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PURIFICATION AND CHARACTERIZATION OF RAT LIVER MICROSOMAL THIOL METHYLTRANSFERASE

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Summary

Thiol methyltransferase (*S*-Adenosyl-L-methionine:thiol *S*-methyltransferase, EC 2.1.1.9), an enzyme which catalyzes the transfer of a methyl group from *S*-adenosylmethionine to the sulfhydryl group of a methyl acceptor, has been solubilized and partially purified from rat liver microsomes. The enzyme methylates xenobiotic sulfhydryl-containing compounds (e.g. β-mercaptoethanol), but not physiological sulfhydryl-containing compounds (e.g. homocysteine, cysteine, etc.). This thiol methyltransferase was inhibited by *N*-ethylmaleimide and *p*-chloromercuribenzoate, suggesting the existence of sulfhydryl groups on the enzyme essential for catalytic activity. The enzyme activity was also inhibited by *S*-adenosyl-L-homocysteine, a product of the enzymatic reaction. Using amino acid, base and sugar modified analogs of *S*-adenosyl-L-homocysteine, the specificity of this protein-ligand interaction was examined. It was observed that thiol methyltransferase exhibits a very high specificity for the structural features of *S*-adenosyl-L-homocysteine. Only analogs of *S*-adenosyl-L-homocysteine having minor base modifications exhibited inhibitory activity (e.g. S-tubercidinyl-L-homocysteine and *S*-3-deazaadenosyl-L-homocysteine). The specificity of the enzyme for the methyl donor, *S*-adenosyl-L-methionine, was also examined. Radiolabelled (*Me*-^{14}C) analogs of *S*-adenosyl-L-methionine were tested as substrates, whereas the unlabelled analogs were evaluated as inhibitors of this enzymatic transmethylation. Consistent with the results observed for the analogs of *S*-adenosyl-L-homocysteine, the enzyme exhibited

Abbreviations used are: SAC, *S*-adenosyl-L-cysteine; SAH, *S*-adenosyl-L-homocysteine; $SAHO_2$, *S*-adenosyl-L-homocysteine sulfone; SAHO, *S*-adenosyl-L-homocysteine sulfoxide; SAM, *S*-adenosyl-L-methionine; SAraAH, *S*-5′-[9-(arabinofuranosyl)adenyl]-L-homocysteine; SAmH, *S*-aristeromycinyl-L-homocysteine; SCH, *S*-cytidyl-L-homocysteine; SCM, *S*-cytidyl-L-methionine; SGH, *S*-guanosyl-L-homocysteine; SGM, *S*-guanosyl-L-methionine; SIH, *S*-inosyl-L-homocysteine; SIM, *S*-inosyl-L-methionine; STH, *S*-tubercidinyl-L-homocysteine; STM, *S*-tubercidinyl-L-methionine; SUH, *S*-uridyl-L-homocysteine; SUM, *S*-uridyl-L-methionine.

a high specificity for the structural features of *S*-adenosyl-L-methionine, both in its ability to donate a methyl group and in its ability to bind to the enzyme.

Introduction

Thiol methyltransferase (*S*-adenosyl-L-methionine : thiol *S*-methyltransferase, EC 2.1.1.9) catalyzes the transfer of a methyl group from *S*-adenosylmethionine to the sulfhydryl group on a methyl acceptor [1,2]. Both soluble [1] and microsomal [2] forms of this enzyme have been reported. Remy [1] has reported a thiol methyltransferase present in the soluble fraction of various tissues including liver, kidney, intestine and spleen which catalyzes the methylation of a variety of thiosubstituted purines and pyrimidines [1,3]. This soluble enzyme plays an integral part in the metabolism of thiopyrimidine type antithyroid drugs [3,4]. The microsomal form of thiol methyltransferase [2] is capable of methylating a variety of non-physiological sulfhydryl compounds (e.g. mercaptoethanol), whereas physiological sulfhydryl compounds (e.g. homocysteine, cysteine) are not substrates for this enzyme. This microsomal enzyme catalyzes the detoxification of many foreign substances which can be converted to, or which contain, a sulfhydryl group. For example, the S-methylation of dialkyldithiocarbamates [5,6] and metabolites of thiamine tetrahydrofuryl disulfide [7,8] in vivo have been attributed to this microsomal enzyme activity.

Considering the importance of thiol methyltransferases in the detoxification of xenobiotic sulfhydryl-containing compounds, we decided to further study the microsomal form of this enzyme. Since our laboratory has been interested in the design of inhibitors of *S*-adenosylmethionine-dependent methyltransferases [9—13], we were particularly interested in the inhibitory effects of analogs of *S*-adenosylhomocysteine on this enzyme. Therefore, we report here the solubilization and partial purification of rat liver microsomal thiol methyltransferase and the characterization of certain of its properties (e.g. substrate specificity, inhibitor specificity, etc.).

