MOLECULAR PHARMACOLOGY, 22:752-759

Differences in the Active-Site Region of Tumor versus Normal Isozymes of Mammalian ATP:L-Methionine S-Adenosyltransferase

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Received March 15, 1982; Accepted May 28, 1982

SUMMARY

ATP:L-Methionine S-adenosyltransferase (MAT) isozymes, which catalyze the formation of the strategic biochemical intermediate, S-adenosyl-L-methionine, have been fractionated from rat liver and Novikoff solid hepatoma and have been studied to determine whether the tumor-derived MAT isozymes provide an exploitable target for cancer chemotherapy. Three rat liver MAT isozymes and two tumor MAT isozymes were obtained, and, under specifically defined assay conditions, seven amino acid analogues (L-norleucine, L-ethionine, seleno-D,L-methionine, seleno-D,L-ethionine, 6-diazo-5-oxo-L-norleucine, L-glutamic acid γ-methyl ester, and D-methionine) have been found to display differential inhibitory activities toward the tumor versus normal MAT isozymes. The inhibitory activities and substrate specificities of α-methyl-D,L-methionine, L-ethionine, S-n-propyl-D,L-homocysteine, seleno-D,L-methionine, seleno-D,L-ethionine, and D-methionine toward these five MAT isozymes have been measured. Significant differences in the substrate activities of these methionine analogues toward tumor versus normal MAT isozymes have been observed. α-Methyl-D,L-methionine can serve as a substrate for both tumor MAT isozymes but not for any of the rat liver isozymes; L-ethionine has significantly higher substrate activity toward the tumor isozymes as compared with the three rat liver isozymes; two of the three rat liver isozymes utilize seleno-D,L-methionine and seleno-D,L-ethionine more effectively as substrates than do the two tumor isozymes; S-n-propyl-D,L-homocysteine appears to be a selective substrate of one of the tumor isozymes; D-methionine has low but detectable levels of substrate activity toward the three rat liver isozymes, whereas it has significantly greater activity toward the tumor isozymes. The results of this study provide preliminary evidence for the existence of exploitable topographic and electronic differences within the active site of tumor versus normal MAT isozymes. These differences appear to involve the specific region which accommodates the sulfur (or hetero) atom and the S-methyl (or S-alkyl) substituent of L-methionine (or substrate analogue).

INTRODUCTION

S-Adenosyl-L-methionine, the principal biological methyl donor, as well as the precursor of aminopropyl groups utilized in polyamine biosynthesis, is a participant in numerous biochemical reactions essential to normal cellular growth and function. In particular, methylation of rRNA is essential to regulation of ribosome production (1); methylation of mRNA plays a critical role in initiation of protein synthesis (2); and methylation of tRNA causes conformational changes in this macromolecule critical to its cellular functioning (3). It has been reported that aberrant methylation of tRNA is associated both with neoplasia (4) and with the transformation of cells by oncogenic viruses (5). In addition, methylation by Ado-Met[1] is essential to the modification, processing, and function of DNA (6). The diverse strategic role of Ado-Met in cellular processes has become more widely appreciated in recent years (7) and has increasingly supported the rationale that selective inhibitors of the enzymatic

This work was supported in part by American Cancer Society Grant CH-141 and the Audrey B. Jones Fund for Cancer Research.

[1] The abbreviations used are: Ado-Met, S-adenosyl-L-methionine; MAT and methionine adenosyltransferase, ATP:L-methionine S-adenosyltransferase (EC 2.5.1.6); rat liver iso-1, rat liver iso-2, and rat liver iso-3, rat liver methionine adenosyltransferase isozymes; tumor iso-1 and tumor iso-2, Novikoff solid hepatoma methionine adenosyltransferase isozymes; cycloleucine, 1-aminocyclopentane-1-carboxylic acid.

0026-895X/82/060752-08$02.00/0

synthesis of Ado-Met might have pharmacological utility, as proposed and studied previously by Talalay and co-workers (8–13). The attempted study of tumor-derived methionine adenosyltransferases as a selective target for cytotoxicity has been hampered over the years by the observation of relatively low levels of the enzyme in malignant tissues (14). Liau *et al.* (15) were the first to characterize a tumor-derived MAT isozyme, isolated from Novikoff ascites hepatoma. These same workers were also the first to observe that rat liver MAT exists in multiple forms (15). Subsequently, fractionation of rat liver into three MAT isozymes has been reported by Hoffman and Kunz (16), and fractionation of rat Novikoff solid hepatoma MAT into two isozymes has been reported by Sufrin and Lombardini (17). Liau *et al.* (15, 18–20) have accumulated evidence that tumor-derived MATs are altered enzyme forms which are also associated with human malignant tumors.

