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# Recognition of Age-damaged (*R*,*S*)-Adenosyl-L-methionine by Two Methyltransferases in the Yeast *Saccharomyces cerevisiae**

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**Chris R. Vinci and Steven G. Clarke**[1]

*From the Department of Chemistry and Biochemistry and the Molecular Biology Institute, UCLA, Los Angeles, California 90095-1569*

**The biological methyl donor *S*-adenosylmethionine (AdoMet) can exist in two diastereoisomeric states with respect to its sulfonium ion. The *S* configuration, (*S*,*S*)-AdoMet, is the only form that is produced enzymatically as well as the only form used in almost all biological methylation reactions. Under physiological conditions, however, the sulfonium ion can spontaneously racemize to the *R* form, producing (*R*,*S*)-AdoMet. As of yet, (*R*,*S*)-AdoMet has no known physiological function and may inhibit cellular reactions. In this study, we found two *Saccharomyces cerevisiae* enzymes that are capable of recognizing (*R*,*S*)-AdoMet and using it to methylate homocysteine to form methionine. These enzymes are the products of the *SAM4* and *MHT1* genes, identified previously as homocysteine methyltransferases dependent upon AdoMet and *S*-methylmethionine, respectively. We found here that Sam4 recognizes both (*S*,*S*)- and (*R*,*S*)-AdoMet, but that its activity is much higher with the *R*,*S* form. Mht1 reacts with only the *R*,*S* form of AdoMet, whereas no activity is seen with the *S*,*S* form. *R*,*S*-Specific homocysteine methyltransferase activity is also shown here to occur in extracts of *Arabidopsis thaliana*, *Drosophila melanogaster*, and *Caenorhabditis elegans*, but has not been detected in several tissue extracts of *Mus musculus*. Such activity may function to prevent the accumulation of (*R*,*S*)-AdoMet in these organisms.**

The aging process, as well as several human diseases, has been linked to the accumulation of spontaneously damaged biomolecules. Cells have evolved several ways of dealing with these altered molecules, including degradation, excretion, and repair pathways (1–6). The balance between the formation of age-altered molecules and the pathways that limit their cellular accumulation has been described as a battle between chemistry and biochemistry, where chemistry ultimately wins (2).

Although enzymes that recognize damaged DNA (3) and proteins (1, 2, 5) have been well characterized, this is not yet the case for spontaneously altered small molecules. Of the large number of metabolites that are produced and used by biological systems, many are unstable, degrading into forms that may have reduced function or that may be toxic. One pathway of small molecule degradation and cellular recognition has been described recently. Here, *trans*-aconitate formed spontaneously from the citric acid cycle intermediate *cis*-aconitate results in the inhibition of at least two steps in the cycle (7, 8). *trans*-Aconitate is then recognized by a specific yeast methyltransferase; the methyl ester formed has reduced inhibitory properties (9).

One of the crucial small molecule metabolites in all organisms is *S*-adenosyl-L-methionine (AdoMet)[2] (10–12). Second to ATP, it is probably the most widely used cofactor in nature (12, 13). Not only does it serve as the primary methyl donor, but it also functions as an amino, adenosyl, and ribosyl donor (11). It also plays a role in the formation of adenosyl radicals (14) and as a precursor of polyamines (15). AdoMet has been shown to be unstable in cells, forming a variety of degradation products. Internal cyclization can form homoserine lactone and 5′-methylthioadenosine; hydrolysis at the glycosidic bond can form adenine and *S*-pentosylmethionine; and racemization at the sulfonium ion can form the *R*,*S* diastereomer (16–18). We have been particularly interested in this latter reaction because pathways for the utilization or metabolism of this diastereomer have not been described.

