions corresponding to peak I and II, respectively, were pooled, taken to dryness under nitrogen and further derivatized with tBDMClS [14]. Aliquots corresponding to 0.5 nmol substance were analyzed on an LKB 2091 gas chromatograph-mass spectrometer, equipped with a fused-silica capillary column with a chemically bonded polydimethylsiloxane phase, CPSil 5 CB (Chrompack, Middelburg, The Netherlands). The column temperature was 225°C and the mass spectra were recorded at an electron energy of 22.5 eV and an ion source temperature of 310°C. The *m/z* 115, 75 and 73 represent fragments from the tBDMS groups. The *m/z* 353 represents the loss of a methyl group (M – 15) and *m/z* 311 is equivalent with loss of the *t*-butyl group (M – 57) from the derivatized acetol. The upper part of (B) shows the spectrum obtained using the authentic compound and the lower part is that of the acetone metabolite.

Table 4

Incorporation of [^{14}C]acetone into low-M_r compounds and carbohydrates in livers from starved and acetone-treated rats

Type of treatment	^{14}C incorporation (cpm/g liver)		
	Low-M_r compound	Osazone derivates of carbohydrate	Glucose
None (4)	23000 ± 4500	4100 ± 900	580 ± 110
Imidazole, 200 mg/kg (4)	11900 ± 3700[a]	430 ± 160[b]	190 ± 60[b]

[a] $p < 0.01$; [b] $p < 0.001$, compared to control

The results are given as mean ± SD from the number of animals given within parentheses. The rats were given acetone intragastrically (5 ml/kg) on day 1 and starved day 1 and 2. Imidazole (100 mg/ml in water) was injected i.p. into one group of rats on day 3. 3 h later, 4 μCi [^{14}C]acetone was injected i.p. into all animals. The rats were killed after a further 3 h period and low-M_r compounds in the livers were analyzed as described in section 2

at a dose of 200 mg/mg (yielding approx. 3–5 mM extracellular concentration) 3 h before [^{14}C]-acetone, inhibited the conversion of radioactive acetone into carbohydrates by 90% and the incorporation of radioactivity into specifically glucose by 67%.

4. DISCUSSION

Our results indicate that acetone- and ethanol-inducible cytochrome P-450 form(s) are responsible for the microsomal metabolism of acetone to oxygenated products being able to undergo further metabolism to intermediates participating in the gluconeogenetic pathway. The extent of inhibition by imidazole in vitro of the cytochrome P-450-dependent acetone oxidation correlated well with the findings in vivo regarding the ability of imidazole, at similar concentrations, to prevent the incorporation of [^{14}C]acetone into low-M_r carbohydrates, such as glucose. The apparent K_m of the reaction in microsomes of about 0.9 mM is lower than the acetone concentrations in plasma reported under starvation and conditions of ketoacidosis [7,8].

The inhibition by aniline, ethanol and benzene of the microsomal acetone oxidation indicates the utilization of a common cytochrome P-450-dependent metabolic site for these substrates. The absence of a stimulatory effect of Fe-EDTA on the reaction, which is in contrast to the situation concerning the cytochrome P-450-dependent ethanol oxidation [2,16], indicates the existence of mechanisms for acetone hydroxylation not involving the participation of free hydroxyl radicals.

The induction of the ethanol-inducible form of cytochrome P-450 by acetone and of similar cytochrome P-450 forms in the rat also by starvation indicates that acetone might be of highly physiological importance as an inducing agent of cytochrome P-450 under starvation and diabetic conditions. Furthermore, it appears that acetone may be one of the quantitatively most important endogenous substrates for cytochrome P-450 under these conditions.

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