The present study was undertaken to determine whether the differences between tumor and normal MAT isozymes include exploitable topographic or electronic differences in the active site. With an approach that was utilized in previous studies by Talalay and co-workers (8–10, 12, 13), a number of amino acids whose kinetic behavior indicates that they are competitive analogues of L-methionine have been examined for their inhibitory and substrate properties toward tumor and normal rat liver MAT isozymes. This study was intended to be a means of detecting possible differences in the topographic features of the active-site region.

EXPERIMENTAL PROCEDURE

Materials

L-[^{14}C-*methyl*]methionine and L-[^{35}S]methionine were purchased from Amersham/Searle (Arlington Heights, Ill.). Aquasol and adenosine 5′-triphosphate, tetrasodium salt (8-^{14}C) were obtained from New England Nuclear Corporation (Boston, Mass.). Cycloleucine (I), L-methionine, seleno-D,L-methionine (IV), seleno-DL-ethionine (V), 6-diazo-5-oxo-L-norleucine (VIII), and L-glutamic acid γ-methyl ester (IX) were purchased from Sigma Chemical Company (St. Louis, Mo.). D-Methionine (X), L-norleucine (VII), and L-ethionine (III) were obtained from Tridom Chemical, Inc. (Hauppague, N. Y.) as Fluka (puriss) grade. α-Methyl,D,L-methionine (II) (Vega Biochemicals, Tucson, Ariz.) was purified by countercurrent distribution using butanol/acetic acid/water (4:1:5) and then recrystallized from ethanol/water (1:3). *S-n*-Propyl-D,L-homocysteine was the gift of Dr. Paul Talalay, The Johns Hopkins University School of Medicine (Baltimore, Md.).

Livers of female (Wistar strain) rats were used as the source of normal MAT isozymes. Novikoff solid hepatoma cells were the source of tumor MAT isozymes and were obtained by harvesting cells from female rats (Wistar strain) which had been inoculated 7 days previously with Novikoff hepatoma cells (obtained from Dr. Douglas Stocco, Texas Tech University Health Sciences Center).

Methods

Fractionation of methionine adenosyltransferase isozymes from Novikoff solid hepatoma and rat liver. Two MAT isozymes were fractionated from Novikoff solid hepatoma cells with the use of hydrophobic chromatography on phenyl-Sepharose according to the procedures of Kunz *et al.* (21). These isozymes were designated tumor iso-1 and tumor iso-2 according to order of chromatographic elution. Three MAT isozymes were fractionated from normal rat liver as described by Hoffman and Kunz (16), using a modification of their initially reported procedure (21). The three rat liver isozymes were designated rat liver iso-1, rat liver iso-2, and rat liver iso-3 according to order of elution during phenyl-Sepharose chromatography. A more detailed description of our separation procedures for rat liver and Novikoff solid hepatoma MAT isozymes is presented elsewhere (22).

Enzymatic assays and determination of inhibitory potency. MAT activity was determined according to the procedure of Chou and Lombardini (23). Assay conditions were optimized to give linear product formation with respect to both time and enzyme protein. Assays were carried out in a final volume of 0.1 ml containing the following components in micromoles: Tris-HCl (pH 8.0), 10; KCl, 30; $MgCl_2$, 2.4; dithiothreitol, 0.4; and ATP, 0.5. The 0.1-ml assay mixture also contained a subsaturating level of [^{35}S] or [^{14}C-*methyl*]L-methionine (at least 50% below that of the K_m value whose micromolar amount is specified in Table 1 for each enzyme preparation), and isoenzyme preparation, 0.4–1 μg. K_m (L-methionine) values for each isozyme were determined; those for the three rat liver isozymes are in good agreement with the values reported by Hoffman and Kunz (16). The K_m value for rat liver iso-3 is approximate, since nonlinear kinetics was observed, precluding accurate determination of this parameter. The recorded inhibitory potency of an amino acid analogue represents the concentration which inhibits the activity of the specific MAT isozyme by 50% (I_{50} value) under the specified assay conditions. The I_{50} values were calculated from Dixon plots (24), using least-squares regression analysis. The use of I_{50} as opposed to K_i values to describe inhibitory potencies is based on the fact that rat liver iso-3, the major liver isozyme (16), displays deviations from Michaelis-Menten kinetics that do not enable accurate determination of K_m (L-methionine) or subsequent K_i values.

