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S-methylation of 2-mercaptopyrazine in rat liver microsomes and cytosol

D. H. LEE and D. H. KIM*

* Bioanalysis and Biotransformation Research Center, Korea Institute of Science and Technology, PO Box 131, Chungryang, Seoul, Korea

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1. 2-(Allylthio)pyrazine (2-AP) has been demonstrated to protect the liver against toxicants by inhibiting CYP2E1 activity. Since 2-mercaptopyrazine (2-MP) is presumed to be a metabolite of 2-AP, the experiments were performed to determine whether rat liver microsomal and/or cytosolic preparations could catalyse the *S*-methylation of 2-MP.

2. It was found that both rat liver microsomes and cytosol could catalyse the *S*-methylation of 2-MP. The microsomal activity displayed biphasic substrate kinetics, with apparent $K_m = 8.44 \pm 2.68$ and 417 ± 74 μM for the high- and low-affinity activities respectively. The high-affinity activity had an apparent K_m for *S*-adenosyl-L-methionine (Ado-Met) of 3.52 μM. The cytosolic activity also displayed biphasic substrate kinetics, with apparent K_m of 3.26 ± 0.62 and 91.6 ± 23.1 μM for the high- and low-affinity activities respectively.

3. The microsomal *S*-methylation of 2-MP was inhibited by 2,3-dichloro-α-methylbenzylamine (DCMB), SKF-525A and benzylamine, known microsomal thiol methyltransferase (TMT) inhibitors, whereas cytosolic activity was inhibited by anisic acid and 3-chlorobenzoate, which also inhibit cytosolic thiopurine methyltransferase (TPMT). Both activities were inhibited by *S*-adenosyl-L-homocysteine (Met-Hcy).

4. These results suggest that both TMT and TPMT may be involved in the *in vivo* methylation of 2-MP.

Introduction

S-Adenosyl-L-methionine (Ado-Met)-dependent sulphur methylation plays a major role in the metabolism and elimination of aliphatic and aromatic sulphydryl drugs such as 6-mercaptopurine, captopril and disulphiram (Remy 1963, Drummer *et al*. 1982, Glauser *et al*. 1993). Thiol methylation in mammals is catalysed by two separate enzymes: cytosolic thiopurine methyltransferase (TPMT) (EC 2.1.1.67) and microsomal thiol methyltransferase (TMT) (EC 2.1.1.9). These two enzymes differ in substrate specificities and inhibitor sensitivities, and regulation. TPMT is selectively inhibited by benzoic acid derivatives and is involved in the methylation of aromatic and heterocyclic sulphydryl compounds (Remy 1963, Woodson and Weinshilboum 1983, Woodson *et al*. 1983). In contrast, TMT preferentially catalyses the *S*-methylation of aliphatic sulphydryl compounds, and is inhibited by 2,3-dichloro-α-methylbenzylamine (DCMB) and SKF-525A (Weinshilboum *et al*. 1979, Keith *et al*. 1984, Glauser *et al*. 1992).

2-(Allylthio)pyrazine (2-AP) has been reported to protect the liver against toxic agents (acetaminophen and carbon tetrachloride) by selectively inhibiting hepatic cytochrome P4502E1 (Kim *et al*. 1997) as well as by directly scavenging oxygen species (Kim *et al*. 1996). This compound also reduced vinyl carbamate- or vinyl carbamate epoxide-induced hepatotoxicity, mutagenicity and tumorigenicity (Surh *et al*. 1998). 2-AP is extensively metabolized and no parent compound was detected

* Author for correspondence; E-mail: dhkim@kist.re.kr

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in urine and bile (Han and Lee 1999). In the preliminary experiments, *S*-methyl-2-mercaptopyrazine was tentatively identified in the urine from the 2-AP treated rat, suggesting that 2-mercaptopyrazine (2-MP) is generated in the course of the metabolism of 2-AP. To understand the fate of 2-MP in the rat is of importance because it is pharmacologically active (Kang and Kim 1998). Accordingly, the purpose of the present investigation was to characterize the *S*-methylation of 2-MP, a metabolite intermediate generated from 2-AP, by microsomal TMT and cytosolic TPMT to evaluate further the potential role of TMT and TPMT in the *in vivo* methylation of 2-MP.

