Suicide Inactivation of Thioether *S*-Methyltransferase by Ethyl Vinyl Sulfide†

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Received January 16, 1996⊗

ABSTRACT: Thioether *S*-methyltransferase is an important enzyme in the metabolism of sulfur and selenium-containing compounds in animals. Ethyl vinyl sulfide was previously shown to be a substrate for this enzyme yielding methyl ethyl vinyl sulfonium ion ($MEVS^+$) upon reaction with S-adenosylmethionine. Since vinyl sulfonium ions are reactive toward nucleophiles, the inactivation of thioether *S*-methyltransferase as a result of its methylation of ethyl vinyl sulfide was investigated. Ethyl vinyl sulfide was found to inactivate thioether *S*-methyltransferase in a time-dependent, pseudo-first-order process with k_{inact} and K_I values of 0.05 min^{-1} and 0.275 mM, respectively. Calculation of the partition ratio revealed one inactivation event for every 100 turnovers. Dimethyl sulfide, an alternate substrate for thioether *S*-methyltransferase which yields the nonreactive product trimethyl sulfonium ion, protected the enzyme from inactivation by ethyl vinyl sulfide. The inactivation is a result of covalent reaction of methyl ethyl vinyl sulfonium ion with the enzyme as shown by comigration of radioactivity with the enzyme during denaturing gel filtration of reaction mixtures containing thioether *S*-methyltransferase, ethyl vinyl sulfide, and *S*-adenosyl[*methyl*-^{3}H]methionine. Using this method the stoichiometry of inactivation was determined to be 1 mol of [^{3}H]-methyl group/mol of thioether *S*-methyltransferase inactivated. Both the alternate substrate, dimethyl sulfide, and the competitive product inhibitor, *S*-adenosylhomocysteine, inhibited such covalent labeling of the enzyme by ethyl vinyl sulfide and *S*-adenosyl[*methyl*-^{3}H]methionine. Chemically synthesized $MEVS^+$ inactivated thioether *S*-methyltransferase, and [*methyl*-^{14}C]$MEVS^+$ covalently labeled the enzyme with ^{14}C. These results reveal a previously unrecognized mechanism for biochemical activation of vinyl thioethers by methylation to form reactive vinyl sulfonium ions.

S-Adenosylmethionine:thioether *S*-methyltransferase (TEMTase)[1] (EC 2.1.1.96) methylates sulfur, selenium, or tellurium atoms contained in ether-type bonds (Mozier et al., 1988). The cDNA for mouse lung thioether *S*-methyltransferase was recently cloned and shown to encode a protein with an M_r of 29 640 (Warner et al., 1995) containing three sequence motifs common to many AdoMet-dependent methyltransferases (Kagan & Clarke, 1994). The physiological role of TEMTase is the detoxification and solubilization for urinary excretion of endogenous and exogenous sulfur- and selenium-containing compounds (Mozier & Hoffman, 1990). Substrates for TEMTase may occur directly or be produced by the microsomal enzyme thiol *S*-methyltransferase (Carrithers & Hoffman, 1994).

An early survey of candidate substrates for TEMTase revealed a possible suicide inhibitor in the compound EVS (Mozier & Hoffman, 1990), whose methylation yields $MEVS^+$ (Figure 1). $MEVS^+$ is a reactive species because of the ability of the sulfonium to stabilize the neighboring carbanion formed during nucleophilic attack on C-2 of the vinyl group (Doering & Schrieber, 1955; Stirling, 1977). Figure 1 shows the proposed mechanism of TEMTase inactivation following methylation of EVS to form $MEVS^+$ which then reacts with nucleophilic amino acid residues in or around the active site of the enzyme. We therefore conducted the present experiments to determine if EVS was in fact a suicide inhibitor of TEMTase.

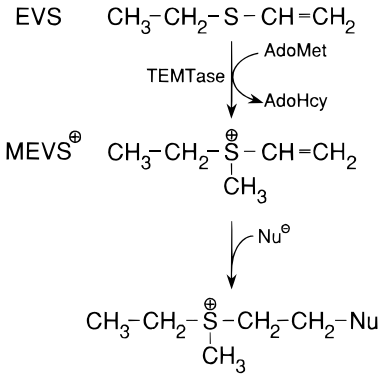

FIGURE 1: Proposed mechanism for TEMTase inactivation by ethyl vinyl sulfide. Abbreviations: EVS, ethyl vinyl sulfide; $MEVS^+$, methyl ethyl vinyl sulfonium ion; Nu^-, nucleophile in the active site of TEMTase.