Materials and Methods

Chemicals. *S*-Adenosyl[*Me*-^{14}C]methionine (New England Nuclear, 55.0 Ci/mol) was diluted to 10 μCi/ml and stored at —21°C. *S*-Adenosylmethionine chloride (Sigma), which was purified by chromatography on Amberlite IRC-50 ion-exchange resin, was stored as a 0.01 M aqueous stock solution. The following compounds were obtained from the indicated sources: *S*-adenosyl-L-homocysteine, β-mercaptoethanol, *N*-ethylmaleimide, Sepharose 4B, Triton X-100 (Sigma). The following compounds were synthesized using published procedures: *S*-3′-deoxyadenosyl-L-[*Me*-^{14}C]methionine, *S*-aristeromycinyl-L-[*Me*-^{14}C]methionine, *S*-tubercidinyl-L-[*Me*-^{14}C]methionine, *S*-3-deazaadenosyl-L-[*Me*-^{14}C]methionine, *S*-N^6-methyl-3-deazaadenosyl-L-[*Me*-^{14}C]methionine, *S*-8-azaadenosyl-L-[*Me*-^{14}C]methionine [15]; the analogs of *S*-adenosyl-L-methionine listed in Table V [15]; and the analogs of *S*-adenosyl-L-homocysteine listed in Table V [9—13]. Protein concentrations were determined using the method of Lowry et al. [17].

Thiol methyltransferase assay. Thiol methyltransferase activity was determined using a modification of the radiochemical assay described earlier by Bremer and Greenberg [2]. A standard incubation mixture contained the following components added in this sequence: water, so that the final volume was 0.25 ml; 0.50 μmol β-mercaptoethanol, 0.05 μ-Ci of *S*-adenosyl[*Me*-^{14}C]methionine, 0.25 μmol *S*-adenosyl-L-methionine, 25 μmol phosphate buffer, pH 7.9, and the enzyme preparation. The reaction was started by addition of the enzyme and incubation was carried out at 37°C. The reaction was stopped by addition of 0.10 ml of 0.5 M borate buffer (pH 10.0) and the methylated product extracted with 10 ml of toluene/isoamyl alcohol (3 : 2, v/v). After centrifugation, an aliquot (5 ml) of the organic layer was transferred to a scintillation vial, a dioxane-based phosphor solution added and the radioactivity measured. The results were corrected with blank values obtained by carrying out the reaction without β-mercaptoethanol. In kinetic experiments, the concentrations of the β-mercaptoethanol or the methyl donor were varied, while the concentration of the other substrate was maintained at a constant saturating level. The kinetic constants (K_m and V) and the inhibition constants (K_i) were calculated according to the methods of Cleland [18] using a Hewlett-Packard 2100A digital computer.

Enzyme subcellular distribution studies. For studying the subcellular distribution of the enzyme activity, tissues were homogenized and then fractionated by differential and density gradient centrifugation. For example, fresh rat livers were homogenized in five volumes of ice-cold 0.25 M sucrose/0.01 M phosphate buffer (pH 7.0), using a glass homogenizer equipped with a loose fitting, motor driven Teflon pestle. The homogenate was filtered through two layers of gauze and the filtrate centrifuged at 900 $\times g$ and the resulting nuclear pellet washed once by resuspension in buffer. The supernatants were combined and centrifuged at 10 000 $\times g$ for 15 min which yielded the crude mitochondrial pellet. The mitochondrial pellet was washed with 0.32 M sucrose three times by resuspension and recentrifugation. The supernatant from the initial 10 000 $\times g$ centrifugation was combined with the first mitochondrial wash and then centrifuged at 100 000 $\times g$ for 1 h yielding the microsomal fraction. Subcellular fractionation of rat heart and brain was achieved using techniques similar to those described above for rat liver, except that hearts (free of aorta and excess fat) were homogenized in five volumes of 5 mM phosphate buffer, pH 7.4 (0.32 M sucrose) and brains were homogenized in 10 volumes of 1 mM phosphate buffer, pH 6.7 (0.32 M sucrose, 1 mM $MgCl_2$). Brain synaptosomes were purified from the mitochondrial fraction using a two-step discontinuous Ficoll-sucrose gradient [19,20] consisting of 20 ml layers of 14% (w/v) and 7.5% (w/v) Ficoll in 0.32 M sucrose. The gradient was centrifuged at 25 000 rev./min (Beckman SW 25.2 rotor) for 45 min and the synaptosomal fraction was obtained from the interface of the 7.5—14% Ficoll sucrose layer.

Enzyme purification. Thiol methyltransferase was solubilized and then purified from liver microsomal vesicles. Purification of the microsomal vesicles was achieved using a three-step discontinuous sucrose gradient consisting of 0.35, 1.4, and 2.0 M sucrose. The gradient was centrifuged at 25 000 rev./min (Beckman SW 25.2 rotor) for 30 min and the sharp band which formed just below the 0.35 and 1.4 M sucrose interface was removed, diluted with 0.25 M

sucrose/0.1 M Tris buffer, pH 7.4, and resedimented by centrifugation (MP2 fraction). This MP2 fraction exhibited the highest thiol methyltransferase activity on the gradient and was used for subsequent purification steps. Solubilization of this thiol methyltransferase activity was achieved by suspending the purified microsomal fraction (MP2) in 10 mM phosphate buffer, pH 7.0, with varying amounts of Triton X-100. The sample were incubated at 4°C for 30 min after which they were centrifuged at 100 000 × *g*. The protein concentration and enzyme activity in the supernatant were then determined. Further purification was attained by chromatography of the Triton X-100-solubilized material on a Sepharose 4B column (1.5 × 40 cm) eluting with 0.1 M phosphate buffer, pH 7.0, containing 0.3% Triton X-100.