As biosynthesized, AdoMet is in the *S*,*S* configuration, where the first *S* corresponds to the stereochemistry at the sulfonium ion and the second to that at the $\alpha$-carbon (19, 20). The *S*,*S* form appears to be the biologically active species (21, 22), and a number of methyltransferases have been shown to use it exclusively (20, 21, 23, 24). However, the instability of the sulfonium center results in spontaneous racemization under biological conditions to form (*R*,*S*)-AdoMet (Fig. 1) (20). If only the *S*,*S* form is used, and the *R*,*S* form is constantly produced by racemization, the levels of biologically inactive (*R*,*S*)-AdoMet should build up over time in cells. This material may not only take up precious cell space, but may also be toxic (24, 25). The racemization of AdoMet may also be a factor in its pharmacology when used as a nutritional supplement (SAMe) (26). These preparations generally contain from 20 to 40% of the *R*,*S* form (27), and there is uncertainty at present about the relative contribution of each form to the therapeutic effect and whether there may be any toxicity associated with the *R*,*S* form.

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[1] To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry and the Molecular Biology Inst., UCLA, 607 Charles E. Young Dr. East, Los Angeles, CA 90095-1569. Tel.: 310-825-8754; Fax: 310-825-1968; E-mail: clarke@mbi.ucla.edu.

[2] The abbreviations used are: AdoMet, *S*-adenosyl-L-methionine; HPLC, high performance liquid chromatography; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; AdoHcy, *S*-adenosyl-L-homocysteine.

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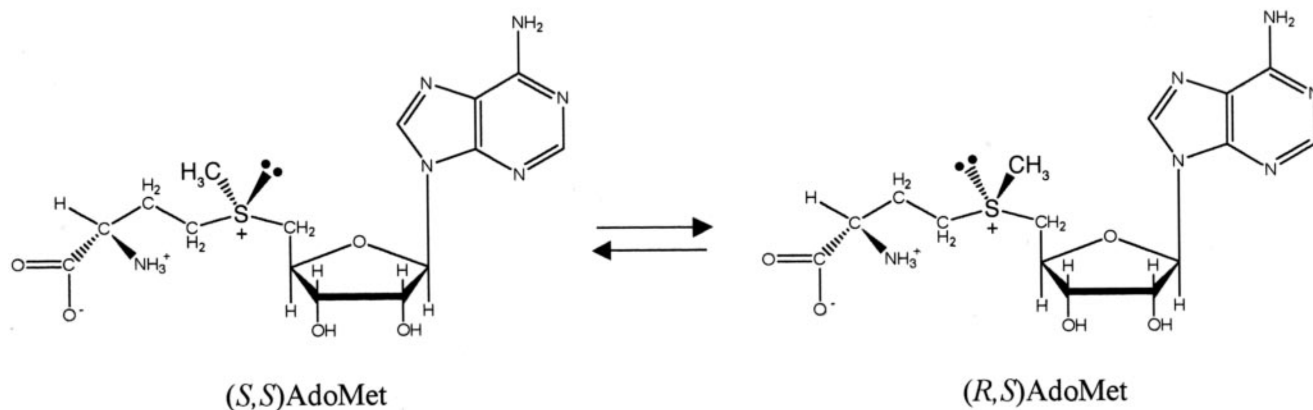


FIGURE 1. ***S,S* and *R,S* diastereomers of AdoMet.** In the *S,S*/*R,S* notation, the first letter designates the stereoconfiguration of the sulfonium ion, and the second letter designates that of the $\alpha$-carbon of the methionine moiety. (*S,S*)-AdoMet is the product of the AdoMet synthetase reaction and is the form predominantly used in enzymatic reactions. (*R,S*)-AdoMet forms spontaneously under physiological conditions from (*S,S*)-AdoMet.

There is some indirect evidence to suggest that (*R,S*)-AdoMet can be metabolized in cells. Based on steady-state calculations for cells at physiological temperature and pH, the ratio of (*R,S*)- to (*S,S*)-AdoMet has been calculated as 19:81 (18). However, the *R,S* form is undetectable in soybean extracts (17), and the *R,S*/*S,S* ratio in mouse liver (18) and rat brain (25) has been measured as 3:97. Thus, there appears to be a mechanism(s) that keeps the intracellular (*R,S*)-AdoMet levels low.