EXPERIMENTAL PROCEDURES

Materials

Specialized Materials. Ethyl vinyl sulfide was purchased from Lancaster Synthesis (Windham, NH), and fresh dilu-

† Supported by Grant No. ES-4887 from the National Institute of Environmental Health Sciences, National Institutes of Health.

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⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1996.

[1] Abbreviations: TEMTase, *S*-adenosyl-L-methionine:thioether *S*-methyltransferase (EC 2.1.1.96); AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; AdoVin, *S*-adenosyl-L-vinthionine; EVS, ethyl vinyl sulfide; $MEVS^+$, methyl ethyl vinyl sulfonium ion; DMS, dimethyl sulfide; TMS^+, trimethyl sulfonium ion; SDS, sodium dodecyl sulfate; MES, 4-morpholinoethanesulfonic acid.

0006-2960/96/0435-4480$12.00/0

tions in buffer were prepared for each experiment; [*methyl*-^{3}H]AdoMet was purchased at 73 Ci/mmol from New England Nuclear; [*methyl*-^{14}C]dimethyl sulfate was purchased as the neat liquid from American Radiolabeled Chemicals at 4.1 Ci/mol; G-75 Sephadex was purchased from Pharmacia; and all other chemicals were purchased from either Sigma Chemical Co. or Fisher Scientific and were of the highest quality available.

Methods

Enzyme Purification and Assay. TEMTase was purified from mouse lungs according to Mozier et al. (1988). Briefly, this procedure consisted of three successive column chromatographic steps: anion exchange on DEAE-Sephacryl, gel filtration on G-75 Sephadex, and chromatofocusing from pH 7 to 4. TEMTase purified in this way had a purity greater than 95% as assessed by Coomassie blue staining of samples separated by SDS−polyacrylamide gel electrophoresis.

The standard assay was also performed as described by Mozier et al. (1988). This assay is based on the methylation by [*methyl*-^{3}H]AdoMet of DMS to form [^{3}H]TMS$^+$ as catalyzed by TEMTase. [^{3}H]TMS$^+$ was isolated by cation exchange high-pressure liquid chromatography and quantitated by liquid scintillation counting. Protein concentrations were measured either by a filter paper dye binding assay (Minamide et al., 1990) or by the bicinchonic acid method (Smith et al., 1985) using reagents purchased from Pierce Chemical Co.

Inactivation Experiments. For inactivation studies using EVS, TEMTase (10 μg) was incubated at 37 °C with 153 μM [*methyl*-^{3}H]AdoMet and the indicated concentrations of EVS in a final volume of 100 μL of assay buffer (25 mM MES at pH 6.3, 2 mM DTT, and 1 mM EDTA). Aliquots of 10 μL were removed at 0, 4, 8, and 12 min and diluted 10-fold into assay mix (35 μM [*methyl*-^{3}H]AdoMet and 500 μM DMS in assay buffer). [^{3}H]TMS$^+$ was measured after 10 min at 37 °C as described previously (Mozier et al., 1988).

For inactivation studies using MEVS$^+$, TEMTase (2 μg) was incubated at 37 °C for various times with the indicated concentrations of MEVS$^+$ in 50 μL of assay buffer. Because MEVS$^+$ is a product and thus a potential inhibitor of TEMTase, it was removed before assay of the enzyme. This was accomplished by passing each 50 μl reaction mixture through a Quick-Spin column of Sephadex G-25 (Boehringer Mannheim) by centrifugation at 1000*g* for 2 min at 5 °C. Before use, the column buffer was changed to assay buffer from that provided by the manufacturer by similar centrifugation twice with 1 mL of assay buffer. Each eluate of 50 μl was then assayed as above for TEMTase activity in a final volume of 100 μl. Control experiments with untreated TEMTase consistently gave more than 90% recovery of activity upon spin column treatment. MEVS$^+$ for these experiments was prepared by reaction of EVS with methyl iodide and was characterized by fast-atom bombardment mass spectrometry as previously described in detail (Mozier & Hoffman, 1990).