Results

Subcellular distribution of thiol methyltransferase

The subcellular distribution of thiol methyltransferase in rat liver, brain and heart is shown in Table I. In each tissue the majority of the enzyme activity appears in the particulate fractions, with only minor amounts present in the soluble fraction. Consistent with the results of Bremer and Greenberg [2], and Fujita and Suzuoki [7], we observed that the microsomal fractions of the tissues examined possessed the highest activity, with the liver microsomal fraction having 30—40 times the activity present in the heart and brain microsomes. The microsomal thiol methyltransferase could not be solubilized by washing in saturated NaCl, suggesting that the enzyme is not ionically bound to the microsomal membrane. The microsomal enzyme activity could, however, be solubilized with non-ionic detergents (see results below). Rat brain synaptosomes prepared on a discontinuous Ficoll gradient exhibited only 5.3% of the total brain activity.

TABLE I

SUBCELLULAR DISTRIBUTION OF THIOL METHYLTRANSFERASE IN RAT LIVER, BRAIN AND HEART

Fractionation procedures are described in detail in Materials and Methods. Assay conditions: β-mercaptoethanol concentration, 2.0 mM; *S*-adenosyl-L-[*Me*-^{14}C]methionine (0.05 μCi) concentration, 1.0 mM; phosphate buffer, pH 7.9 (100 mM). For more details see Materials and Methods. Total enzyme activities expressed as percentage of total thiol methyltransferase activity recovered after differential centrifugation. Specific activities (in parentheses) are expressed as nmol of product per mg protein per min. Protein concentrations were determined by the method of Lowry et al. [17].

Subcellular fraction	Percent total enzyme activity (specific activity)		
	Liver	Heart	Brain
Nuclear	21 (0.15)	27 (0.006)	20 (0.008)
Mitochondrial	10 (0.13)	29 (0.018)	16 (0.005)
Microsomal	66 (0.82)	38 (0.019)	61 (0.03)
Soluble *	3 (0.008)	6 (0.0006)	3 (0.008)
Synaptosomes	—	—	5.3 (0.009)

* Soluble fraction refers to the supernatant from the 100 000 × *g* centrifugation.

Enzyme isolation and purification

Earlier we have reported [21] that rat liver microsomal thiol methyltransferase could be solubilized using Triton X-100. In the present study we have used this solubilization technique in the purification of this microsomal enzyme. Pure microsomal vesicles were obtained from the crude microsomal fraction using a two-step discontinuous sucrose gradient [21]. The thiol methyltransferase present in these purified rat liver microsomal vesicles was then solubilized with Triton X-100. Shown in Fig. 1 is a solubilization curve for the rat liver microsomal enzyme, where the percentage of solubilized enzyme activity is plotted as a function of Triton X-100 concentration present in the preincubation mixture. Complete solubilization of the liver microsomal enzyme was achieved by incubation at 4°C for 30 min with 0.3% Triton X-100. Using similar conditions the thiol methyltransferase activities present in purified rat heart and brain microsomes could also be solubilized.

Chromatography of the solubilized liver microsomal enzyme on Sephadex G-200 resulted in the activity eluting with the void volume indicating a molecular weight in excess of $2 \cdot 10^5$ for these solubilized fragments. Chromatography of the solubilized liver microsomal thiol methyltransferase on Sepharose-4B allowed for further purification of the enzyme activity (Table II). Electron micrographs of the thiol methyltransferase containing fractions obtained after Sepharose 4B chromatography showed uniform fragments with an average size of approx. 200 Å. The isolation steps outlined in Table II afforded a 21-fold purification of the liver microsomal thiol methyltransferase activity. This solubi-

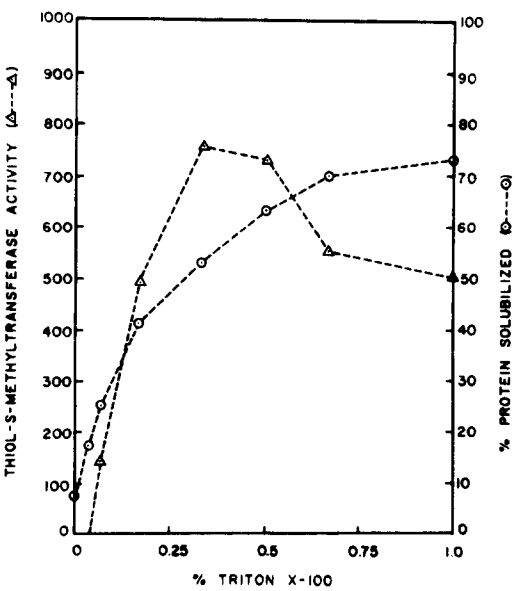


Fig. 1. Triton X-100 solubilization of rat liver microsomal thiol methyltransferase. Aliquots of 0.6 ml (4 mg protein) of the liver microsomal fractions (MP2) were treated with varied amounts of Triton X-100 at 4°C for 30 min and then centrifuged at 100 000 × *g* for 30 min. Enzyme activity expressed as nmol/ml per h (Δ———Δ), and protein content expressed as percent solubilized (⊙———⊙) were then determined in the supernatant fraction. The methods used to measure enzyme activity and protein content are described in detail in Materials and Methods.

