THE FAD-CONTAINING MONOOXYGENASE-CATALYZED N-OXIDATION AND DEMETHYLATION OF TRIMETHYLAMINE IN RAT LIVER MICROSOMES

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SUMMARY

Trimethylaminuria (TMAuria), the excessive urinary excretion of the odorous trimethylamine (TMA), accompanies elimination of TMA in sweat and corresponding "fish-odor" syndrome. TMA was oxidized *in vitro* in rat liver microsomes from male Sprague-Dawley rats to TMA N-oxide and N-demethylated to dimethylamine (DMA). Both reactions were inhibited to 1-3% of normal activity by preincubation of microsomes without NADPH-generating system at 37°C for 10 minutes indicating the FAD-containing monooxygenase-catalyzed reactions. On the other hand, the reactions were not inhibited by gas phase containing up to 80% carbon monoxide/20% oxygen mixture. The results are compatible with the hypothesis that in rat liver microsomes the N-oxygenation and N-demethylation of TMA are catalyzed only or predominantly by FAD-containing monooxygenases, and the cytochrome P-450 monooxygenases play a negligible, if any, role.

KEY WORDS

trimethylamine, dimethylamine, trimethylamine N-oxide, FAD-containing monooxygenases

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INTRODUCTION

Trimethylaminuria is an excessive urinary elimination of trimethylamine (TMA) with accompanying unpleasant body odor caused by simultaneous elimination of TMA in sweat. It was originally described in a patient with the inherited Turner's syndrome /1/, but another patient with the similar Noonan's syndrome was not affected by this "fish-odor syndrome" /2/. Trimethylaminuria (TMAuria) is supposed to be related to insufficient capacity of body enzymes to N-oxygenate TMA to non-odorous trimethylamine N-oxide (TMAO). In a British population this defect appeared to be inherited as a recessive trait under diallelic control of a single gene /3-6/.

TMA (minor) and TMAO (predominant) are present in many organisms from prokaryotes to complex eukaryotes, especially in marine organisms, with elasmobranchs containing TMAO at up to 1.5% of muscle weight /7/. TMA level in sea-food may increase during storage at above zero temperatures and indicates bacterial spoilage, since various marine (and intestinal) bacteria efficiently reduce TMAO to TMA /8/. Rats and humans eliminate TMA and especially TMAO in urine (usual ratio 1:10 to 1:30) and excrete also dimethylamine (DMA) and monomethylamine (MMA) which seem to be little related to TMA and TMAO levels /9,10/, although DMA can also be formed from TMA. Healthy male volunteers given [^{14}C]TMA (61.8 mg of free base) excreted 95% radioactivity as TMAO, less than 5% as TMA and less than 0.1% as DMA, with exponential elimination of 95% of the given radioactivity in urine within 24 hours /3/. Healthy humans excreted 0-25 μmol TMA/24 h, whereas heterozygous "carriers" excreted 130-160 μmol/24 h and homozygous subjects even 750 μmol/24 h, and responded by a 20-fold increase of TMA excretion after oral intake of TMA.HCl (300 mg of free base), whereas healthy subjects continued to excrete normal levels. The heterozygotes responded in an intermediate way /4,5/. Humans given choline (0.4 mmol/kg) orally excreted 67% of the dose as TMA (TMAO was not measured), whereas rats given 15 mmol/kg excreted 2.3-4.3% as TMA, 7.6-23% as TMAO and 0.2-0.3% as DMA /9-10/, indicating that choline is the source of TMA, and the capacity for TMA oxidation (especially the N-oxygenation) can also be exceeded after high choline dose. Humans convert more choline to TMA and TMAO than rats /9,10/. Phosphocholine was metabolized to TMA and TMAO in rats similarly to choline, but betaine and other potential amines only to

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homogenate in 154 mM KCl/50 mM Tris.HCl, pH 7.4 for 60 min at 105,000 *g*. The pellet was resuspended in 154 mM KCl/10 mM EDTA and re-centrifuged and final resuspension was made in 0.25 M sucrose at 15-20 mg of protein/ml. The microsomes were stored at -70°C.