Gel Filtration of TEMTase−MEVS$^+$ Complex. To test for covalent labeling by MEVS$^+$ generated *in situ*, TEMTase (130 μg) was incubated for 15 min at 37 °C in a volume of 200 μL containing 103 μM [*methyl*-^{3}H]AdoMet and 25 mM EVS in assay buffer. To quench the reactions, SDS was added to a final concentration of 0.1%. Reaction mixtures were fractionated on a 15 mL bed volume of Sephadex G-75 equilibrated and eluted with 0.1% SDS in 0.1 M ammonium bicarbonate at pH 8.1. Fractions of 0.5 mL were collected with a Bio-Rad model 2110 fraction collector, diluted into 8 mL EcoLite scintillation fluid, and counted for one min in a Beckman LS-3150P liquid scintillation counter.

During attempts to obtain labeled tryptic peptides from TEMTase, reaction mixtures identical to the above were fractionated by gel filtration in 0.1 M ammonium bicarbonate at pH 8.1 lacking SDS. Peak fractions of labeled TEMTase were digested with trypsin, and digests were fractionated by reversed-phase HPLC on a 0.46 × 25 cm C18 column eluted for 100 min at 1 mL/min with a gradient from water to acetonitrile both containing 0.1% trifluoroacetic acid. Fractions of 0.5 mL were collected and mixed with 8 mL of Ecolite scintillation fluid, and the ^{3}H content was determined by liquid scintillation counting.

A sample of [*methyl*-^{14}C]MEVS was chemically synthesized to study covalent binding to TEMTase. The batch of 250 μCi of [*methyl*-^{14}C]dimethyl sulfate was mixed with 20 μL of EVS in 80 μL of methanol, and this mixture was allowed to react overnight at room temperature. After dilution with water to 1 mL, the reaction mixture was extracted twice with 5 mL of diethyl ether. The aqueous phase containing the cationic [*methyl*-^{14}C]MEVS$^+$ was applied to a 0.5 mL column bed of S-Sepharose Fast Flow cation exchanger (Pharmacia) equilibrated with water. The column was washed with water until ^{14}C in the eluate was near background. The [*methyl*-^{14}C]MEVS$^+$ was then eluted from the column with 0.3 M NaCl, and fractions of 0.1 mL were collected. Samples of 1 μL of these fractions were assayed for ^{14}C by liquid scintillation counting to determine which contained the [*methyl*-^{14}C]MEVS$^+$ product. The two fractions with the highest radioactivity totalling approximately 150 μCi were pooled, and a 10 μL portion was mixed with 50 nmol of authentic nonradioactive MEVS$^+$ in 10 μL of water. This mixture was analyzed by ion chromatography as described previously using solvent A (Hoffman, 1991). Both the ^{14}C and conductivity peaks (MEVS$^+$) eluted at 4.9 min, verifying the identity of the [*methyl*-^{14}C]MEVS$^+$ which was used in the following experiment.

A mixture of 100 μg of TEMTase and 200 nmol (0.82 μCi; 1 mM) of [*methyl*-^{14}C]MEVS$^+$ in 200 μL of assay buffer was incubated at 37 °C for 30 min. An otherwise identical reaction mixture contained in addition 25 mM TMS$^+$. Reaction mixtures were quenched with SDS and analyzed as above by gel filtration under denaturing conditions. The eluates were collected and assayed for ^{14}C by liquid scintillation counting.

RESULTS

TEMTase was incubated with EVS and AdoMet and then tested for its ability to methylate DMS to TMS$^+$. The data in Figure 2 show that TEMTase was inactivated by EVS in a time-dependent, pseudo-first-order process, a basic criterion for a suicide inhibitor. TEMTase activity disappeared logarithmically for 12 min, but the loss of activity began to level off after 15 min and no further loss of activity occurred after 30 min. A likely explanation for the inability to achieve further inactivation is that the AdoHcy generated after 15 min by methylation of EVS with AdoMet inhibited and