TABLE II

PURIFICATION OF RAT LIVER MICROSOMAL THIOL METHYLTRANSFERASE

Fractionation procedures are described in detail in Materials and Methods. Protein concentrations were determined by the method of Lowry et al. [17]. Assay conditions are described in detail in Table I and Materials and Methods. Specific activity expressed as nmol of methylated product formed per mg of protein per min.

Fraction	Total protein (mg)	Specific activity (nmol/mg protein/min)	Purification	Recovery (%)
Homogenate	943	0.14	—	—
100 000 × *g* sediment	82	0.82	5.8	51
Sucrose gradient (MP2)	54	1.01	7.2	41
Supernatant fluid after solubilization with Triton X-100 *	32	1.44	10.3	35
Sepharose 4B chromatography **	15	2.94	21	33

* See Fig. 1 for details concerning the solubilization with Triton X-100.

** The supernatant obtained after solubilization of the MP2 fraction with 0.3% Triton X-100 was applied to a 1.5 × 40 cm column of Sepharose 4B. The enzyme was eluted with 0.01 M phosphate buffer, pH 7.0, which contained 0.3% Triton X-100 and 1.5-ml fractions were collected.

lized and partially purified enzyme preparation was used for subsequent studies.

Properties of microsomal thiol methyltransferase

Consistent with the results of Bremer and Greenberg [2], the solubilized liver microsomal thiol methyltransferase catalyzed the transfer of a methyl group from *S*-adenosylmethionine to non-physiological sulfhydryl-containing compounds (e.g. β-mercaptoethanol, mercaptoacetic acid, etc.), The solubilized liver microsomal enzyme exhibited optimal activity for the S-methylation of β-mercaptoethanol in the pH range of 7.8—9.0. Because of the unstable nature of *S*-adenosylmethionine at high pH values, subsequent kinetic experiments were carried out at pH 7.9. Bremer and Greenberg [2] and Fujita and Suzuoki [7] observed similar pH profiles for the enzyme activity in the intact microsomal vesicles. Addition of various monovalent (e.g. Na^+, K^+) and divalent cations (e.g. Mg^{2+}, Mn^{2+}) failed to stimulate the catalytic activity. Similarly, addition of EDTA didn't affect the activity of the solubilized thiol methyltransferase, further suggesting the lack of a metal requirement for catalysis. As shown in Table III, the solubilized microsomal thiol methyltransferase is inhibited by *N*-ethylmaleimide and *p*-chloromercuribenzoate. Fujita and Suzuoki [7] had previously reported that *p*-chloromercuribenzoate, but not *N*-ethylmaleimide, inhibited this enzyme activity. The experimental procedures used in their inactivation studies [7] would not have prevented the interaction of *p*-chloromercuribenzoate with the sulfhydryl-containing substrate rather than with the enzyme. Our data reported in Table III was obtained by first preincubating the enzyme and the sulfhydryl reagent, followed by dialysis or dilution to remove the unreacted sulfhydryl reagent, before assaying for residual enzyme activity. Under these conditions both *N*-ethylmaleimide and *p*-chloro-

TABLE III

EFFECTS OF *N*-ETHYLMALEIMIDE AND *p*-CHLOROMERCURIBENZOATE ON SOLUBILIZED MICROSOMAL THIOL METHYLTRANSFERASE

The liver microsomal thiol methyltransferase was solubilized and purified by Sepharose 4B chromatography as outlined in Table II. Preincubation mixtures consisted of the enzyme preparation, inhibitor (*N*-ethylmaleimide or *p*-chloromercuribenzoate) and phosphate buffer, pH 7.6. Incubations were carried out at 25°C for the indicated times after which the samples were dialyzed (0.02 M phosphate buffer, pH 7.6). Residual activity was determined using the assay outlined in Table I. The inhibition is expressed as percent inhibition of the S-methylation of β-mercaptoethanol using as controls samples which were preincubated and dialyzed under identical conditions.

Inhibitor	Concentration (mM)	Preincubation time (min)	Inhibition (%)
N-Ethylmaleimide	0.2	10	56
N-Ethylmaleimide	0.2	45	79
p-Chloromercuribenzoate	0.1	10	55
p-Chloromercuribenzoate	0.1	40	77

mercuribenzoate produced enzyme inactivation, suggesting the existence of free sulfhydryl groups on the enzyme which are required for catalytic activity.