Materials and methods

Materials

2-MP with a chemical purity >98% was provided by Professor Nak Doo Kim, Seoul National University. [^{14}C-methyl]Ado-Met (spec. act. 59 μCi/μmol) was from New England Nuclear (Boston, MA, USA). SKF-525A and DCMB were from Research Biochemicals, Inc. (Natrick, MA, USA). Ado-Met, Ado-Hcy, benzylamine, 3,4-dimethoxybenzoate, *m*-anisic acid and 3-chlorobenzoate were from Sigma (St Louis, MO, USA). All other chemicals used were highest grade commercially available.

Preparation of subcellular fraction.

Male Sprague–Dawley rats (200–220 g) from Dae Han (Taejon, Korea) were used for the preparation of subcellular fractions. Immediately following perfusion of livers with phosphate-buffered saline, the livers were removed, washed twice and suspended in 0.1 M Tris-acetate (pH 7.4) containing 0.1 M KCl, 1 mM EDTA and 20 μM butylated hydroxytoluene to a final concentration of 30% (v/v). The mixture was homogenized (Ultra-turrax T25, IKA Lab) and centrifuged at 10000*g* for 30 min at 4 °C. The supernatant was re-centrifuged at 100000*g* for 60 min at 4 °C (CENTRIKON T-2180, Kontron, Milano, Italy) and the cytosolic fraction was recovered. The microsomal pellet was resuspended in 0.1 M potassium pyrophosphate (pH 7.4) containing 1 mM EDTA and 20% (w/v) glycerol and centrifuged again at 100 000*g* for 60 min at 4 °C. The resulting pellet was suspended in 10 mM Tris-acetate (pH 7.4) containing 1 mM EDTA and 20% (w/v) glycerol and aliquots were stored at –80 °C until use. Pooled microsomes and cytosol from 10 different rats were used for 2-MP methyltransferase assay.

2-MP methyltransferase assay

2-MP methyltransferase activity was assayed by measuring the conversion of 2-MP to radioactively labelled Me-MP (figure 1) with [^{14}C-methyl]Ado-Met as the methyl donor, as described by Glauser *et al.* (1993) with slight modification. In the microsomal assay, the reaction mixture consisted of 0.1 M potassium phosphate buffer (pH 7.5) containing 0.1 mg microsomal protein, 30 μM Ado-Met (9 μCi/μmol) and various concentrations of 2-MP in a final volume of 200 μl. Samples without 2-MP were used as blank for the assay. In the cytosolic assay, 0.1 mg cytosolic protein was added to the reaction mixture instead of microsomes. The mixtures were incubated for 30 min at 37 °C and the reaction was terminated by the addition of 200 μl 0.1 N HCl. The radioactive product was separated from [^{14}C-methyl]Ado-Met by solvent extraction with 2 vols toluene, and radioactivity was measured in a liquid scintillation counter (2000CA, Packard, Meriden, CT, USA).

Hplc analysis of methylated products

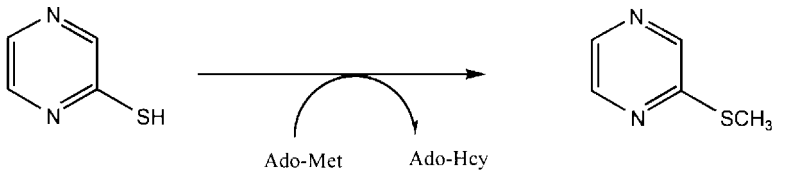
The residue extracted with toluene was analysed on a reverse-phase C_{18} hplc column (Ultrasphere ODS, 4.6× 250 mm, 5 μm; Beckman), using a Waters hplc system. The column was eluted at a flow rate of 1.0 ml/min with 10% methanol (v/v) in distilled water with a linear gradient to 70% methanol over 26 min. The eluent was monitored at 320 nm and radioactivity was monitored using a ß-RAM radioactivity flow detector (IN/US Corporation, Tampa, FL, USA) equipped with solid phase cell.