Enzyme assays

The incubation mixtures contained, in a final volume of 2.5 ml, final concentrations of 154 mM KCl, 50 mM KCl-Tris, pH 7.4, 10 mM $MgCl_2$, 0.4 mM NADP, 10 mM glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 0.5 I.U./ml, microsomal protein 1 or 1.5 mg/ml and TMA.HCl in 0.01 M HCl at 250 μM concentration to secure V_{max} conditions. In most assays all components were mixed on ice in Perkin-Elmer head-space vials, preincubated for 2 min at 37°C in a water shaking bath, capped with PTFE-lined septa, and TMA.HCl in 0.01 M HCl was injected through the septum to start the reaction. After 10 min the incubation was stopped by chilling the samples on ice and injecting 1/10 volume of 3M HCl followed with 1/10 volume of 12% sodium tungstate for precipitation of proteins for TMA, DMA and MMA analysis. For TMAO analysis the reaction was terminated by injecting 25% $ZnSO_4$ and a saturated solution of $Ba(OH)_2$ for precipitation of protein (1/10 volume each).

Analysis

For the gas chromatographic head space analysis of TMA, DMA and MMA the method described for urine /16,5/ was modified. An aliquot of the clear acidic supernatant for TMA, DMA and MMA analysis was put into a clean head-space vial, frozen, overlaid with 0.01 M HCl, refrozen. An aliquot of the alkaline supernatant for TMAO analysis was made more alkaline with NaOH solution, evaporated at 80-85°C under vacuum, the dry evaporate was dissolved in 1 M HCl and TMAO was reduced by $TiCl_3$ under nitrogen in head space vials sealed by PTFE-lined septa essentially as described /9,10/ but without HCHO, which proved to be unnecessary. The samples were frozen at -70°C, overlaid with 0.01 M HCl and re-frozen. Before the analysis, the sample was taken from the freezer, dry K_2CO_3 and 1 ml 80% K_2CO_3 were added, the head-space vial was sealed, the sample was

heated in a 100°C water bath and 2 ml of head-space gas was injected onto the Perkin-Elmer 8500 series gas chromatograph with a glass column (2 m, 2 mm i.d.) packed with Carbopack B/4% Carbowax 20M/0.8% KOH (Supelco, Belafonte, PA). The amines were eluted with hydrogen, 15 ml/min. The temperatures were: injection port 250°C, column 65°C, NPD detector 300°C. For the quantitative evaluation, the peaks integrated by the built-in integrator were compared with TMA, DMA or TMAO standards which were incubated and processed in the same way as the samples.

RESULTS AND DISCUSSION

In order to select a suitable TMA concentration for V_{max}, TMA metabolism was followed by substrate disappearance (measuring residual substrate) and TMAO formation. Both assays gave the K_m value for TMA of 15 μM, using Lineweaver-Burk analysis (double reciprocal plot).

It is known that in the absence of NADP or NADPH, the FAD-containing monooxygenases (FMO) are irreversibly inactivated at 37°C during 30 minutes. In order to inactivate FMO without affecting cytochrome P-450 monooxygenases, the microsomes were preincubated without the NADPH-generating system (NADPH-GS) at 37°C for only 10 min. A possible minor decrease in P-450 activity during incubation of another series of samples at 37°C with the NADPH-GS was checked in a third series of samples preincubated at 37°C with NADPH-GS for 2 min. It became obvious that at the saturating TMA concentration of 250 μM, an appreciable P-450 catalysis could be ruled out, since there was no difference between the 2 min and 10 min preincubation with NADPH-GS. FMO catalysis was apparent in the almost complete inactivation of the microsomal metabolism of TMA to TMAO and DMA (Table 1): there was between 1 and 3% of residual activity.

The lack of catalysis by cytochrome P-450 monooxygenases was also checked in looking for the effect of carbon monoxide on TMA oxidation. The incubation in 100% oxygen did not influence TMA demethylation in most experiments, but doubled the N-oxygenation (Table 2) and even 50% oxygen/50% CO mixture significantly stimulated the rate of N-oxygenation in control microsomes. In the 80% CO/20% O_2 gas mixture in the incubation, the rate of demethylation and N-oxygenation were similar to that in air. These

TABLE 1

Thermal inactivation of the oxidation of trimethylamine to trimethylamine N-oxide (TMAO) and dimethylamine (DMA) by rat liver FAD containing monooxygenases, by preincubation of the microsomes at 37°C in the absence of the NADPH generating system