Determination of substrate activity. The assays were performed using [^{14}C]ATP (0.2 mM) in the system described above. Substrate analogues were tested at a concentration of 10 mM for D- or L-amino acids and 20 mM for DL-amino acids. Reaction velocities were compared with that observed when 10 mM L-methionine was used as substrate.

RESULTS

Determination of inhibitory activities. The inhibitory potencies of nine amino acid analogues are presented in Table 1. Cycloleucine (I), an antitumor agent first observed by Lombardini *et al.* (8) to be a potent inhibitor of isofunctional MATs, was used as a standard for comparison of inhibition of other amino acid analogues. α-Methyl-D,L-methionine (II), L-ethionine (III), seleno-

TABLE 1

Inhibitory potencies of amino acid analogues on ATP:L-methionine adenosyltransferase isozymes of rat liver and Novikoff solid hepatoma

Data were calculated from Dixon plots (24) and are expressed as the concentration of analogue needed to inhibit the MAT reaction by 50% (I_{50}). The enzyme activity of each isozyme was measured at an L-methionine concentration chosen to be at least 50% below its K_m (L-methionine) value and according to described procedures (23).

	Rat liver			Tumor	
	Iso-1	Iso-2	Iso-3	Iso-1	Iso-2
K_m (L-methionine) (μM)	34.5 ± 4.4	11.1 ± 3.3	~93 ± 5	11.0 ± 1.6	5.78 ± 0.24
L-methionine assay concentration (μM)	5	1	37.5	1	1
Compound	Concentration required for 50% inhibition				
			mM		
I. Cycloleucine[a]	0.29 ± 0.02	0.18 ± 0.01	2.50 ± 0.14[b]	0.19 ± 0.03	0.14 ± 0.01
II. α-Methyl-D,L-methionine	68.7 ± 0.7	52.5 ± 5.3	*I*[c]	32.3 ± 7.2	30.9 ± 3.1
III. L-Ethionine	1.64 ± 0.27	0.79 ± 0.17	15.4 ± 3.2	0.21 ± 0.02	0.25 ± 0.01
IV. Seleno-D,L-methionine	0.12 ± 0.04	0.087 ± 0.004	0.24 ± 0.04	0.0060 ± 0.0006	0.0059 ± 0.0003
V. Seleno-D,L-ethionine	4.28 ± 0.59	1.98 ± 0.34	11.1 ± 1.9	0.22 ± 0.02	0.21 ± 0.01
VI. *S-n*-Propyl-D,L-homocysteine	68 ± 6[d]	56 ± 3[d]	113[d]	12.1[d]	41.4[d]
VII. L-Norleucine	1.9	1.0	20.2	4.2	5.0
VIII. 6-Diazo-5-oxo-L-norleucine	4.0	2.4	1.0	10.5	10.6
IX. L-Glutamic acid γ-methyl ester	1.25 ± 0.08	0.9 ± 0.36	9.90 ± 2.20	2.54 ± 0.18	2.43 ± 0.11
X. D-Methionine	8.1	7.9	*I*	2.6	1.9

[a] The number of experiments utilizing cycloleucine in the three rat liver isozymes was 19; cycloleucine was tested in 10 experiments in the two tumor isozymes. Data are presented as means ± standard error of the mean for all other compounds tested in two or three separate experiments.

[b] Hoffman and Sullivan (25) have reported *in vitro* activation, rather than inhibition, of rat liver iso-3 by cycloleucine. This apparent discrepancy between our *in vitro* observations and theirs most likely reflects differences in handling, storage, and assay conditions for this isozyme.

[c] The designation *I* (for inactive) indicates that at a maximal concentration of 120 mM less than 10% inhibition was observed.

[d] These values were determined by extrapolation and are not bracketed by experimental observations.

D,L-methionine (IV), seleno-D,L-ethionine (V), and *S-n*-propyl-D,L-homocysteine (VI) comprise a group of L-methionine analogues for which inhibitory activity was measured in order to complement and compare the corresponding determinations of their substrate activities, as given in Table 2. L-Norleucine (VII), 6-diazo-5-oxo-L-norleucine (VIII), and L-glutamic acid γ-methyl ester (IX) (as well as IV, V, and X) are amino acid analogues for which detectable (at least 2-fold) differences in inhibitory activity between tumor MAT isozymes and normal MAT isozymes were observed.