Kinetic analysis

Initially the kinetic data were analysed using the Eadie–Hofstee equation. Owing to the non-linear nature of 2-MP methylation, Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were calculated using a non-linear regression model derived from the Michaelis–Menten equation:

$$V_{total} = V_{max\,1}[S]/(K_{m1} + [S]) + V_{max\,2}[S]/(K_{m2} + [S]),$$

where V_{total} and $[S]$ are the rate of *S*-methylation and substrate concentration respectively.



2-Mercaptopyrazine (2-MP) 2-(Methylthio)pyrazine (Me-MP)

Figure 1. Scheme for 2-MP *S*-methylation. Ado-Met is *S*-adenosyl-L-methionine; Ado-Hcy is *S*-adenosyl-L-homocysteine.

Regression analyses were performed using WinNonlin (v.4.2; Scientific Consults, Lexington, KN, USA).

Results

Identification of methylation products

The product of the 2-MP methyltransferase reaction catalysed by rat liver microsomes was identified by hplc. Me-MP eluted at 19.3 min and the radioactive reaction product co-eluted at the same retention time (figure 2). The radioactive peak was fractionated and its structure was confirmed on the basis of mass spectral data (data not shown). Of the radioactivity applied to the column, 95% was recovered and 98% of the recovered radioactivity was associated with Me-MP.

S-methylation of 2-MP by rat liver microsomes

The effect of 2-MP concentration on hepatic microsomal 2-MP methyltransferase activity was investigated to determine kinetic profile of the rat enzyme (figure 3A). As shown in the Eadie–Hofstee plot (figure 3B), *S*-methylation of 2-MP in the presence of rat liver microsomes displayed biphasic kinetics at 2-MP concentrations from 2 to 2000 μM. Low and high K_m calculated by non-linear regression were 8.44 ± 2.68 and 417 ± 74 μM respectively with corresponding V_{max} = 0.175 ± 0.035 and 1.219 ± 0.048 nmol/min/mg protein. The effects of Ado-Met concentration on 2-MP methylation were determined in the presence of 5 μM 2-MP and an Eadie–Hofstee plot of the data is shown in figure 4. The apparent K_m for Ado-Met was 3.52 ± 0.42 μM. A similar K_m was calculated when the reaction was analyzed in the presence of 500 μM 2-MP.

S-methylation of 2-MP by rat liver cytosol

To determine whether *S*-methylation of 2-MP occurred in rat liver cytosol, cytosolic 2-MP methyltransferase activity was assayed with various concentrations of 2-MP (figure 5A) and an Eadie–Hofstee plot of the data is shown figure 5B. *S*-methylation of 2-MP in the presence of rat liver cytosol also displayed biphasic kinetics at 2-MP concentrations from 2 to 2000 μM. Low and high K_m calculated by non-linear regression were 3.26 ± 0.62 and 91.6 ± 23.1 μM respectively with corresponding V_{max} = 0.025 ± 0.002 and 0.056 ± 0.004 nmol/min/mg protein. The effects of Ado-Met concentration on 2-MP methylation were determined in the presence of 5 μM 2-MP and the apparent K_m for Ado-Met was 4.34 ± 0.63 μM, which is similar to that in microsomes. Cytosolic 2-MP methyltransferase activity was $\sim$ 9% compared with microsomal activity (table 1).