Microsomes	Conditions of preincubation (min / NADPH)	Metabolic rates (nmol/mg protein/min) TMAO formation	DMA formation
Control	2/yes	2.467 ± 0.256	0.932 ± 0.028
	10/yes	2.150 ± 0.147	0.886 ± 0.022
	10/no	0.084 ± 0.007	0.009 ± 0.009
PCN	2/yes	2.053 ± 0.090	1.173 ± 0.088
	10/yes	1.986 ± 0.110	1.048 ± 0.072
	10/no	0.060 ± 0.001	0.033 ± 0.027

Control microsomes were from untreated male Sprague-Dawley rats. Pregnenolone 16-α-carbonitrile pretreatment (PCN) was i.g. in corn oil five times, 25 mg/kg each, twice daily and rats were sacrificed 12 h after the 5th dose. The results are means ± S.E.M. of three samples. The concentrations of NADPH-generating mixture cofactors were identical in all samples incubated for 10 min at 37°C.

TABLE 2

Lack of the effect of carbon monoxide on the oxidation of trimethylamine to trimethylamine N-oxide (TMAO) and dimethylamine (DMA) by control and PCN rat liver microsomes

The incubation mixture gas phase	Metabolic rates (nmol/mg protein/min) TMAO formation	DMA formation
Control microsomes		
Air	3.013 ± 0.149	2.691 ± 0.267
100% O_2	5.480 ± 1.002	2.816 ± 0.045
50%/50% CO/O_2	3.803 ± 0.114	3.132 ± 0.702
80%/20% CO/O_2	2.796 ± 0.366	3.038 ± 0.024
PCN microsomes		
Air	2.278 ± 0.015	1.878 ± 0.355
100% O_2	4.827 + 0.584	2.394 ± 0.458
50%/50% CO/O_2	2.365 ± 0.273	2.154 ± 0.153
80%/20% CO/O_2	2.161 ± 0.022	2.470 ± 0.175

The microsomes with NADPH generating system in head space glass vials on ice were flushed with the above gas mixtures for 90 sec and sealed with PTFE coated septa, preincubated for 2 min at 37°C and the reaction started with TMA.HCl injection through the septum to make up 250 μM TMA. For other details see text. The results are means ± S.E.M. of three samples.

results confirmed that P-450-catalysis of TMA oxidation is negligible or non-existent.

Our results agree with the data of Poulsen and Ziegler /14/ and Sabourin and Hodgson /15/ that FAD-containing monooxygenases catalyze the oxidation of TMA. Moreover, the data have shown that both N-oxygenation and demethylation to DMA are catalyzed by these enzymes in the liver microsomes. In contrast to these authors, the K_m we found in the rat microsomes was much lower than with pig or mouse purified FMO. We have also demonstrated that the rates of TMA demethylation (DMA formation) at saturating TMA concentration are 40 - 70% of N-oxygenation. It is markedly different from the observation /3/ that the TMA demethylation rate in man *in vivo* is only a fraction of N-oxygenation.

The N-dealkylation is believed to be catalyzed by cytochromes P-450 /16/. However, tertiary amines are often N-oxygenated by FAD-containing monooxygenases. TMA appears to be the first significant example of N-demethylation catalyzed by FMO.

The evidence that FMO catalyzed TMA oxidation indicates that it might be difficult to influence the fish-odor syndrome by enzyme induction, since these enzymes have never been successfully stimulated. The only successfully applied prevention or diminishing of the fish-odor syndrome smell so far appears to be in controlling the diet by avoiding food containing trimethylamine N-oxide (sea-food and some freshwater fish) and to avoid food containing excessive amounts of choline or lecithin (meat, eggs and some "health foods", to which choline and lecithin are frequently added artificially).

The pronounced difference between the low rate of TMA N-demethylation in humans *in vivo* and our *in vitro* rate for this pathway in rat liver microsomes needs further analysis. There could be a species difference between man and rat. It is important in view of the fact that DMA is the precursor of the potent carcinogen N-nitroso dimethylamine. Moreover, it should be clarified whether the rat is a good experimental model for the study of the fish-odor syndrome.

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