The observed I_{50} values for Compounds I–X have been examined to determine whether significant differences exist in the inhibitory properties of any of these molecules toward tumor MAT isozymes versus normal rat liver MAT isozymes. Such differential susceptibilities to inhibition might provide evidence for exploitable differences between the active-site regions of tumor isozymes and those of normal isozymes. A comparison of I_{50} values has been made between the two low-K_m (L-methionine) rat liver isozymes (rat liver iso-1 and rat liver iso-2) and the two tumor isozymes (tumor iso-1 and tumor iso-2), because the small differences in K_m (L-methionine) values among these four isozymes initially suggested a possible correspondence of the normal isozymes with those derived from tumor. Of particular importance from a chemotherapeutic viewpoint is the comparison between rat liver iso-2 and the two tumor isozymes, since it has been established that rat liver iso-2 is the predominant form of methionine adenosyltransferase in non-liver tissues (16). Excluded from this specific comparison is rat liver iso-3, a MAT isozyme that is virtually unique to liver and which has properties that distinguish it from the remaining liver isozymes. These features include its high apparent K_m (L-methionine) value and nonlinear kinetic behavior (16), its *in vitro* activation by dimethyl sulfoxide (26), and its sulfhydryl requirement for activity (18). In fact, the high I_{50} values of compounds recorded for rat liver iso-3 appear to be a general reflection of the lower binding affinity (and high apparent K_m) of this specific isozyme. An exception is the inhibitory activity of Compound VIII, 6-diazo-5-oxo-L-norleucine, which is significantly greater toward rat liver iso-3 than toward the remaining four isozymes. This result is of some interest, since Compound VIII has been well studied by others (27) and has a wide range of pharmacological effects.

Differences in the susceptibility of tumor iso-1 and iso-2 versus rat liver iso-1 and iso-2 to inhibition by Compounds I–IX can be classified as follows: (a) no significant differences between tumor isozymes and normal isozymes—Compounds I and II; (b) small but detectable (2-fold) differences between tumor isozymes and normal isozymes—Compounds VI (tumor iso-1 versus rat liver iso-1 and iso-2), VII, IX, and X; (c) significant differences between tumor isozymes and normal isozymes—Compounds III (Tumor iso-1 and iso-2 versus rat liver iso-1),

TABLE 2

Analogues of methionine as substrates of ATP:L-methionine adenosyltransferase isozymes of rat liver and Novikoff solid hepatoma

The assays were performed using [^{14}C]ATP (0.2 mM) in the system described under Methods. Results are given as a percentage of activity compared with that obtained when 10 mM L-methionine was used as substrate. D-Methionine and L-methionine were also tested at lower concentrations and their activities were compared. The quantity of *S*-adenosyl-L-methionine formed for each of the rat liver and tumor isozymes in a 30-min control incubation with L-methionine (10 mM) as substrate was approximately 1.36 nmoles. The amounts of isozyme protein utilized in the control incubations were as follows: rat liver iso-1 and iso-2, 4 μg; rat liver iso-3, 2 μg; tumor iso-1 and iso-2, 3 μg.

Compound	Concentration	Relative substrate activity				
		Rat liver			Tumor	
		Iso-1	Iso-2	Iso-3	Iso-1	Iso-2
	mM			%		
L-Methionine	10	100	100	100	100	100
II. α-Methyl-D,L-methionine	20	0	0	0	27.7 ± 2.5	8.7 ± 0.3
III. L-Ethionine	10	32.5 ± 4.3[a]	40.0 ± 1.2	15.0 ± 2.0	81.1 ± 3.4	61.3 ± 14.9
IV. Seleno-D,L-methionine	20	170 ± 11	104.6 ± 2.6	18.9 ± 1.7	74.3 ± 11.5	76.8 ± 5.7
V. Seleno-D,L-ethionine	20	72.3 ± 3.8	61.0 ± 1.7	8.9 ± 2.2	29.7 ± 6.6	34.9 ± 0.7
VI. *S*-*n*-Propyl-D,L-homocysteine	20	1.5 ± 0.9	1.3 ± 1.0	1.0 ± 0.6	0	10.4 ± 3.6
X. D-Methionine	10	10.3 ± 3.7	12.2 ± 3.9	2.5 ± 1.4	56	28.4 ± 6.7
	1	6.2	3.5	5.0	—	18.2
	0.1	6.1	3.4	7.5	26	—

[a] Recorded values, except those for D-methionine at 1 mM and 0.1 mM concentrations, are the means of three independent determinations ± standard error of the mean.

