Biochemical Pharmacology, Vol. 32, No. 24, pp. 3723–3728, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

HEPATIC MICROSOMAL CYTOCHROME P-450-DEPENDENT N-DEMETHYLATION OF METHYLGUANIDINE

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(*Received* 4 *February* 1983; *accepted* 7 *May* 1983)

Abstract—Cytochrome P-450-dependent N-demethylation of methylguanidine, a uremia toxin, was investigated. Methylguanidine was stoichiometrically converted into equal amounts of guanidine and formaldehyde by aerobic incubation with phenobarbital-induced microsomes and NADPH. The guanidine formation in the incubation mixture followed Michaelis–Menten kinetics and required the presence of molecular oxygen and NADPH. Methimazole, a non-formaldehyde-producing substrate specific for FAD-containing monooxygenase, did not inhibit significantly formaldehyde formation, suggesting that microsomal FAD-containing monooxygenase does not play a significant role in N-demethylation of methylguanidine. The direct involvement of cytochrome P-450 in the N-demethylation is supported by the observations that addition of methylguanidine to purified cytochrome P-450$_1$ preparation caused a type I spectral change and that inhibitors of cytochrome P-450, such as carbon monoxide and metyrapone, markedly decreased the rate of demethylation. Neither superoxide anion nor hydrogen peroxide was directly involved in the demethylation reaction. In addition, guanidine formation was observed in the reconstituted system containing purified cytochrome P-450$_1$. Thus, these findings indicate that the hepatic microsomal mixed function oxidase system catalyzes N-demethylation of methylguanidine to guanidine.

Methylguanidine is a guanidine derivative that has been reported to be one of the toxins in the pathogenesis of uremia [1–3]. Many toxic effects of methylguanidine have been described, including an inhibitory effect on oxidative phosphorylation [4] and on the growth of cells in culture [5], as well as acceleration of hemolysis [6]. Although creatinine has been proposed to be one of the precursors of methylguanidine [7, 8], the metabolic fates of this compound have not been clarified.

Cytochrome P-450 participates in the oxidative metabolism of xenobiotic compounds such as drugs and carcinogenic chemicals [9]. Its role in the metabolism of endogenous substances has been implicated in steroid metabolism [9] and in the syntheses of vitamin D [10] and bile acids [11, 12]. The involvement of the cytochrome has also been observed in the metabolic breakdown of fatty acids including arachidonic acid [13–15]. However, whether or not cytochrome P-450 participates in the metabolism of "endogenous toxic substances" formed during pathological processes, such as methylguanidine in uremia, has not been studied. Thus, we investigated the participation of hepatic microsomal cytochrome P-450 in the metabolism of methylguanidine and observed that the microsomal cytochrome P-450-dependent mixed function oxidase system catalyzed N-demethylation of methylguanidine to guanidine.

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MATERIALS AND METHODS

Preparation of microsomes. New Zealand male rabbits (weighing 2 to 2.5 kg) were injected intraperitoneally with 80 mg phenobarbital/kg body weight once daily for 4 days. The animals were fasted for 20 hr before they were killed. The livers were perfused with 0.25 M sucrose solution and homogenized in 8 vol. of the same solution. After centrifugation at 10,000 *g* for 20 min, the microsomal pellet was obtained by further centrifugation at 105,000 *g* for 90 min. After washing once with 1.15% KCl solution, the pellet was frozen and stored at −80°. The microsomes thus obtained were used for assay of methylguanidine demethylase activity within 2 weeks. The activity was conserved completely during this period.