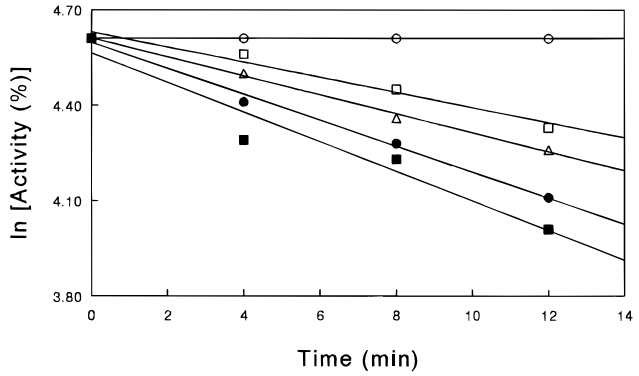

FIGURE 2: Inactivation of TEMTase by EVS. TEMTase was incubated with 153 μM AdoMet and EVS at the following concentrations: 0 (○), 0.25 (□), 0.5 (△), 1 (●), or 5 (■) mM. Samples were incubated for 0−12 min at 37 °C and diluted 10−fold into assay mix, and the production of TMS^+ was measured. The data are the average of at least two duplicate analyses. Ranges for each sample were less than 10%.

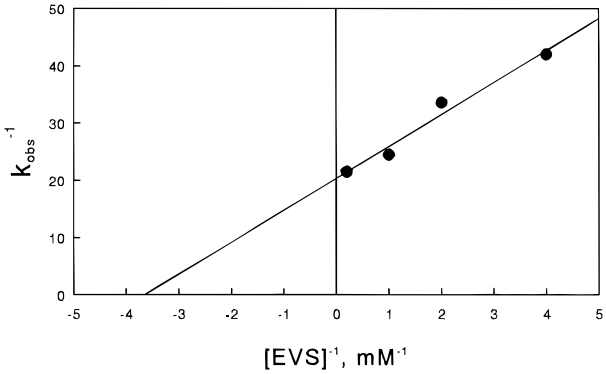

FIGURE 3: Reciprocal plot of k_{obs} vs EVS concentration. The data from Figure 2 were replotted to determine k_{inact} and K_I values.

protected the remaining active TEMTase. (The lower panel of Figure 5 shows that AdoHcy protects TEMTase from radioactive labeling with EVS and [*methyl*-^{3}H]AdoMet.) A control experiment (not shown) demonstrated that a 10-fold dilution of the labeling reaction into assay mixture was sufficient to eliminate inhibition of TEMTase by the remaining EVS during the activity assay itself. Reductions in enzyme activity by EVS, therefore, reflect inactivation during the labeling reaction and not inhibition during the subsequent activity measurement. The slopes of the lines in Figure 2 are the observed rate constants (k_{obs}).

When the reciprocals of k_{obs} are plotted vs 1/[EVS] as in Figure 3, the inactivation rate constant (k_{inact}) and inhibition constant (K_I) can be determined. From this plot the k_{inact} was calculated to be 0.05 min^{-1}, and the K_I was 0.275 mM. These data demonstrate that EVS must first form a Michaelis complex with TEMTase, another basic feature of suicide inhibitors. The partition ratio was estimated from a procedure described by Funaki et al. (1991) to be one inactivation event for every 100 catalytic turnovers.

DMS is a substrate for TEMTase and is routinely used in our laboratory to measure TEMTase activity. If EVS must first bind to the active site of TEMTase to generate $MEVS^+$, as is suggested from the kinetics of inactivation, DMS should compete with EVS for this site and block inactivation by EVS. DMS proved to be an effective competitor of EVS inactivation (Table 1) in a concentration-dependent manner. Replacement of DMS by 10 mM L-methionine, a thioether

Table 1: Protection by DMS against TEMTase Inactivation by EVS[a]

addition	% activity remaining	n
no addition	65 ± 9	9
5 mM DMS	83 ± 6	2
10 mM DMS	93 ± 3	2
10 mM L-methionine	63 ± 9	3

[a] TEMTase (10 μg) was incubated at 37 °C with 5 mM EVS, 153 μM AdoMet, and the additional reagents as indicated. After 12 min, samples were assayed for TEMTase activity by production of TMS^+ as described in Methods. The data are expressed as the percentage of activity remaining after 12 min of incubation with inhibitor. TEMTase activity of 100% was that found after 12 min of incubation with 153 μM AdoMet alone. Where $n = 2$, data are expressed as the mean ± range. Otherwise, the data are expressed as the mean ± standard deviation.