IV, and V. The most significant differences are found for Compounds IV and V—the I_{50} values of Compounds IV and V are 20-fold higher for rat liver iso-1 and 10-fold higher for rat liver iso-2 than for both tumor isozymes.

Taken as a whole, this comparison of I_{50} values between tumor MAT isozymes and normal MAT isozymes strongly suggests that the observed differences reflect alterations in the active-site regions of the tumor isozymes. The greatest differences were detected with the seleno analogues, Compounds IV and V. Furthermore, because of the generally high stereospecificity of MAT for the L-isomer of methionine, it is assumed that the observed I_{50} values of Compounds II, IV, V, and VI, all of which are racemic amino acids, are 2 times higher than that which would be observed for the L-isomer alone. When this is taken into account, the apparent I_{50} value of (L)-IV toward tumor iso-1 and tumor iso-2 is <0.003 mM. This indication that seleno-L-methionine is an extremely potent competitive inhibitor of L-methionine, with apparent differential effects toward the tumor MAT isozymes, provides a significant selective difference that might be exploited chemotherapeutically.

Compounds II, III, IV, V, VI, and X differ from Compounds I, VII, VIII, and IX in an important respect: the former are theoretically capable of substrate activity, whereas the latter are not. Thus, the inhibitory potencies of Compounds II–VI and X are simply a measure of their ability to compete with the natural substrate L-methionine for binding to the active site, and do not address the question of whether their binding results in substrate or inhibitory activity. In fact, examination of the substrate activities of Compounds II–VI and X has provided an additional means with which to probe the active sites of tumor versus normal MAT isozymes.

Substrate specificities of rat liver and Novikoff solid hepatoma methionine adenosyltransferase isozymes. Earlier studies of the substrate specificities of α-methylmethionine, L-ethionine, seleno-D,L-methionine, and other L-methionine analogues for rat liver methionine adenosyltransferase have been made (8, 28–30). However, when these observations were recorded, it was not appreciated that rat liver has multiple forms of MAT and that, in fact, many of these *in vitro* observations were obtained with enzyme preparations of unknown isozymic composition. On this basis, some inconsistencies in these earlier observations can now be better understood. For example, Lombardini *et al.* (8) were unable to substantiate Stekol's observation (29) that *S*-trifluoromethyl-L-homocysteine can serve as a substrate of rat liver MAT, suggesting that, if reexamined, this methionine analogue might have significantly different substrate specificities toward the three isozymes.

Table 2 presents the substrate properties of the following analogues of L-methionine: α-methyl-D,L-methionine (II), L-ethionine (III), seleno-D,L-methionine (IV), seleno-D,L-ethionine (V), *S*-*n*-propyl-D,L-homocysteine (VI), and D-methionine (X). Several significant observations have emerged from this study of substrate analogues and are listed as follows:

1. α-Methylmethionine is devoid of substrate activity toward normal rat liver MAT isozymes, but can serve as a substrate for both tumor MAT isozymes. To our knowledge, this is the first observation of substrate activity for α-methylmethionine toward mammalian MAT, although Schlenk *et al.* (31) have observed similar substrate capabilities of Compound II in yeast systems.

2. L-Ethionine has significantly higher substrate activity toward tumor MAT isozymes as compared with normal rat liver MAT isozymes.

3. Seleno-D,L-methionine and seleno-D,L-ethionine display significantly higher substrate activity toward rat liver iso-1 and iso-2 as compared with tumor iso-1 and tumor iso-2.

4. *S*-*n*-Propyl-D,L-homocysteine, although virtually

devoid of substrate activity toward the three rat liver MAT isozymes as well as tumor iso-1, has detectable levels of substrate activity toward tumor iso-2.

5. A comparison of the three rat liver isozymes shows that L-ethionine, seleno-D,L-methionine, and seleno,D,L-ethionine have significantly greater substrate capabilities toward rat liver iso-1 and rat liver iso-2 than toward rat liver iso-3. In other words, rat liver iso-3, which has a high apparent K_m (L-methionine) value, indicating a low binding affinity for the natural substrate (L-methionine), nevertheless under these *in vitro* conditions displays relatively higher substrate specificity for L-methionine than do the two corresponding low-K_m rat liver isozymes. This observation might have implications with regard to the specific roles that each of these rat liver MAT isozymes play in the metabolic functions of this organ. It should be noted that rat liver iso-3 is not only the major MAT rat liver isozyme, but is found almost exclusively in liver (16).