We were thus interested in an older report that suggested that (*R,S*)-AdoMet might be utilized by a yeast methyltransferase (23). In this work, homocysteine methyltransferase activity was observed at twice the rate when an *S,S*/*R,S* mixture of AdoMet was used compared with that seen with the *S,S* form at an equal total concentration (23). Previous studies have shown that there are at least two homocysteine methyltransferase enzymes present in *Saccharomyces cerevisiae* (28, 29). These are encoded by the *SAM4* and *MHT1* genes and utilize AdoMet and *S*-methylmethionine, respectively, as methyl donors (29). It is not clear whether either of these enzymes was responsible for the utilization of (*R,S*)-AdoMet seen initially (23).

We were intrigued by the apparent lack of cellular logic in encoding an AdoMet-utilizing homocysteine methyltransferase. Homocysteine methyltransferases function to make methionine, which is then the precursor of AdoMet. The source of the methyl group can be $N^5$-methyltetrahydrofolate, *S*-methylmethionine, or betaine (12, 29). If AdoMet itself were used as the donor, the reaction would appear to be a futile cycle, resulting in the hydrolysis of 3 ATP eq (Fig. 2). However, such a "futile cycle" could be justified if the AdoMet-dependent homocysteine methyltransferase utilized the *R,S* form rather than the *S,S* form. In this scenario, the inactive *R,S* form, resulting from the unwanted racemization of the *S,S* form, would be converted to methionine, which could in turn be converted to the active *S,S* form of AdoMet. Such a mechanism could explain the low cellular levels of (*R,S*)-AdoMet observed (17, 18, 25).

In this work, we demonstrate that Sam4 and Mht1 in *S. cerevisiae* are both in fact capable of using (*R,S*)-AdoMet as a methyl donor. Sam4 has a higher specificity for (*R,S*)- than for (*S,S*)-AdoMet, and Mht1 uses (*R,S*)-AdoMet in exclusion of the *S,S* form. Thus, these two enzymes may work to prevent the accumulation of (*R,S*)-AdoMet within cells.

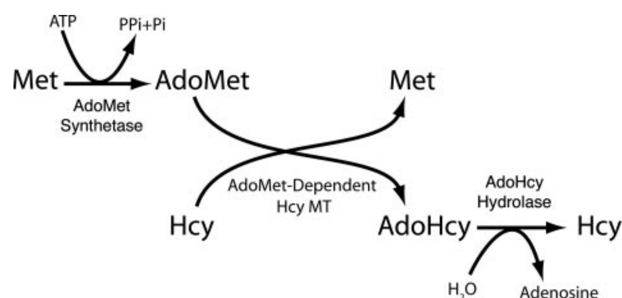


FIGURE 2. **AdoMet-dependent homocysteine methyltransferase, in combination with AdoMet synthetase and AdoHcy hydrolase, catalyzes a potential futile cycle.** The methionine used to make AdoMet would be regenerated by the methyltransferase activity, and the homocysteine (*Hcy*) used by the methyltransferase (*MT*) would be regenerated by the hydrolase. The combination of activities of these three enzymes would be expected to result in the net hydrolysis of ATP to form adenosine. Adenosine requires three ATP molecules to be regenerated as ATP.