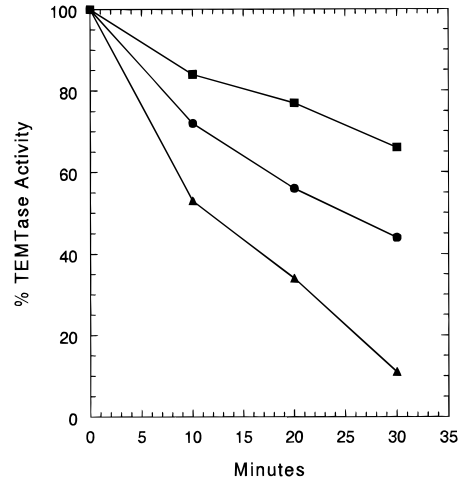

FIGURE 4: Inactivation of TEMTase by $MEVS^+$. Samples of 2 μg of TEMTase were incubated with $MEVS^+$ at the times and concentrations indicated, separated from $MEVS^+$ by spin column gel filtration, and assayed for activity. $MEVS^+$ concentrations: 100 μM (■); 1 mM (●); 10 mM, (▲).

which is not a substrate for TEMTase (Mozier et al., 1988), gave no protection from inhibition by EVS. These data demonstrate that EVS must bind to the active site of TEMTase to cause inactivation.

The assumption that $MEVS^+$ generated *in situ* from AdoMet and EVS inactivates TEMTase was tested by studies of direct inactivation with chemically synthesized $MEVS^+$ alone. The data in Figure 4 show that $MEVS^+$ inactivated TEMTase in a time- and concentration-dependent fashion. The nearly complete inactivation of TEMTase after 30 min at the highest concentration of $MEVS^+$ as opposed to the more limited inactivation with AdoMet and EVS again suggests a role for AdoHcy protection in the latter case.

Covalent modification of TEMTase during methylation of EVS was demonstrated by gel filtration chromatography. The top panel of Figure 5 shows the chromatographic fractionation of two labeling reactions with [*methyl*-^{3}H]AdoMet, one containing 25 mM EVS and the other containing 25 mM DMS. When EVS was used, a peak of ^{3}H eluted at 8 mL which was absent in samples incubated with DMS. We have shown previously that TEMTase elutes at 8 mL from this column (Warner, 1992). Free [*methyl*-^{3}H]AdoMet eluted at the column volume of 15 mL determined by calibration runs with radioactive AdoMet alone. From these results it can be calculated that the enzyme becomes covalently bound to