6. A comparison of rat liver iso-2 and the two tumor isozymes indicates substantial differences in the substrate activities of α-methyl-D,L-methionine, L-ethionine, seleno-D,L-methionine, and seleno-D,L-ethionine. This comparison is of particular importance, since it clearly demonstrates that rat liver iso-2, the major form of methionine adenosyltransferase in non-liver tissues, differs from the tumor-derived forms in a manner that might be exploited chemotherapeutically.

7. The substrate specificity of D-methionine was examined to determine whether tumor MAT isozymes maintain the strict stereospecificity for the L-enantiomer of methionine generally displayed by isofunctional MAT enzymes. The yeast enzymes which have been found to convert D-methionine to *S*-adenosyl-D-methionine are an exception (31). Pan and Tarver (28) originally reported D-methionine to be a substrate of rat liver MAT with a relative activity of 13%, but this result was based on the premise that their rat liver MAT was a homogeneous preparation with a K_m (L-methionine) of 0.91 mM. The presence of undetected low-K_m (L-methionine) MAT isozymes in their preparation as well as the possible undetected contamination of their sample of D-methionine (obtained by optical resolution of *N*-acetyl-D,L-methionine using acylase-1) with as little as 0.1% L-methionine, and assayed at a 10 mM concentration, alternatively could account for the observed substrate activity. In an attempt to resolve this ambiguity we have retested the substrate activities of D-methionine at 10 mM, 1 mM, and 0.1 mM concentrations and in every instance have observed detectable levels of substrate activity toward all tumor and rat liver MAT isozymes. It also appears that, under these *in vitro* conditions, D-methionine has greater substrate activity toward the tumor isozymes than toward the rat liver isozymes.

DISCUSSION

A study of the substrate activities of the methionine analogues II–VI and X has revealed significant differences in their behavior toward the tumor versus normal MAT isozymes. We propose that these altered substrate specificities, as well as observed differences in inhibitory activity, in turn reflect differences in both the topographic and electronic features of specific regions of the active site.

Topographic differences between the active site of tumor versus normal methionine adenosyltransferase isozymes. The binding of L-methionine to the enzyme surface has been described (12) as a two-step process in which the first step is molecular recognition of the properly oriented amino and carboxyl groups by the enzyme through long-range coulombic forces which initiate the interaction. Subsequently, van der Waals and hydrophobic forces become effective at shorter range, and complete the binding event. The sulfur atom of L-methionine as well as its *S*-methyl substituent need not be present for binding of an amino acid to occur, as illustrated, for example, by the inhibitory activity of Compounds I and VII. However, the presence of a sulfur atom and its alkyl substituent are stringent requirements for substrate activity. It is apparent that there is an accessory binding site for the sulfur atom on the enzyme surface which contributes significantly to the binding affinity of L-methionine, as evidenced by the diminished binding capacity of L-norleucine (VII). It is also apparent that the sulfur atom and its *S*-methyl substituent must be properly oriented in order for L-methionine to undergo initial activation to the transition state intermediate and eventual transformation into Ado-Met. However, it is entirely possible that the sulfur atom and *S*-methyl group of a methionine analogue with a specific conformational or steric restraint might be unable to assume this critical orientation necessary for substrate activity, but nevertheless could still be accommodated at the L-methionine binding site and thus display inhibitory activity only. It appears that, when α-methylmethionine binds to the enzyme surface, steric accommodation of the α-methyl substituent is made at the expense of conformational changes in the remaining $—CH_2—CH_2—S—CH_3$ portion of the molecule: for the rat liver methionine adenosyltransferase isozymes, these induced conformational changes displace the sulfur atom and *S*-methyl substituent from the precise orientation that is essential for substrate activity; for the tumor isozymes, there appears to be less drastic displacement of the sulfur atom and its *S*-methyl substituent, since the molecule can still serve as substrate.