Analytical methods. The content of cytochrome P-450 was determined as described by Omura and Sato [16], using an extinction coefficient of 91 mM^{-1} cm^{-1}. Protein concentration was measured by the method of Lowry *et al.* [17], using bovine serum albumin as a standard. Separation and quantitation of guanidine were performed with a Shimadzu model LC-3A high pressure liquid chromatograph (HPLC), equipped with a fluorometric detection system by a modification of the method described by Hiraga and Kinoshita [18]. The separation between methylguanidine and guanidine was performed on a strong cation-exchange resin column (ICS-05/SO504, 100 mm × 4 mm i.d., Shimadzu Sei-

sakusho). The mobile phase was 0.2 N sodium borate solution containing 0.35 N sodium citrate and 0.8 M sodium chloride (pH 12.0). After the separation, guanidine compounds were reacted with 1% ninhydrin in the reaction coil and were detected at 500 nm with the fluorometric detector using an excitation wavelength of 395 nm. The areas under the chromatographic peaks were integrated with an electronic integrator (Chromatopak RIA, Shimadzu Seisakusho).

Enzyme assay in microsomal preparation. The rate of methylguanidine N-demethylation was determined by measuring guanidine, or formaldehyde, according to the method of Nash [19]. The reaction mixture contained microsomes corresponding to 3 mg protein, 5 mM methylguanidine and 1 mM NADPH in a final volume of 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.4). The inhibition of the reaction by methimazole was performed at a concentration of 0.25 or 1.0 mM of the drug at pH 7.4 or 8.4. The reaction was started by addition of the substrate and terminated after 30 min by adding 0.25 ml of 20% trichloroacetic acid solution. The results obtained by using freshly prepared microsomes were the same as those obtained by using frozen-stored microsomes.

Enzyme assay in the reconstituted system. The activity of purified NADPH–cytochrome P-450 reductase was assayed by measuring NADPH–cytochrome *c* reductase activity by the method of Mihara and Sato [20]. One unit of the enzyme was defined as the amount catalyzing the reduction of 1 μmole of the acceptor per min at 25°. The reaction mixture for the assay of mixed function oxidase activity contained 3 μM cytochrome P-450$_1$, 1.2 units of NADPH–cytochrome P-450 reductase, Triton X-100 (0.05%, w/v), 1 mM NADPH, 0.1 M potassium phosphate buffer (pH 7.4), and 5 mM methylguanidine as substrate in a final volume of 1.0 ml, as previously described [21–23]. The reaction mixture was incubated at 37° for 20 min. The rate of methylguanidine N-demethylation was determined by measuring guanidine or formaldehyde formation.

Carbon monoxide inhibition. Inhibition of cytochrome P-450 action by carbon monoxide was studied in a closed vessel (2.0 ml) equipped with a galvanic-type oxygen electrode and a thermostatic water bath. The reaction mixture was saturated with carbon monoxide by bubbling thoroughly, and then oxygen was introduced to an appropriate tension by monitoring with the electrode in the liquid phase as previously described by Sugiyama *et al.* [21].

Spectral change by addition of methylguanidine to purified cytochrome P-450 preparation. Equal amounts of purified cytochrome P-450$_1$, 7 μM in 0.1 M potassium phosphate buffer (pH 7.4), were placed in sample and reference cuvettes, and difference spectra were measured by adding, successively, small aliquots of 1 M methylguanidine (dissolved in the same buffer) to the sample cuvette. The same amounts of the buffer were added to the reference cuvette. The extent of the spectral change was measured as the difference in absorbance at two fixed wavelengths, with a Hitachi 557 dual wavelength spectrophotometer. The dissociation constant (K_s) was determined from a reciprocal plot of the absorbance changes as described by Schenkman *et al.* [24].

Preparation of microsomal enzymes. Cytochrome P-450$_1$ was purified from liver microsomes of phenobarbital-pretreated rabbits, using ω-aminooctyl Sepharose, hydroxylapatite and CM-sepharose chromatographies by a modification of the procedure reported by Imai *et al.* [25], to a concentration of 17.2 nmoles/mg protein. Detergent-solubilized NADPH–cytochrome P-450 reductase was purified by the method of Yasukochi and Masters [26] using 2′,5′-ADP-Sepharose affinity chromatography, and a specific activity of 36 units/mg protein was obtained as previously described [21].