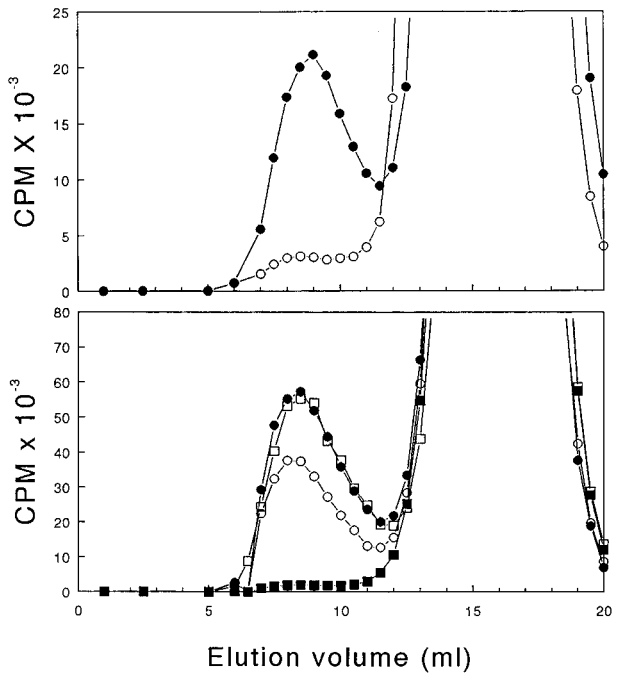

FIGURE 5: Gel filtration of TEMTase reacted with [*methyl*-^{3}H]-AdoMet and EVS**.** **(**Upper panel) TEMTase (130 μg, 22 μM) was incubated with 105 μM [methyl-^{3}H]AdoMet (187 cpm/pmol) and either 25 mM EVS (●) or 25 mM DMS (○) for 15 min at 37 °C. The reaction was quenched with 0.1% SDS and loaded onto a column of G-75 resin (15 mL bed volume) and eluted with 0.1M ammonium bicarbonate in 0.1% SDS. Fractions of 0.5 mL were collected and assayed for ^{3}H by liquid scintillation counting. (Lower panel) TEMTase (130 μg, 22 μM) was incubated as in the upper panel with 105 μM [*methyl*-^{3}H]AdoMet (222 cpm/pmol) and no EVS (■), 25 mM EVS (□), 25 mM EVS and 1 mM D-AdoHcy (●), or 25 mM EVS and 1 mM L-AdoHcy (○). Samples were analyzed as in the upper panel.

0.35−0.4 mol of methyl groups per mol of TEMTase. This correlates well with the 35% loss of activity as shown in Table 1.

If covalent labeling of TEMTase requires AdoMet methylation of EVS to form MEVS$^+$, then AdoHcy, which is a product inhibitor (Mozier et al., 1988), should inhibit labeling of TEMTase. The bottom panel of Figure 5 shows the results of such an experiment. TEMTase was incubated with 25 mM EVS in the presence of a 10-fold excess (in relation to [*methyl*-^{3}H]AdoMet) of the biologically active L-isomer or the inactive D-isomer of AdoHcy. When L-AdoHcy was included in the labeling reaction, the peak of ^{3}H was decreased by approximately 30%, while the D-isomer had no effect on ^{3}H labeling of TEMTase. When TEMTase was incubated with [*methyl*-^{3}H]AdoMet alone, no ^{3}H was associated with TEMTase, ruling out direct methylation of the enzyme by AdoMet.

The results in Figure 6 show that chemically synthesized [*methyl*-^{14}C]MEVS$^+$ covalently labeled TEMTase. It was calculated that 0.62 mol of methyl ^{14}C bound per mol of enzyme when incubated for 30 min with 1 mM [*methyl*-^{14}C]-MEVS$^+$ alone. Since this is close to the 56% inactivation of TEMTase found in Figure 4 under these conditions, this again indicates that reaction with one molecule of MEVS$^+$ inactivates one molecule of TEMTase. The alternate product, TMS$^+$, protected TEMTase from labeling by [*methyl*-^{14}C]-MEVS$^+$, suggesting that the inactivation required binding to the active site of the enzyme.

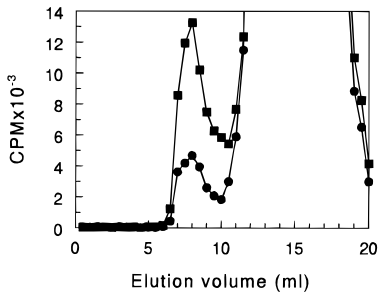

FIGURE 6: Gel Filtration of TEMTase reacted with [*methyl*-^{14}C]-MEVS$^+$. Samples of TEMTase were incubated at 37 °C for 30 min with 1 mM [*methyl*-^{14}C]MEVS$^+$ alone (■) or with 25 mM TMS$^+$ (●) and analyzed as described in the legend to Figure 5 with the exception that ^{14}C was counted rather than ^{3}H.

DISCUSSION

EVS inactivated TEMTase in a pseudo-first-order, time-dependent process and covalently labeled the enzyme in a reaction that required binding of both EVS and AdoMet to the active site. The methylated product of EVS, MEVS$^+$, also inactivated TEMTase and covalently labeled the enzyme.

MEVS$^+$ belongs to the general class of vinyl sulfonium compounds which are known to form adducts with nucleophiles as illustrated in Figure 1. In this case the nucleophiles are presumed to be amino acid residues in and around the active site of TEMTase. Although EVS was not a particularly effective inhibitor, with approximately 1% of the catalytic events resulting in a covalent modification and a K_I of 0.275 mM, it was specific in its action and apparently reacted with only one amino acid residue per TEMTase molecule. We have made preliminary attempts to purify affinity-labeled tryptic peptides following incubation of TEMTase with EVS and [*methyl*-^{3}H]AdoMet. The results of these experiments were not shown because they were unsuccessful, apparently due to the instability of the TEMTase−MEVS$^+$ adduct under conditions necessary for complete proteolysis of labeled protein.