The methionine analogue, L-ethionine, has substrate specificities which strongly suggest that, for the rat liver MAT isozymes, steric accommodation of the larger *S*-ethyl substituent requires partial displacement of this moiety from the critical orientation needed for substrate activity; alternatively, the tumor isozymes appear to accommodate the *S*-ethyl substituent very well, since L-ethionine has almost complete substrate activity toward tumor iso-1 and tumor iso-2. The ability of Compound VI, the *S*-*n*-propyl analogue, to act selectively as a substrate for tumor iso-2 lends further support to the idea that the tumor isozymes have greater tolerance for the increased steric bulk of these two methionine analogues, Compounds III and VI. It is our hypothesis that the differing substrate specificities of α-methyl-D,L-methionine, L-ethionine, and *S*-*n*-propyl-D,L-homocysteine are indications of significant topographic differences between tumor and rat liver methionine adenosyltransferase iso-

zymes in the region where the sulfur atom and its *S*-alkyl substituent are accommodated at the active site.

Electronic differences between the active site of tumor versus normal methionine adenosyltransferase isozymes. Among the large group of amino acids that can generally be classified as methionine analogues, a molecule whose physical properties and chemical behavior closely resemble those of L-methionine is its seleno analogue, seleno-L-methionine. A comparison of the van der Waals radii (32) of sulfur (1.85 Å) and selenium (2.0 Å), and of the average C—S (1.81 Å) and C—Se (1.98 Å) bond lengths (32), indicates that the respective amino acids are almost isosteric. However, selenium is less electronegative than sulfur, and as a result has a lesser tendency to form hydrogen bonds. On a strictly mechanistic basis, one would expect seleno-L-methionine to be a more effective substrate for isofunctional methionine adenosyltransferases than L-methionine itself: *S*-adenosyl-L-methionine is a high-energy, positively charged chemical species. In contrast, *Se*-adenosyl-seleno-L-methionine is expected to be more energetically stable because the reduced electronegativity and greater metallic character of selenium should allow more effective dispersion of its positive charge. Accordingly, this should lower the activation energy of the reaction. It is therefore not surprising that seleno-D,L-methionine is a better substrate for rat liver iso-1 than is L-methionine itself.

It is interesting to note that L-ethionine is a more effective substrate of the tumor MAT isozymes, whereas the seleno analogues IV and V are more effective substrates of the normal rat liver isozymes. (The exceptional behavior of rat liver iso-3 toward the seleno amino acids has been noted under Results and is an apparent indication of the higher substrate selectivity of this specific isozyme.)

We propose that the significant differences observed between tumor and normal MAT isozymes in both the substrate and inhibitory properties of selenomethionine and selenoethionine provide preliminary evidence for electronic differences between tumor and rat liver methionine adenosyltransferase isozymes in the region of the active site at which the sulfur atom and its *S*-alkyl substituent bind. Although highly speculative, these electronic differences suggest the possibility of a specific amino acid modification within the protein sequence of the tumor isozymes.

D-Methionine. Although it is generally recognized that isofunctional methionine adenosyltransferases maintain a high stereospecific preference for the L-enantiomer of methionine, this in itself does not preclude the possibility of either substrate or inhibitory activity for the D-enantiomer of methionine or a methionine analogue. As noted, Schlenk *et al.* (31) have observed that D-methionine is a substrate of yeast MAT. Furthermore, Coulter *et al.* (10) have reported equipotent inhibitory activity for the D- and L-isomers of *S*-phenylhomocysteine and related analogues toward rat liver methionine adenosyltransferase. The question of whether D-methionine can serve as a substrate for rat liver and tumor MAT isozymes not only is of enzymological interest but also may have pharmacological implications. Our initial observations that D-methionine is a better substrate of tumor versus normal MAT isozymes appears consistent with the idea that the tumor MAT isozymes have greater tolerance for the differing steric requirements of analogues of L-methionine (e.g., Compounds II, III, and VI), since they more easily allow these compounds to assume a conformation critical for their activity as substrates. It is obvious that the enzyme-bound conformation of D-methionine which produces substrate activity differs greatly from that of L-methionine. For this reason, the observation of substrate activity for D-methionine toward rat liver and tumor MAT isozymes could provide new information about the complementary topographic features of the L-methionine binding site(s) of these hepatic MAT isozymes.