## EXPERIMENTAL PROCEDURES

*Preparation and Purification of (R,S)- and (S,S)-[$^3$H]AdoMet*—*S*-Adenosyl-L-[*methyl*-$^3$H]methionine (referred to throughout as [$^3$H]AdoMet; 1 mCi/ml, 79.0 Ci/mmol, in dilute HCl/ethanol (9:1, v/v) at pH 2–2.5; GE Healthcare) was diluted 10-fold in 0.1 M HCl and divided into two aliquots. One aliquot was incubated for 8 days at 37 °C, resulting in an *R,S*-enriched [$^3$H]AdoMet sample, whereas the other was kept at −80 °C. An aliquot (100 $\mu$l) of each preparation was then fractionated with a cation-exchange HPLC column (Whatman Partisil SCX, 10-$\mu$m bead diameter, 4.6 mm, inner diameter, × 250 mm) using a method similar to that described previously (18). Briefly, the column was equilibrated and eluted with 80% buffer A (0.7 ml of concentrated $NH_4OH$ added to 1000 ml of 20% acetonitrile in $H_2O$ and brought to pH 3.0 with 88% acetic acid) and 20% buffer B (buffer A adjusted to 50 mM $(NH_4)_2SO_4$ and then to pH 3.0 with concentrated sulfuric acid). The column was eluted at room temperature at a flow rate of 1 ml/min. The absorbance of the eluate was monitored at 254 nm, and 500-$\mu$l fractions were collected. An aliquot (50 $\mu$l) of each fraction was mixed with 2 ml of fluor (Safety-Solve, Research Products International Corp., Mount Prospect, IL) to measure radioactivity. The fractions corresponding to the *R,S* peak of the incubated sample and to the *S,S* peak of the non-incubated sample were each

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**TABLE 1**
**Yeast strains**

| Strain | Genotype | Source |
|---|---|---|
| BY4741 | *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* | SGDP[a] |
| *mht1*⁻ (BY4741) | BY4741, *Δyll062c::Kan*ʳ | SGDP[a] |
| BY4742 | *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* | SGDP[a] |
| *sam4*⁻ (BY4742) | BY4742, *Δypl273w::Kan*ʳ | SGDP[a] |
| *mht1*⁻ (BY4742) | BY4742, *Δyll062c::Kan*ʳ | SGDP[a] |
| *sam4*⁻/*mht1*⁻ | *MATα Δypl273w::Kan*ʳ *Δyll062c::Kan*ʳ *LYS*⁺ *MET*⁺ | This study |

[a] Strains prepared by the *Saccharomyces* Genome Deletion Project (SGDP) and purchased from Invitrogen.

pooled, divided into 200-μl aliquots, and stored at −80 °C. [$^3$H]AdoMet concentrations were calculated using a specific activity of 79,000 cpm/pmol provided by the manufacturer.

*NMR Analysis of AdoMet Stereochemistry*—AdoMet (chloride salt; purity of ~70% with 1 mol/mol $H_2O$ and 4.6% methanol; Sigma) was dissolved in 0.1 M HCl. One aliquot was incubated at 37 °C for 160 h, whereas the other was not incubated. Both were then dried and dissolved in $D_2O$ to a final concentration of 10 mg/ml. The $^1$H NMR spectrum for 500 μl of each sample was determined using a Bruker ARX400 spectrometer operating at 400.13 MHz as described previously (27).

*Isoaspartyl Protein Methyltransferase Assay*—Human recombinant L-isoaspartyl protein methyltransferase (0.13 μg, specific activity of 20,990 pmol/min/mg of protein) was incubated with 0.016 pmol of either (*R*,*S*)- or (*S*,*S*)-[$^3$H]AdoMet in the presence or absence of 3.5 nmol of the isoaspartyl-containing peptide KASA(isoD)LAKY (California Peptide Research, Inc., Napa, CA). The reaction was buffered in 50 mM BisTris-HCl (pH 6.4) in a final volume of 40 μl. After incubation for 45 min at 37 °C, methylation of the peptide was determined as described previously (30).