Substrate specificity of thiol methyltransferase

Bremer and Goldberg [2] and Fujita and Suzuoki [7] have reported data on the methyl acceptor substrate specificity of the microsomal thiol methyltransferase. Our results were consistent with the earlier data [2,7] suggesting that the enzyme plays a role in the detoxification of xenobiotic sulfhydryl-containing compounds. The solubilized liver microsomal enzyme was unable to catalyze the methylation of physiological sulfhydryl-containing compounds such as

TABLE IV

SUBSTRATE KINETIC PROPERTIES FOR RAT LIVER MICROSOMAL THIOL METHYLTRANSFERASE

The liver microsomal thiol methyltransferase was solubilized with Triton X-100 and purified by Sepharose 4B chromatography as outlined in Table II.

Substrate	K_m ± S.E. (μM)	V ± S.E. (nmol product/mg protein per min)
β-Mercaptoethanol *	2369 ± 590	0.351 ± 0.069
S-Adenosyl-L-methionine **	105.8 ± 18	2.18 ± 0.177
S-Tubercidinyl-L-methionine **	154 ± 10	1.37 ± 0.05
S-3-Deazaadenosyl-L-methionine **	224 ± 46	1.44 ± 0.17
S-N^6-Methyladenosyl-L-methionine **	1017 ± 77	1.24 ± 0.07
S-8-Azaadenosyl-L-methionine **	719 ± 330	1.83 ± 0.70
S-3′-Deoxyadenosyl-L-methionine **	120.5 ± 3	0.053 ± 0.001
S-Aristeromycinyl-L-methionine **	60 ± 4.4	0.051 ± 0.001

* Assay conditions were similar to those outlined in Table I, except β-mercaptoethanol concentration was varied; *S*-adenosylmethionine concentration, 1.0 mM.

** β-Mercaptoethanol concentration, 2.0 mM; methyl donor concentration variable.

homocysteine, cysteine or glutathione. As shown in Table IV, the methyl acceptor substrate used in our studies, β-mercaptoethanol, had a K_m = 2.37 ± 0.59 mM. *S*-Adenosyl-L-methionine, the methyl donor for this reaction, exhibited a K_m = 106 ± 18 μM. Using various analogs of *S*-adenosyl-L-methionine synthesized earlier in our laboratory [15], we determined the structural features of this molecule which are needed to produce maximal methyl donor activity. The K_m and V values for those analogs exhibiting methyl donor activity are listed in Table IV.

Inhibitor specificity of thiol methyltransferase

A general characteristic of most *S*-adenosylmethionine-dependent methyltransferases is the inhibition produced by the demethylated product *S*-adenosyl-L-homocysteine [16]. Similarly, we have observed in this study that solubilized liver microsomal thiol methyltransferase is also sensitive to product inhibition by *S*-adenosyl-L-homocysteine. As shown in Fig. 2, when *S*-adenosyl-L-methionine is the variable substrate, *S*-adenosyl-L-homocysteine exhibits competitive inhibitory kinetics, K_i = 144 ± 12 μM. The competitive kinetics suggest that *S*-adenosyl-L-homocysteine is competing for the *S*-adenosyl-L-methionine binding site, a phenomenon consistent with other *S*-adenosyl-L-methionine-dependent methyltransferases [9—16].

In an effort to characterize the *S*-adenosyl-L-methionine binding site on thiol methyltransferase, we have evaluated a series of *S*-adenosyl-L-methionine and *S*-adenosyl-L-homocysteine analogs for their abilities to inhibit this transmethylation. The *S*-adenosyl-L-methionine and *S*-adenosyl-L-homocysteine analogs (Table V) had modifications in either the amino acid, base or sugar portions of these molecules. In preliminary experiments the effects of these synthetic analogs of *S*-adenosyl-L-methionine and *S*-adenosyl-L-homocysteine on the thiol methyltransferase-catalyzed methyl transfer from *S*-adenosyl[*Me*-^{14}C]-

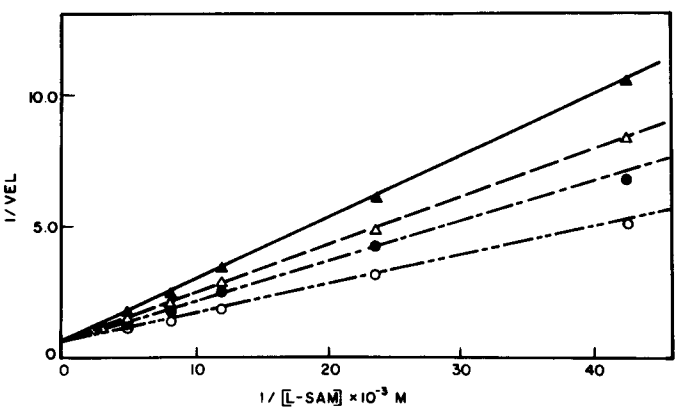


Fig. 2. *S*-Adenosyl-L-homocysteine (SAH) inhibition of thiol methyltransferase activity. Plots of reciprocal velocity vs. reciprocal *S*-adenosylmethionine (SAM) concentration in the presence of various concentrations of *S*-adenosyl-L-homocysteine: ○———○, [SAH] = 0; ●———●, [SAH] = 0.04 mM; △———△, [SAH] = 0.08 mM; ▲———▲, [SAH] = 0.16 mM; β-mercaptoethanol concentration, 2.0 mM. Vel = nmol of product/mg protein per min.