This is the second description of the bioactivation of a vinyl thioether to form a reactive vinyl sulfonium ion. Ethionine is carcinogenic (Farber, 1963), and Leopold et al. (1979) investigated the biological properties of vinthionine (*S*-vinyl homocysteine) as a potential metabolite of ethionine. Although vinthionine does not appear to be a metabolite of ethionine, it was found to be mutagenic in the Ames test. In this first report vinthionine was also tested as a carcinogen in mice and did not cause lung adenomas. However, in a second report Leopold et al. (1982) found vinthionine to be a liver carcinogen in mice and rats. They also synthesized [*vinyl*-^{14}C]vinthionine and tested for the covalent binding of radioactivity to macromolecules from rats treated in vivo. Liver protein, DNA, and RNA all became highly labeled to an extent comparable to that for other strongly alkylating carcinogens. These authors postulated two possible mechanisms for such covalent labeling by the vinyl group. The first involved the γ-cleavage of vinthionine releasing vinyl mercaptan, which was proposed to form 2-mercaptoethyl adducts with macromolecules. The second involved the reaction of vinthionine and ATP to form the vinyl sulfonium compound, AdoVin, catalyzed by AdoMet synthetase. AdoVin could then react with macromolecular nucleophiles through its vinyl group similar to MEVS$^+$ in Figure 1. Leopold et al. tested for AdoVin biosynthesis using a yeast

extract capable of AdoMet synthesis with ATP and methionine but found no activity with vinthionine. However, they did report a personal communication from Sullivan and Hoffman describing the biosynthesis of AdoVin in vitro by mouse liver extracts and the appearance of AdoVin in livers of mice treated with vinthionine. This provided support for the second proposed mechanism of bioactivation of vinthionine.

While we have emphasized the mechanism shown in Figure 1 for covalent inactivation of TEMTase by $MEVS^+$, an alternative explanation is that $MEVS^+$ inactivated TEMTase by methylation of the enzyme. However, the studies by Doering and Schreiber (1955) of the reactivity of vinyl sulfonium ions support the mechanism proposed in Figure 1. They found that dimethyl vinyl sulfonium ion reacted with nucleophiles such as hydroxide ion, ethoxide ion, and the C2 carbanion of diethyl malonate almost exclusively by nucleophilic addition to the C2 atom of the vinyl group. If methylation was favored, these strong nucleophiles would have yielded methanol, methyl ethyl ether, and diethyl methylmalonate, respectively, along with methyl vinyl sulfide, but these products were not found. Combined with the results of Leopold et al. (1982) described above showing that the vinyl group of vinthionine becomes covalently attached to macromolecules, this suggests that the mechanism of inactivation of TEMTase by $MEVS^+$ occurs as in Figure 1. However, definitive proof of this awaits experiments on radioactive labeling of TEMTase using $MEVS^+$ with radioactive atoms other than those in the methyl group.

Another potential route for formation of vinyl sulfonium ions comes from the methylation of sulfur mustards (2-chloroethyl sulfides) as recently proposed by Hoffman (1994). Mozier and Hoffman (1990) found that TEMTase acted in vitro and in vivo to detoxify 2-chloroethyl ethyl sulfide by methylating the sulfur atom and thus inhibiting the formation of the reactive episulfonium intermediate. However, the product of this reaction, 2-chloroethyl ethyl methyl sulfonium ion, can eliminate HCl to form $MEVS^+$. The extent to which this type of reaction might occur in vivo and its role in the acute and systemic toxicity of sulfur mustards are unknown at present.

While the present report examined only a single specific enzyme target, TEMTase, for covalent reaction with its vinyl sulfonium ion product $MEVS^+$, the previously published work with [*vinyl*-^{14}C]vinthionine suggests that vinyl sulfonium ions react generally with nucleophiles in cellular macromolecules. It is not known at this point if $MEVS^+$ has other targets in addition to TEMTase which would lead to more widespread cellular damage after EVS exposure, but it appears that bioactivation to form vinyl sulfonium ions may be a route to mutagenicity and carcinogenicity. In addition, due to their sulfonium ion character similar to AdoMet, vinyl sulfonium ions may be useful inhibitory and affinity reagents in studying the mechanisms and physiological roles of the many enzymes which utilize or synthesize AdoMet.

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BI9600815