*Yeast Strains*—Table 1 lists the yeast strains used in this study. The *sam4*⁻ and *mht1*⁻ single knock-out mutant strains were generated by the *Saccharomyces* Genome Deletion Project (www-sequence.stanford.edu/group/yeast_deletion_project/available.html) and purchased from Invitrogen. The *sam4*⁻/*mht1*⁻ double knock-out strain was created by mating the *MATα* strain of *sam4*⁻ with the *MAT***a** strain of *mht1*⁻ and selecting for cells able to grow on lysine- and methionine-deficient plates. The resulting diploid strain was induced to sporulate, and the haploid spores containing both gene deletions were identified by screening on kanamycin plates for the non-parental ditype. The deletion of both the *SAM4* and *MHT1* genes was confirmed by PCR analysis using flanking TAG1 and TAG2 primers (www-sequence.stanford.edu/group/yeast_deletion_project/PCR_strategy.html).

*Extract Preparation*—Yeast extracts were prepared by modification of a previous method (31). Briefly, a single colony was inoculated in 6 ml of YPD medium (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose) and incubated overnight with shaking at 30 °C. One ml of the culture was then transferred to 250 ml of YPD medium and grown to an absorbance of 1.0 at 600 nm. Cells were pelleted at 5000 × *g* for 5 min and washed twice with 5 ml of water. The cell pellet volume was estimated, and 2 volumes of lysis buffer (0.1 M sodium phosphate (pH 7.0) and 1 mM phenylmethylsulfonyl fluoride) and 1 volume of baked zirconium beads (BioSpec Products, Inc., Bartlesville, OK) were added. Cells were then lysed via six cycles of alternate 1-min periods of vortexing and incubating on ice. The lysate was removed from the beads and centrifuged at 14,000 × *g* for 50 min at 4 °C. The supernatant was then divided into aliquots of 500 μl and kept at −80 °C until needed. The total protein concentration for each lysate was determined using the method of Lowry *et al.* (32) after precipitation in 10% trichloroacetic acid and using bovine serum albumin as a standard.

*Arabidopsis* seed extract prepared as described previously (33) was provided by Sarah Villa (UCLA). Mouse liver and brain extracts were prepared as described previously (34) and provided by Jonathan Lowenson (UCLA). An extract of *Caenorhabditis elegans* prepared as described previously (35) was provided by Tara Gomez (UCLA). *Drosophila* embryonic extract prepared as described previously (36) was provided by Thomas Fellner (UCLA).

*Homocysteine Methyltransferase Assays*—A modification of the method of Shapiro *et al.* (37) was used. Unless indicated otherwise, 0.2 pmol of purified (*R*,*S*)- or (*S*,*S*)-[$^3$H]AdoMet was incubated with or without the specified amount of DL-homocysteine (Sigma) in 0.15 M sodium phosphate (pH 7.0) with ~0.1 mg of extract protein in a total volume of 200 μl. The temperature of incubation was dependent on the type of extract used: 37 °C for mouse; 30 °C for yeast, *Drosophila* and *C. elegans*; and 40 °C for *Arabidopsis*. At the end of each reaction incubation, [$^3$H]methionine was separated from [$^3$H]AdoMet by cation-exchange chromatography on Dowex 50WX8-400 columns (0.5 cm, inner diameter, × 2 cm; Sigma). Prior to use, the Dowex resin was washed alternately with 1 M HCl and 1 M NaOH, each step being separated by an $H_2O$ wash. This washing sequence was repeated a second time, after which the resin was finally equilibrated with 0.1 M sodium phosphate (pH 7.0). After the reaction mixtures were allowed to flow through the columns, the columns were eluted with 2 ml of $H_2O$, and the total effluent was collected in scintillation vials, mixed with 15 ml of Safety-Solve fluor, and counted on a Beckman LS6500 counter. The amount of [$^3$H]methionine produced was calculated from this radioactivity using a specific activity of 79,000 cpm/pmol.