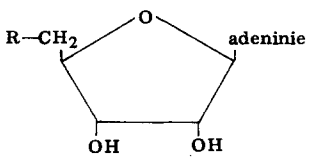
methionine to β-mercaptoethanol were determined. The results listed in Table V are expressed as the percent inhibition of the S-methylation of β-mercaptoethanol as compared to control experiments. In discussing their inhibitory activities, the analogs have been divided into three general classes: (a) amino acid-modified derivatives; (b) base-modified derivatives; and (c) sugar-modified derivatives.

TABLE V

INHIBITION OF RAT LIVER MICROSOMAL THIOL METHYLTRANSFERASE BY ANALOGS OF *S*-ADENOSYL-L-HOMOCYSTEINE AND *S*-ADENOSYL-L-METHIONINE

The liver microsomal thiol methyltransferase was solubilized with Triton X-100 and purified by Sepharose 4B chromatography as outlined in Table II. Assay conditions are similar to those outlined in Table I except inhibitors added at concentrations of 0.2 mM and 2.0 mM. Expressed as percent inhibition of the S-methylation of β-mercaptoethanol.

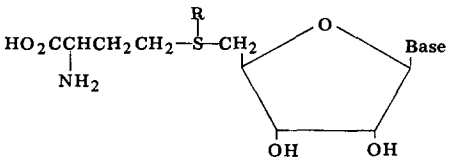
Amino acid modifications



Compound	R=	Inhibition (%)		Inhibition constants *
		I = 0.2 mM	*I* = 2.0 mM	K_{is} ± S.E. (μM)
L-SAH	—S—$CH_2CH_2CH(NH_2)CO_2H$(L)	9	61	144 ± 12
D-SAH	—S—$CH_2CH_2CH(NH_2)CO_2H$(D)	1	15	—
D-SAM	$-\overset{(+)}{\underset{\vert}{S}}(CH_3)-CH_2CH_2CH(NH_2)CO_2H$(D)	10	13	716 ± 127
L-SAC	—S—$CH_2CH(NH_2)CO_2H$	4	7	—
S-Methyl-L-SAC	$-\overset{(+)}{\underset{\vert}{S}}(CH_3)-CH_2CH(NH_2)CO_2H$	0	8	—
Decarboxylated SAH	—S—$CH_2CH_2CH_2NH_2$	3	12	—
Decarboxylated SAM	$-\overset{(+)}{\underset{\vert}{S}}(CH_3)-CH_2CH_2CH_2NH_2$	0	14	—
Deaminated SAH	—S—$CH_2CH_2CH_2CO_2H$	3	12	—
Deaminated SAM	$-\overset{(+)}{\underset{\vert}{S}}(CH_3)-CH_2CH_2CH_2CO_2H$	2	12	—
N^{α}-acetyl-L-SAH	—S—$CH_2CH_2CH(NHAc)CO_2H$	0	4	—
N^{α}-acetyl-L-SAM	$-\overset{(+)}{\underset{\vert}{S}}(CH_3)-CH_2CH_2CH(NHAc)CO_2H$	0	0	—
L-SAHO	$-S(=O)-CH_2CH_2CH(NH_2)CO_2H$	7	28	—
L-SAHO$_2$	$-S(=O)_2-CH_2CH_2CH(NH_2)CO_2H$	6	17	—

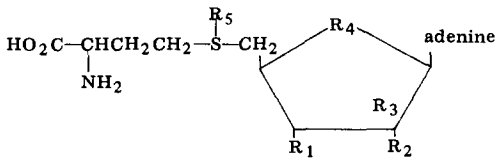
TABLE V (continued)

Base modifications



Compound	Base	R	Inhibition (%)		Inhibition constants
			I = 0.2 mM	I = 2.0 mM	K_{is} ± S.E. (μM)
SGH	guanine	—	7	16	—
SGM	guanine	CH_3	11	36	—
SIH	hypoxanthine	—	7	19	—
SIM	hypoxanthine	CH_3	4	17	—
SUH	uracil	—	8	15	—
SUM	uracil	CH_3	0	13	—
SCH	cytosine	—	10	15	—
SCM	cytosine	CH_3	4	9	—
STH	7-deazaadenine	—	36	87	59.7 ± 5.9
STM	7-deazaadenine	CH_3	25	75	94.5 ± 12.9
N^6-methyl-SAH	N^6-methyladenine	—	9	28	1274 ± 152
N^6-methyl-SAM	N^6-methyladenine	CH_3	1	38	1159 ± 173
N^6-dimethyl-SAH	N^6-dimethyl-SAH	—	3	25	—
N^6-dimethyl-SAM	N^6-dimethyl-SAM	CH_3	0	16	—
3-Deaza-SAH	3-deazaadenine	—	14	49	420.5 ± 51
3-Deaza-SAM	3-deazaadenine	CH_3	16	64	270 ± 23
8-Aza-SAH	8-azaadeine	—	5	39	2041 ± 262
8-Aza-SAM	8-azaadenine	CH_3	1	10	7461 ± 1865