L-Ethionine, selenomethionine, and selenoethionine. Our present findings on the differing *in vitro* substrate and inhibitory activities of L-ethionine, selenomethionine, and selenoethionine toward the separated rat liver MAT isozymes complement earlier studies of Stekol (29) as well as those of Pan and Tarver (28), carried out at a time when the existence of three rat liver MAT isozymes was not recognized. Stekol (29) found that the *in vivo* utilization of selenomethionine is much more extensive than that of ethionine and selenoethionine, suggesting that this might be responsible for the more acute toxicity of Compound IV to rats as compared with that of Compounds III and V. Our *in vitro* studies indicating that Compound IV is more effective than either Compound III or V as a substrate of all three rat liver isozymes are consistent with these *in vivo* observations of Stekol (29). Pan and Tarver (28), in examining the *in vitro* substrate capabilities of rat liver MAT (unknown isozymic composition), established the following order of decreasing activity: L-methionine > seleno-L-methionine > L-ethionine, seleno-D,L-methionine, D-methionine, and seleno-D-methionine. These results correlate with our observations for rat liver iso-3. Since rat liver iso-3 is the major isoform in liver, it is possible that the MAT preparation of Pan and Tarver (28) did not contain significant amounts of rat liver iso-1 or iso-2. This is not unlikely, since Hoffman and Sullivan (25) have reported recently that the liver contains only moderate amounts of rat liver iso-1 and very small amounts of rat liver iso-2.

α-Methylmethionine and S-n-propylhomocysteine. We have observed that α-methylmethionine and *S-n*-propylhomocysteine, under specifically defined *in vitro* conditions, display selective substrate activity toward tumor MAT isozymes. This suggests that the design of selective substrates of tumor MAT isozymes might provide an alternative strategy in the rational design of methionine analogues with potential tumor-specific cytotoxic action. Thus, pharmacological doses of a specific amino acid analogue with selective substrate activity toward tumor MAT isozymes, and one which is a relatively poor competitive inhibitor of tumor and normal MAT isozymes, might have little effect on normal cells. However, the selective synthesis, *in vivo*, of even small amounts of an *S*-adenosylmethionine analogue within the tumor cell could, in turn, have specific cytotoxic effects. These effects might be mediated in the tumor cell through subsequent interactions of the Ado-Met analogue with *S*-adenosylmethionine decarboxylase or other Ado-Met-

utilizing enzymes involved in transmethylation reactions. The potential of this approach is supported by the studies of Abdel-Monem and co-workers (33, 34) as well as those of Nakamura and Schlenk (35). Abdel-Monem *et al.* have synthesized *S*-adenosyl-D,L-α-methylmethionine (33) and have observed that this Ado-Met analogue is a potent irreversible inhibitor of mammalian *S*-adenosyl-L-methionine decarboxylase (33) with selective inhibitory effects on spermine and DNA biosynthesis (34). Nakamura and Schlenk (35) likewise have synthesized *S*-adenosyl-D,L-α-methylmethionine and have found it to be a substrate for a number of transmethylase enzymes.

CONCLUSIONS

Although a primary concern in the design and synthesis of cancer chemotherapeutic agents has been the achievement of selective tumor cytotoxic action, the inability to identify clearly the selective lethal targets within the tumor cell has generally made this goal elusive. We propose that tumor-specific methionine adenosyltransferases meet the stringent criteria for a selective target for cytotoxic action: (a) The strategic biochemical importance of Ado-Met to cellular functions indicates that selective impairment of its synthesis should induce preferential cytotoxicity. (b) Tumor-derived methionine adenosyltransferases have been reported by Liau *et al.* (15, 18–20) to be altered enzyme forms. On the basis of our observations, it now appears that these modifications include topographic and electronic differences in a region of the L-methionine binding site of the tumor isozymes. More specifically, this is the region where the sulfur atom and the *S*-methyl substituent of L-methionine are accommodated.

The observations of this study appear highly relevant to the development of cancer chemotherapeutic agents for the treatment of human malignancies. Liau *et al.* (20) have examined methionine adenosyltransferase isozymes of a wide variety of human malignant tumors xenografted into athymic nude mice and in all cases have detected an altered tumor-specific enzyme. If tumor-specific methionine adenosyltransferases prove to be consistently associated with human malignant tumors, as Liau *et al.* (20) have initially observed, then the design of selective inhibitors of these tumor-specific enzymes could assume significance in the potential treatment of human malignancies. It is not known at this time whether methionine adenosyltransferases derived from human tumors possess the specific alterations in their active site that we propose for tumor-derived hepatic MAT isozymes of rats. However, the availability of an animal model system in which exploitable differences have been detected between tumor versus normal methionine adenosyltransferases provides an opportunity for the design, synthesis, and initial investigation of the pharmacological potential of such tumor-specific inhibitors.

ACKNOWLEDGMENTS

We thank Dr. Paul Talalay for his continuing interest and encouragement, Dr. Garland Marshall for many helpful discussions, and S. Paulette Decker for expert technical assistance.

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