## RESULTS

*Preparation of (R,S)- and (S,S)-[$^3$H]AdoMet*—In preparing (*R*,*S*)-AdoMet, we took advantage of the fact that (*S*,*S*)-AdoMet can spontaneously racemize to the *R*,*S* form *in vitro* under physiological conditions to produce an *S*,*S*/*R*,*S* mixture that is roughly 50:50 (16–18). In Fig. 3, we compare the $^1$H NMR spectra of a commercial preparation of AdoMet with and without incubation to induce racemization, which was performed at pH 1.0 to minimize the formation of other AdoMet degradation products such as methylthioadenosine and homoserine lactone (18). Clear peaks associated with the methyl group of AdoMet were found at 2.93 ppm in (*S*,*S*)-AdoMet and at 2.89 ppm in (*R*,*S*)-AdoMet (27). Under our conditions, we obtained a 49:51 mixture that was close to the equilibrium distribution. We then used these conditions to generate (*R*,*S*)-[$^3$H]AdoMet from a preparation of (*S*,*S*)-[$^3$H]AdoMet (Fig. 4). We were able to cleanly separate the isotopically labeled *R*,*S* and *S*,*S* forms by

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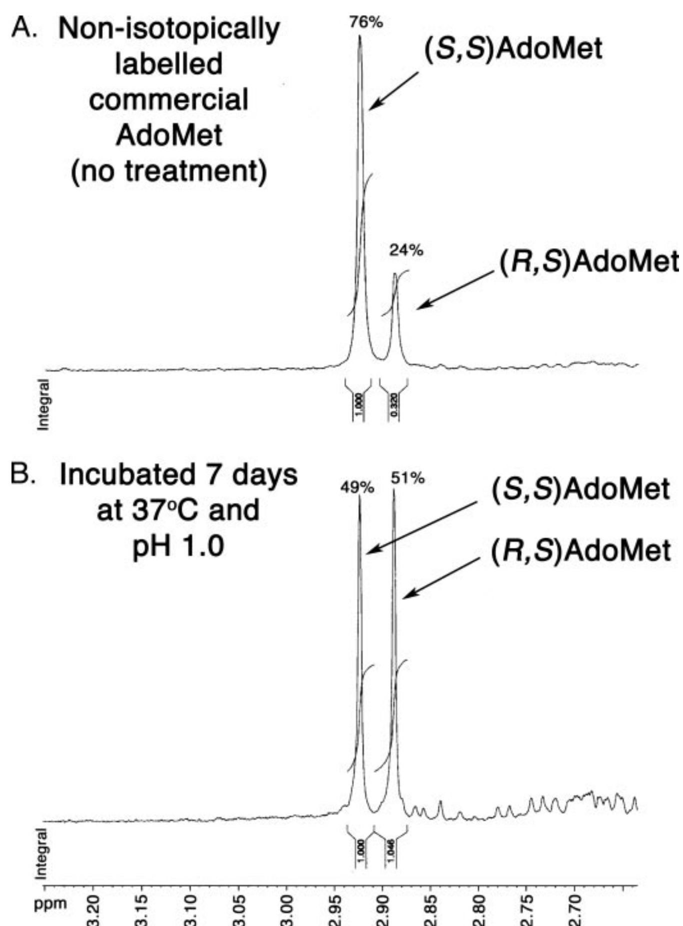


FIGURE 3. **400 MHz $^{1}$H NMR spectra of commercial and racemized AdoMet.** *A*, AdoMet was analyzed directly. The peaks at 2.93 and 2.89 ppm correspond to the hydrogens of the sulfonium-bound methyl groups of (*S*,*S*)- and (*R*,*S*)-AdoMet, respectively. *B*, AdoMet was analyzed after incubation in 0.1 M HCl for 160 h at 37 °C.