Sugar modifications



Compound	R_1	R_2	R_3	R_4	R_5	Inhibition (%)		Inhibition constants
						I = 0.2 mM	I = 2.0 mM	K_{is} ± S.E. (μM)
2'-deoxy-SAH	OH	H	H	O	—	0	7	—
2'deoxy-SAM	OH	H	H	O	CH_3	15	54	382 ± 72.5
3'-deoxy-SAH	H	OH	H	O	—	8	32	—
3'deoxy-SAM	H	OH	H	O	CH_3	20	98	172 ± 16
SAraAH	OH	H	OH	O	—	0	31	942 ± 71
SAmH	OH	OH	H	CH_2	—	9	83	73 ± 9

* Each inhibitor exhibited linear competitive kinetics when *S*-adenosyl-L-methionine was the variable substrate.

As is evident from the data shown in Table V, microsomal thiol methyltransferase exhibits very high specificity for the structural features of the homocysteine portions of *S*-adenosyl-L-homocysteine and *S*-adenosyl-L-methionine. Because of the weak inhibitory activity of the specific structural analogs listed below in parentheses, we have concluded that the structural features of primary importance in the binding of *S*-adenosyl-L-homocysteine and *S*-adenosyl-L-methionine include: (1) the chirality of the amino acid asymmetric carbon (D-SAH and D-SAM); (2) the terminal amino group (deaminated SAH, deaminated SAM, N^{α}-acetyl-L-SAM); (3) the terminal carboxyl group (decarboxylated SAH and decarboxylated SAM); (4) the 3-carbon distance between the sulfur atom and the terminal amino and carbonyl groups (L-SAC and *S*-methyl-L-SAC); and (5) the sulfur atom (L-SAHO and L-$SAHO_2$).

Of the base-modified analogs, STH, STM, 3-deaza-SAH and 3-deaza-SAM were inhibitors of the solubilized microsomal thiol methyltransferase. These results suggest that minor changes in selected positions on the adenine ring of *S*-adenosyl-L-methionine or *S*-adenosyl-L-homocysteine can be tolerated in the enzymatic binding process. However, more drastic changes such as replacement of the adenine moiety with a pyrimidine base (e.g. SUH, SUM, SCH, SCM) or purine bases which lack a 6-amino group or possess a modified 6-amino group (e.g. SGH, SGM, SIH, SIM, N^6-methyl-SAH, N^6-methyl-SAM, N^6-dimethyl-SAH, N^6-dimethyl-SAM) result in loss of inhibitory activity. Thus the presence of a purine base containing a 6-amino group appears to be a minimum requirement for the binding of *S*-adenosyl-L-homocysteine or *S*-adenosyl-L-methionine to microsomal thiol methyltransferase.

The sugar-modified analogs of *S*-adenosyl-L-methionine and *S*-adenosyl-L-homocysteine exhibited interesting inhibitory activities toward this enzyme (Table V). 2′-Deoxy-SAH, 3′-deoxy-SAH and the arabinose derivative (SAra-AH) of *S*-adenosyl-L-homocysteine were rather poor inhibitors. In contrast, the corresponding 2′-deoxy-SAM and 3′-deoxy-SAM were quite potent inhibitors. These results would suggest that slight differences might exist in the binding requirements for *S*-adenosyl-L-methionine vs. *S*-adenosyl-L-homocysteine at this enzymatic site. The most potent inhibitor of the thiol methyltransferase was *S*-aristeromycinyl-L-homocysteine (SAmH).

For analogs showing inhibitory activity against thiol methyltransferase in the preliminary studies, kinetic experiments were performed and inhibition constants (K_i) were calculated (Table V). With each of the active *S*-adenosyl-L-methionine or *S*-adenosyl-L-homocysteine analogs studied, we observed competitive inhibitory kinetic patterns, similar to those observed for *S*-adenosyl-L-homocysteine. The most potent inhibitors of this enzyme were STH, STM, 3′-deoxy-SAM and SAmH.

Discussion

In 1961 Bremer and Greenberg [2] reported the existence of a microsomal thiol methyltransferase, which catalyzed the S-methylation of non-physiological sulfhydryl-containing compounds. Subsequently, the in vivo S-methylation of dialkyldithiocarbamates [5,6] and metabolites of thiamine tetrahydrofuryl disulfide [7,8] have been attributed to this microsomal enzyme.

In this study we have examined the subcellular distribution of this enzyme activity, observing that in addition to its localization in the microsomal fraction of liver, brain and heart homogenates, it also can be found in significant amounts in the mitochondrial and nuclear fractions. Using Triton X-100, we have solubilized the thiol methyltransferase from rat liver microsomes and purified this solubilized enzyme by chromatography on Sepharose 4B. This solubilized enzyme appears to be attached to high molecular weight membrane fragments.