Reagents and biochemicals. NADH and NADPH were obtained from the Oriental Yeast Co. Methylguanidine, guanidine and methimazole were purchased from the Sigma Chemical Co. Triton X-100 was purchased from Wako Pure Chemical Industries Ltd., and Emulgen 913 was from the Kao-Atlas Co., Ltd. Cytochrome *c* (Type III, from horse heart) was purchased from the Sigma Chemical Co. and 2′,5′-ADP-Sepharose was from Pharmacia Fine Chemicals. Metyrapone was a gift from Ciba-Geigy (Japan), Ltd. Phenyl isocyanide was synthesized by the method of Schmidt and Stern [27]. Catalase from bovine liver and superoxide dismutase from bovine erythrocytes were purified according to Sumner and Dounce [28] and McCord and Fridovich [29] respectively. Purified D-amino acid oxidase was a gift from Dr. Hiromasa Tojo of the Department of Biochemistry, Osaka University Medical School.

RESULTS

N-Demethylation of methylguanidine to guanidine by the mixed function oxidase system in liver microsomes from phenobarbital-pretreated rabbits. Aerobic incubation of methylguanidine with the microsomes isolated from phenobarbital-pretreated rabbits and NADPH resulted in the formation of guanidine, as illustrated in Fig. 1. Incubation of methylguanidine with microsomes isolated from untreated rabbits did not produce a significant amount of guanidine in comparison with incubation with phenobarbital-induced microsomes. No destruction of cytochrome P-450 was observed during the incubation. The formation of guanidine in the incubation mixture was concomitant with an approximately equimolar production of formaldehyde and required the presence of molecular oxygen and NADPH, as shown in Table 1. The rate of guanidine formation decreased to 24% of control activity when NADPH was replaced by NADH as the donor of reducing equivalents. These observations suggest that the N-demethylation of methylguanidine to guanidine was catalyzed by the microsomal mixed function oxidase system containing cytochrome P-450. However, N-demethylation of secondary N-methylamines such as methylguanidine can be catalyzed by a FAD-containing monooxygenase in liver microsomes [30, 31] as well as by the cytochrome P-450-dependent oxidase. The rate of formaldehyde formation during N-demethylation of methylguanidine was determined in the presence of methimazole, a non-formaldehyde-producing substrate specific for the monooxygenase [31], as shown

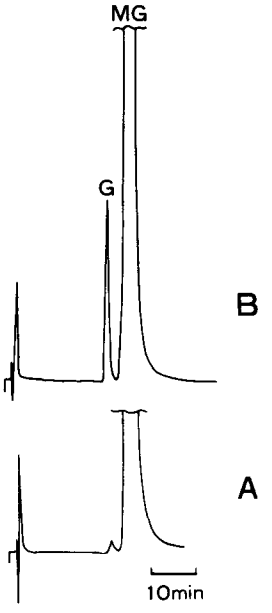

Fig. 1. HPLC profiles of methylguanidine and guanidine in the reaction mixture. The mixture contained microsomes corresponding to 3 mg protein (2.5 nmoles P-450/mg protein), 5 mM methylguanidine and 1 mM NADPH in a final volume of 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.4). Key: (A) reaction terminated at 0 min; (B) reaction terminated after 30 min of incubation at 37°; MG, methylguanidine; and G, guanidine.

in Table 2. No significant decrease in formaldehyde formation was observed after the addition of 0.25 mM methimazole. Since FAD-containing monooxygenase is less stable than cytochrome P-450 in the absence of NADPH or substrate, the effect of methimazole was examined by using freshly prepared, as well as stored, microsomes. No significant effect was observed even in the presence of 1.0 mM methimazole. This finding suggests that the FAD-containing monooxygenase did not participate significantly in the formation of formaldehyde during N-demethylation of methylguanidine in the microsomes.