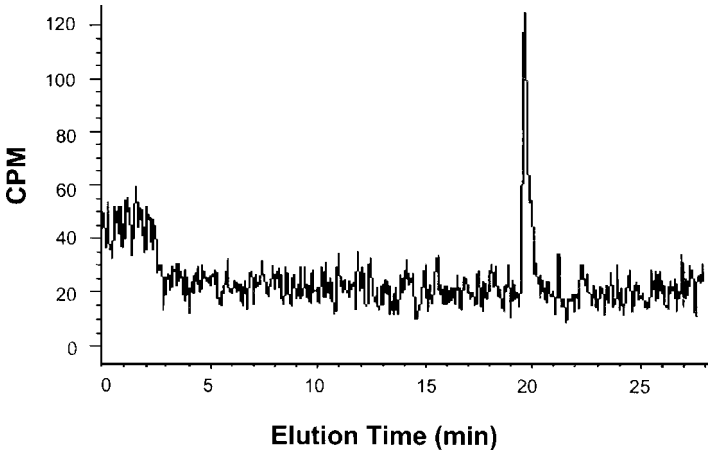


Figure 2. Hplc radio-chromatogram of the product of 2-MP *S*-methylation catalysed by rat liver microsomes.

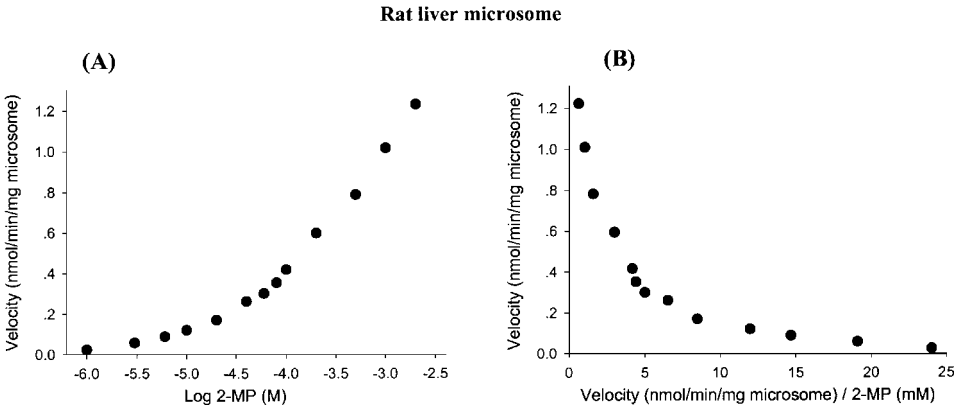


Figure 3. Rat liver microsomal 2-MP *S*-methylation. (A) Effect of 2-MP concentration on 2-MP *S*-methylation. (B) Eadie–Hofstee plot of the data shown in (A). Each point is the mean of at least three determinations.

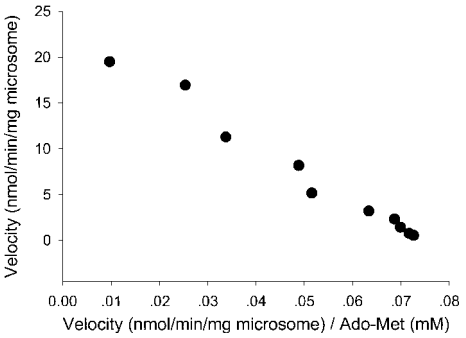


Figure 4. Eadie–Hofstee plot of rat liver microsomal 2-MP *S*-methylation. Each point is the mean of at least three determinations.

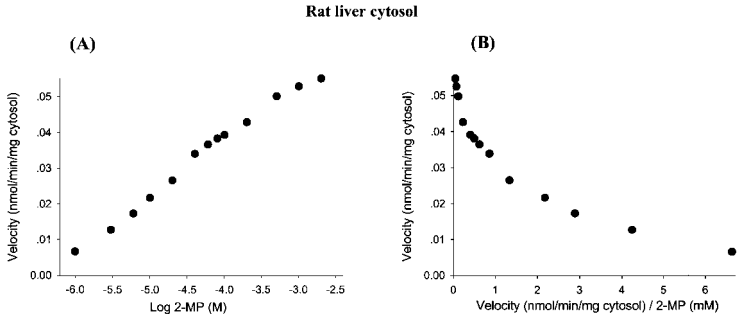


Figure 5. Rat liver cytosolic 2-MP *S*-methylation. (A) Effect of 2-MP concentration on 2-MP *S*-methylation. (B) Eadie–Hofstee plot of the data shown in (A). Each point is the mean of at least three determinations.