The properties of the solubilized microsomal thiol methyltransferase reported here are similar to those reported earlier for the enzyme activity present in the intact microsomes [2,7]. The specificities of the enzymes for non-physiological sulfhydryl compounds and the pH optimums for the enzymatic reactions are identical. Being careful to avoid the possible interaction of the *N*-ethylmaleimide (or *p*-chloromercuribenzoate) with the sulfhydryl-containing substrate, we have demonstrated in this study that the enzyme itself appears to have a sulfhydryl group essential for catalytic activity. This observation is consistent in part with the result reported earlier by Fujita and Suzuoki [7] for the intact microsomal enzyme.

In this study, we have also attempted to map the *S*-adenosyl-L-methionine binding site on microsomal thiol methyltransferase by evaluating the substrate and/or inhibitor properties of analogs of *S*-adenosyl-L-methionine and *S*-adenosyl-L-homocysteine. The results obtained using *Me*-^{14}C-labelled analogs of *S*-adenosyl-L-methionine as potential substrates and unlabelled analogs of *S*-adenosyl-L-methionine and analogs of *S*-adenosyl-L-homocysteine as potential inhibitors are fairly consistent with each other. The results suggest that thiol methyltransferase exhibits high specificity for most of the structural features of *S*-adenosyl-L-homocysteine and *S*-adenosyl-L-methionine. For example, the enzyme is unable to tolerate any changes in the structural features of the amino acid portion of *S*-adenosylmethionine or *S*-adenosyl-L-homocysteine, since amino acid modified analogs exhibited little or no affinity for the enzymatic binding site. Only limited changes can be made in the base portion of *S*-adenosyl-L-methionine. For example, only *S*-tubercidinyl-L-[*Me*-^{14}C]methionine (STM) and *S*-3-deazaadenosyl-L-[*Me*-^{14}C]methionine (3-deaza-SAM) exhibited methyl donor activity of a magnitude comparable to *S*-adenosyl-L-[*Me*-^{14}C]methionine. *S*-N^6-Methyladenosyl-L-[*Me*-^{14}C]methionine and *S*-8-azaadenosyl-L-[*Me*-^{14}C]methionine were also methyl donors for the reaction with *V* similar to the natural donor, but with substantially higher Km values (Table IV). In general, comparable results were also obtained for the corresponding base modified analogs of *S*-adenosyl-L-methionine and *S*-adenosyl-L-homocysteine as inhibitors for this *S*-methyltransferase. Of the base modified analogs, STM and STH were found to be the most potent inhibitors. It should be noted, however, that STH and STM are general inhibitors of all methyltransferases examined to date [12,15,16,22], so that their effects on thiol methyltransferase are not unique.

The solubilized microsomal thiol methyltransferase appears able to tolerate certain changes in the sugar portion of *S*-adenosyl-methionine, since both *S*-3'-deoxyadenosyl[*Me*-^{14}C]methionine and *S*-aristeromycinyl-L-[*Me*-^{14}C] methionine exhibited methyl donor activity. Both of these analogs had K_m values similar to the natural methyl donor, but with substantially smaller *V* values (Table IV).

This observation would suggest that the 3′-hydroxyl and the 1′,5′-oxygen bridge of the ribose portion of *S*-adenosylmethionine are not crucial factors for producing maximum enzyme-ligand binding, but instead perhaps affect the orientation of the sulfonium center and thereby affect the methyl donor capability of the analogs. It is interesting to note that 2′-deoxy-SAM is a better inhibitor than 2′-deoxy-SAH and that 3′-deoxy-SAM is a better inhibitor than 3′-deoxy-SAH, suggesting that the 2′- and 3′-hydroxyl groups are more important in the binding of *S*-adenosyl-L-homocysteine than in the binding of *S*-adenosyl-L-methionine. This observation may also signify an increased importance of the sulfonium center in the binding of *S*-adenosyl-L-methionine, compared to the sulfur atom in the binding of *S*-adenosyl-L-homocysteine to this thiol methyltransferase.

From the results of these studies, it can be concluded that microsomal thiol methyltransferase exhibits very strict specificity for the structural features of *S*-adenosyl-L-methionine and *S*-adenosyl-L-homocysteine. Most modifications of the structure of *S*-adenosyl-L-homocysteine result in a decreased ability of the ligand to interact with the enzymatic binding site. Since other *S*-adenosylmethionine-dependent methyltransferases show less specificity for *S*-adenosyl-L-homocysteine [9,16] it may, therefore, be possible to design analogs of *S*-adenosyl-L-homocysteine as specific inhibitors of other methyltransferases, which don't inhibit thiol methyltransferase. This is a point worthy of consideration, because of the importance of this microsomal S-methylation in the detoxification of xenobiotic sulfhydryl-containing compounds and the undesirable consequences of inhibiting such a detoxification mechanism.

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