To evaluate the role of cytochrome P-450 in the reaction, the effects of various inhibitors of cytochrome P-450 on the rate of the N-demethylation were examined (Table 3). Marked inhibition was caused by 0.1 mM metyrapone or 20 μM phenyl isocyanide. Under an atmosphere of 90% carbon monoxide and 10% oxygen, the rate of N-demethylation decreased to 35% of that under an atmosphere of 90% nitrogen and 10% oxygen. Table 4 shows the effects of addition of superoxide dismutase and catalase on the rate of N-demethylation. Taking the experimental errors into consideration, the effects of these agents seemed not to be significant. Therefore, superoxide and hydrogen peroxide may not be involved directly in the N-demethylation. These observations suggest that the N-demethylation of methylguanidine to guanidine was catalyzed by the

Table 1. Rate of methylguanidine N-demethylation in liver microsomes from phenobarbital-pretreated rabbits*

Conditions	Rate		
	Guanidine [nmole·(mg protein)$^{-1}$·min^{-1}]	Formaldehyde [nmole·(mg protein)$^{-1}$·min^{-1}]	% of Control activity
(1) Complete system	0.54	0.48	100
(2) Minus O_2†	0.02		4
(3) Minus NADPH	0.01		2
(4) Minus NADPH plus NADH (1 mM)	0.14		24

* The demethylation rate in the reaction mixture was determined by measuring guanidine and formaldehyde as described in Materials and Methods.

† When anaerobic conditions were used, a closed vessel equipped with a galvanic-type oxygen electrode was initially deaerated with N_2. Next, the residual O_2 was removed by preincubation of the system with DL-alanine and D-amino acid oxidase. Catalase (0.05 mg) was added to remove the hydrogen peroxide formed by the D-amino acid oxidase reaction.

Table 2. Effect of an inhibitor of microsomal FAD-containing monooxygenase on the rate of methylguanidine N-demethylation in liver microsomes from phenobarbital-pretreated rabbits*

Addition	Rate: Formaldehyde [nmole·(mg protein)$^{-1}$·min^{-1}]	
	pH 7.4	pH 8.4
None	0.50	0.35
Methimazole (0.25 mM)	0.51	0.33

* The reaction mixture contained microsomes corresponding to 3 mg protein, 5 mM methylguanidine, and 1 mM NADPH in a final volume of 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.4 or 8.4) with or without methimazole (0.25 mM).

Table 3. Effect of inhibitors of cytochrome P-450 on the rate of methylguanidine N-demethylation in liver microsomes from phenobarbital-pretreated rabbits*

Addition	Rate	
	Guanidine [nmole·(mg protein)$^{-1}$·min^{-1}]	% of Control activity
Experiment I		
None	0.56	100
Metyrapone (0.1 mM)	0.22	31
Phenyl isocyanide (20 μM)	0.06	10
Experiment II		
None (O_2:N_2 = 10:90)	0.52	100
Carbon monoxide (O_2:CO = 10:90)	0.18	35

* The reaction mixture contained the components described in Materials and Methods, except for addition of inhibitor. In experiment II, the gas phase was prepared as described in the text.

Table 4. Effects of addition of superoxide dismutase and catalase on the rate of methylguanidine *N*-demethylation in liver microsomes from phenobarbital-pretreated rabbits*

Addition	Rate	
	Guanidine [nmole·(mg protein)$^{-1}$·min^{-1}]	% of Control activity
None	0.51	100
Superoxide dismutase	0.47	92
Catalase	0.43	84

* Superoxide dismutase (0.3 mg) or catalase (0.05 mg) was added to the reaction mixture containing the components described in Materials and Methods.

microsomal mixed function oxidase system which contains cytochrome P-450.

Kinetics of N-demethylation of methylguanidine. Guanidine formation during aerobic incubation of methylguanidine with phenobarbital-induced microsomes and NADPH followed Michaelis–Menten kinetics (Fig. 2). The apparent K_m calculated from Lineweaver–Burk plots was 1.9 mM for the microsomes, while V_{max} was 0.75 nmole guanidine formed·min^{-1}·(mg microsomal protein)$^{-1}$ or 0.3 nmole guanidine formed·min^{-1}·(nmole cytochrome P-450)$^{-1}$.