Table 1. Subcellular distribution of 2-MP *S*-methylation

Protein source (μg)	*n*	Specific activity (nmol/min/mg)	% Microsomal activity
Cytosol 100	8	0.039±0.001	9.3
Microsomes 100	8	0.417±0.015	100

Microsomal and cytosolic 2-MP *S*-methyltransferase activities were measured at a concentration of 100μM 2-MP

Table 2. Effects of various methyltransferase inhibitors on 2-MP *S*-methylation mediated by rat liver microsomes and cytosol.[a]

Inhibitors	Concentration (mM)	Microsomes		Cytosol	
		High-affinity	Low-affinity	High-affinity	Low-affinity
DCMB	1.0	21.7±2.2[b]	23.4±2.5	78.1±4.7	60.3±4.6
SKF-525A	0.5	63.3±6.2	56.2±18.8	96.3±2.8	102.6±10.8
benzylamine	1.0	48.5±7.8	73.7±13.9	86.8±2.1	95.0±0.6
aniline	1.0	45.1±6.5	95.2±7.1	96.1±3.3	90.4±6.0
3,4-dimethoxybenzoate	1.0	101.8±3.4	86.2±1.7	22.8±3.7	41.8±2.5
3-chlorobenzoate	1.0	87.3±3.0	95.7±9.2	11.4±3.8	23.8±8.3
m-anisic acid	1.0	95.2±10.6	88.1±6.5	22.5±3.9	37.1±5.5
Ado-Hey	0.1	22.5±7.4	33.8±8.0	8.3±7.8	16.4±1.8

Values are % basal activity[c].

[a] Microsomal high- and low-affinity 2-MP methyltransferase activities were measured at 5 and 500 μM 2-MP respectively; cytosolic activities were measured at 5 and 200 μM 2-MP respectively.

[b] Data are mean±SD of three determinations.

[c] The basal activities at a substrate concentration of 5 μM were 0.065 and 0.016 nmol/min/mg protein for microsomes and cytosol respectively.

Effects of methyltransferase inhibitors

DCMB, benzylamine and SKF-525A are effective inhibitors of microsomal TMT (Glauser *et al.* 1993) and benzoic acid derivatives and anisic acid preferentially inhibit cytosolic TPMT (Woodson *et al.* 1983). Ado-Hcy, a substrate analogue, completely blocks all Ado-Met-mediated *S*-methylation reactions (Borchardt 1977). The *S*-methylation of 2-MP was monitored in the presence of various inhibitors known to inhibit TMT and TPMT activities to evaluate the role of TMT and TPMT in this reaction and the results were summarized in table 2. Ado-Hcy, 0.1 mM, inhibited high- and low-affinity microsomal methylation activities of 2-MP by

77 and 66% respectively. Cytosolic 2-MP methylation activity was abolished by 92% in the presence of 0.1 mM Ado-Hcy. DCMB, SKF-525A, benzylamine and aniline inhibited 22–78% of the microsomal activity, whereas the cytosolic activity was only inhibited by DCMB to a lesser degree. 3,4-Dimethoxybenzoate, 3-chlorobenzoate and *m*-anisic acid selectively inhibited the cytosolic *S*-methylation of 2-MP. These compounds in the experiments did not affect the microsomal activity.