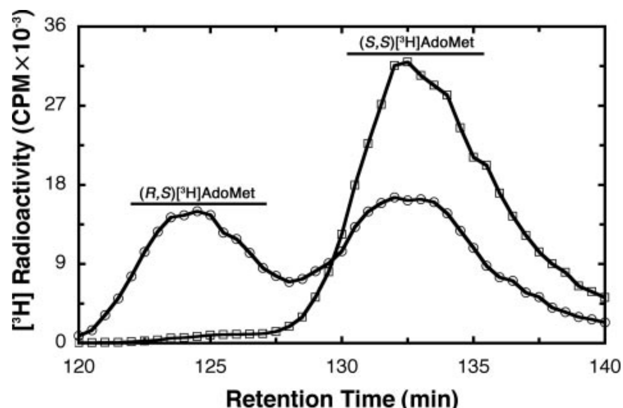


FIGURE 4. **Purification of (*S*,*S*)- and (*R*,*S*)-[$^{3}$H]AdoMet.** (*S*,*S*)-[$^{3}$H]AdoMet and *R*,*S*-enriched [$^{3}$H]AdoMet were fractionated by cation-exchange chromatography using a Partisil SCX HPLC column (4.6 mm, inner diameter, ×250 mm). *R*,*S*-Enriched [$^{3}$H]AdoMet was prepared by incubation at 37 °C for 8 days at pH 1.0. Fractions (1 ml) were collected, and 50 $\mu$l was counted in 2 ml of scintillation fluor. □, radioactivity from the *S*,*S* sample; ○, radioactivity from the *R*,*S*-enriched sample. Fractions corresponding to the *S*,*S* and *R*,*S* peaks were pooled and stored at −80 °C.

cation-exchange HPLC (Fig. 4) (*cf.* Ref. 18). To confirm the identity of the labeled (*R*,*S*)- and (*S*,*S*)-AdoMet samples obtained from the HPLC purification, we tested their ability to serve as substrates for a typical AdoMet-dependent methyltransferase that would be expected to use the *S*,*S* form. Fig. 5 shows that the human recombinant L-isoaspartyl protein repair methyltransferase uses purified (*S*,*S*)-[$^{3}$H]AdoMet essentially to completion (94%), whereas no use is made of purified (*R*,*S*)-[$^{3}$H]AdoMet.

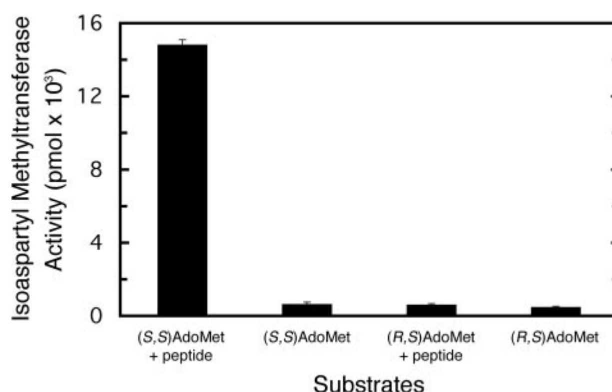


FIGURE 5. **Confirmation of the configuration of purified (*S*,*S*)- and (*R*,*S*)-[$^{3}$H]AdoMet by their utilization by the protein L-isoaspartyl *O*-methyltransferase.** Human recombinant L-isoaspartyl protein methyltransferase activity was measured in the presence and absence of the peptide substrate KASA(isoD)LAKY using 0.016 pmol of either (*S*,*S*)- or (*R*,*S*)-[$^{3}$H]AdoMet prepared as described in the legend to Fig. 4. Assays were done in triplicate, and *error bars* represent S.D.