Interaction of methylguanidine with cytochrome P-450$_1$. The addition of methylguanidine to 0.1 M

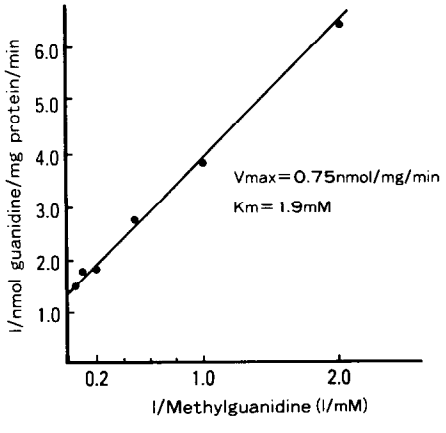

Fig. 2. Lineweaver–Burk plot of N-demethylation of methylguanidine measured by guanidine formation in the reaction mixture. This mixture contained the components described in Materials and Methods.

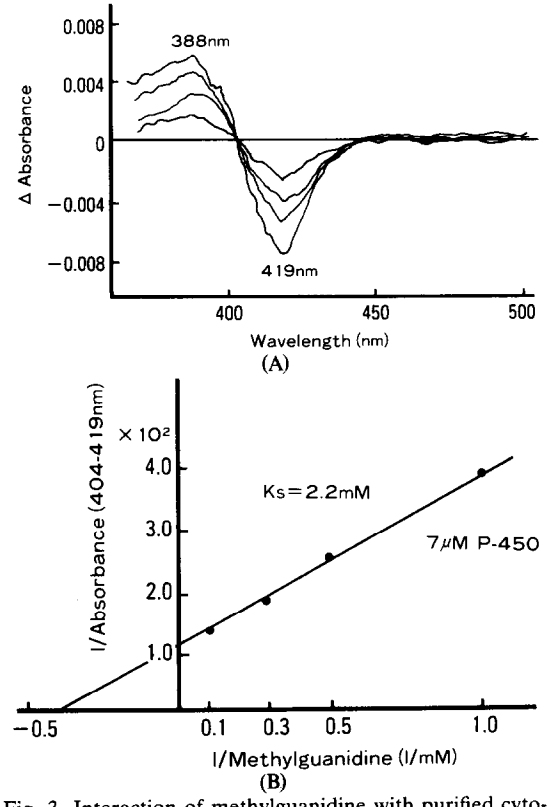

Fig. 3. Interaction of methylguanidine with purified cytochrome P-450$_1$. (A) Spectral change obtained by successive addition of small aliquots of 1 M methylguanidine solution to 0.1 M potassium phosphate buffer (pH 7.4) containing 7 μM cytochrome P-450$_1$ at 37°. (B) Reciprocal plot of changes in absorbance at 419 nm relative to 404 nm.

Table 5. Rate of methylguanidine N-demethylation in the reconstituted system containing purified cytochrome P-450$_1$ from phenobarbital-pretreated rabbits*

Conditions		Rate: Guanidine [nmole·min^{-1}·(nmole P-450)$^{-1}$]
(1)	Complete system	0.65
(2)	Minus P-450$_1$	0.02
(3)	Minus NADPH–cytochrome P-450 reductase	0.03
(4)	Minus NADPH	0.03

* The reaction mixture contained 3 μM cytochrome P-450$_1$, 1.2 units of NADPH–cytochrome P-450 reductase, Triton X-100 (0.05%, w/v), 1 mM NADPH, 0.1 M potassium phosphate buffer (pH 7.4), and 5 mM methylguanidine. The reaction mixture was incubated at 37° for 20 min.

potassium phosphate buffer (pH 7.4) containing 7 μM cytochrome P-450$_1$ caused a type I spectral change characterized by the appearance of a trough at 419 nm and an absorption peak at 388 nm (Fig. 3A). The dissociation constant (K_s) based on the difference spectra was calculated as 2.2 mM from a reciprocal plot of the absorbance changes at 419 nm relative to 404 nm (Fig. 3B).