Discussion

2-AP is a pyrazine derivative of allylsulphide synthesized for use as a chemopreventive agent. This compound was an efficient and selective inhibitor of cytochrome P4502E1 and, hence, was effective in protecting against hepatic damage induced by chemical toxicants (Kim *et al.* 1997). 2-MP, a potential metabolic intermediate of 2-AP, is presumed to be pharmacologically active. The suppression of lipopolysaccharide (LPS)-induced expression of nitric oxide synthase and liver damage by 2-MP have been characterized (Kang and Kim 1998)

2-AP is known to be extensively metabolized in rat and *S*-methyl mercaptopyrazine has been tentatively identified in the urine from the 2-AP-treated rat (Han and Lee 1999). Therefore, the *in vitro S*-methylation of 2-MP was examined to understand the *in vivo* metabolism and enzymes involved in its metabolism. Hplc analysis confirmed that the radiolabelled product from the incubation of 2-MP with rat liver microsomes or cytosol in the presence of ^{14}C-Ado-Met was methyl 2-MP. Under the hplc conditions used, recovery of radioactivity was quantitative and no other significant metabolites were found. Therefore, rat liver microsomes and cytosol are capable of catalysing the conversion of 2-MP to *S*-methyl-2-MP *in vitro*. The subcellular distribution of 2-MP methyltransferase activity revealed that ~ 90% of the activity was found in the microsomes, suggesting that microsomal TMT preferentially catalyses the *S*-methylation of 2-MP. This results is somewhat unexpected because cytosolic TPMT is primarily involved in the *S*-methylation of aromatic and heterocyclic sulphydryl compounds in human (Remy 1963, Woodson *et al.* 1983) and there may be species difference in thiol methyltransferse enzymes.

The microsomal 2-MP methyltransferase activity displayed biphasic substrate kinetics. The phenomenon of biphasic substrate kinetics has been evaluated extensively with both human liver microsomes and human RBC membrane TMT activities (Weinshilboum *et al.* 1979, Keith *et al.* 1983, Glauser *et al.* 1992). This phenomenon was also demonstrated in the *S*-methylation of DDC by rat liver microsomes (Lill *et al.* 1996). The K_m for Ado-Met of high- and low-affinity microsomal 2-MP methyltransferase were similar, indicating that no differences exist in the affinity for Ado-Met of the two microsomal components of methyltransferase. The cytosolic 2-MP methyltransferase activity also displayed biphasic substrate kinetics with a methyl acceptor substrate but not with Ado-Met. This behaviour has not been described with other methyl acceptor substrates either in human liver cytosol or in rat liver cytosol, perhaps because high concentrations of substrates had not been tested.

Effects of various methyltransferase inhibitors of TMT and TPMT were assessed to determine the role of TMT and TPMT in the *S*-methylation of 2-MP. The *in vitro* methylation of diethyldithiocarbamate by human liver microsomes was inhibited by 100% using 1 mM DCMB (Glauser *et al.* 1993), whereas maximum

inhibition of methylation of diethyldithiocarbamate by rat liver microsomes was ~ 60% at the same concentration of DCMB (Lill *et al*. 1996). The present results revealed that DCMB did not completely inhibit both high- and low-affinity microsomal *S*-methylation of 2-MP, consistent with the degree of inhibition of DDC methylation by rat liver microsomal TMT (Lill *et al*. 1996). SKF-525A, a selective inhibitor of TMT, only marginally inhibited the cytosolic *S*-methylation of 2-MP, whereas it inhibited 60% of the high- and low-affinity microsomal *S*-methylation of 2-MP. The *S*-methylation of 2-MP by rat liver cytosol was very sensitive to benzoic acid derivatives in the present experiments. These results are consistent with benzoic acid derivative-induced inhibition of human and mouse TPMT (Woodson *et al*. 1983).

The results collectively demonstrate that 2-MP could be methylated by both rat liver microsomes and cytosol with a methyl donor, Ado-Met, and the *S*-methylation of 2-MP could be mediated by TMT and TPMT.

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