N-Demethylation of methylguanidine in a reconstituted system containing purified cytochrome P-450$_1$. N-Demethylation of methylguanidine was studied in a reconstituted system containing cytochrome P-450$_1$ and purified NADPH–cytochrome P-450 reductase. The rate of methylguanidine N-demethylation was 0.65 nmole guanidine formed·min^{-1}·(nmole cytochrome P-450)$^{-1}$ (Table 5). The turnover number for cytochrome P-450$_1$ in the reconstituted system was two times larger than that per nmole of cytochrome P-450 in the original liver microsomes. Thus, the system seemed to be well reconstituted. From these observations, it was concluded that cytochrome P-450, NADPH–cytochrome P-450 reductase, and NADPH were required for the demethylation in the reconstituted system.

DISCUSSION

Methylguanidine, a uremia toxin, was stoichiometrically converted into equal amounts of guanidine and formaldehyde by aerobic incubation with phenobarbital-induced microsomes and NADPH. The formation of guanidine and formaldehyde required molecular oxygen and NADPH and followed Michaelis–Menten kinetics. Evidence for the involvement of cytochrome P-450 in N-demethylation of methylguanidine was obtained by the observations that the addition of methylguanidine to the cytochrome P-450$_1$ preparation caused a type I spectral change, which has been considered to be related to the formation of an enzyme–substrate complex [24], and that inhibitors of the cytochrome P-450-linked hydroxylase system, such as carbon monoxide and metyrapone, markedly decreased the demethylation rate. In addition, neither superoxide dismutase nor catalase had a significant effect on the rate of the demethylation, suggesting that neither superoxide nor hydrogen peroxide is directly involved in the demethylation reaction. Moreover, guanidine formation was observed in a reconstituted system containing cytochrome P-450$_1$ and purified NADPH–cytochrome P-450 reductase. Thus, we concluded that a cytochrome P-450-containing hepatic microsomal mixed function oxidase system catalyzed N-demethylation of methylguanidine to guanidine.

The oxidative metabolism of secondary N-methylamine by liver microsomes can be catalyzed by either or both of two mixed function oxidase systems: the cytochrome P-450-dependent oxidase and the FAD-containing monooxygenase [30, 31]. A possibility was that N-demethylation of methylguanidine, a secondary N-methylamine, may be catalyzed by the FAD-containing monooxygenase on the pathway for the metabolism of N-methylamines in liver microsomes, as proposed by Prough and Ziegler [31]. However, the rate of guanidine and formaldehyde formation was higher in the microsomes from phenobarbital-treated rabbits than in those from untreated rabbits, whereas FAD-containing monooxygenase activities are not induced by the treatment [32]. Moreover, methimazole, a non-formaldehyde-producing substrate specific for FAD-containing monooxygenase, could not suppress formaldehyde formation in the N-demethylation reaction by the microsomes. Thus, these observations suggest that a FAD-containing monooxygenase did not play a significant role in the N-demethylation of methylguanidine.

The metabolic pathways of guanidine have not been elucidated although enzymatic splitting of canavanine to guanidine and homoserine has been observed in liver homogenate [33]. From our observation *in vitro* that methylguanidine was converted into guanidine by cytochrome P-450-dependent mixed function oxidase system, guanidine in the organism may be derived, at least in part, from methylguanidine.

Cytochrome P-450-dependent mixed function oxidase system catalyzes the oxidative metabolism of xenobiotics and endogenous substances such as steroids [9]. In this paper we describe another functional aspect of the cytochrome P-450 system, which is that cytochrome P-450 may participate in the metabolism of "endogenous toxic substances" formed in pathological conditions such as uremia. However, whether or not methylguanidine N-demethylation is actually catalyzed *in vivo* by the cytochrome P-450 system remains to be elucidated. Further investigation regarding *in vivo* significance is required.

Acknowledgements—A part of this work was supported by research grants from the Ministry of Education, Science and Culture, and the Science and Technology Agency of Japan.

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