*Yeast Sam4 and Mht1 Catalyze R,S-Dependent Homocysteine Methyltransferase Activity*—We first investigated whether the extracts of wild-type *S. cerevisiae* could use (*R*,*S*)-AdoMet as a methyl donor in the conversion of homocysteine to methionine. We found that this was the case; after 60 min of incubation, we could quantitatively convert (*R*,*S*)-[$^{3}$H]AdoMet to a neutral species consistent with [$^{3}$H]methionine (Fig. 6). We found a similar result for (*S*,*S*)-[$^{3}$H]AdoMet, indicating that both diastereomers could be utilized as methyl donors for yeast homocysteine methyltransferases. Control reactions in the absence of homocysteine revealed little or no methylation activity (Fig. 6), confirming the specificity of the assay. We also confirmed that the product was [$^{3}$H]methionine in each case by co-chromatography of the product with a methionine standard by thin-layer chromatography using silica plates and a solvent system of *n*-butyl alcohol/acetic acid/water (4:1:1) (data not shown).

Two homocysteine methyltransferases have been described in yeast, the products of the *SAM4* and *MHT1* genes (29). Thus, we measured the contribution of each of these enzymes to (*R*,*S*)-AdoMet utilization. When extracts of a *sam4*$^{-}$ knock-out strain were incubated with (*S*,*S*)-[$^{3}$H]AdoMet, we found no homocysteine-dependent methyltransferase activity (Fig. 6), suggesting that (*S*,*S*)-AdoMet utilization in homocysteine methylation in yeast is limited to the Sam4 enzyme. On the other hand, homocysteine-dependent methyltransferase activity with (*R*,*S*)-[$^{3}$H]AdoMet, although decreased, was still present (Fig. 6). This result provides the first direct evidence that the "degraded" *R*,*S* form of AdoMet may be utilized in a specific methyltransferase reaction. This result also suggests that, although Sam4 is responsible for most of the (*R*,*S*)-AdoMet-dependent homocysteine methyltransferase activity, there is at least one additional activity present that can utilize the *R*,*S* diastereomer, but not the *S*,*S* diastereomer, of AdoMet for methionine synthesis.

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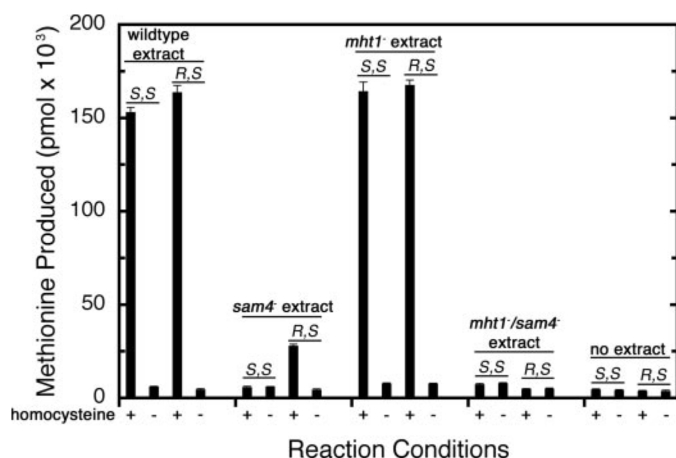
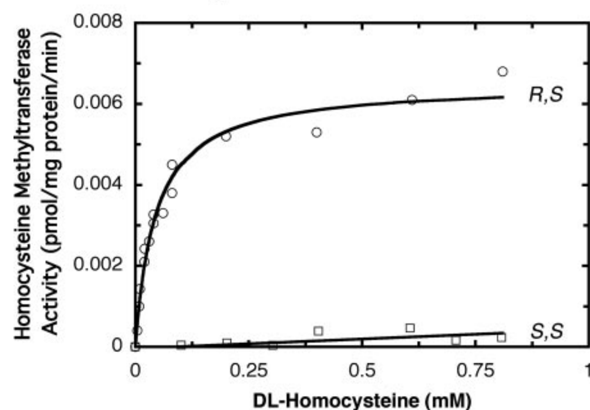



FIGURE 6. **Substrate specificity of yeast Sam4 and Mht1 homocysteine methyltransferases for (*S,S*)- and (*R,S*)-AdoMet.** Homocysteine methyltransferase activity was measured in wild-type, *sam4*$^-$, *mht1*$^-$, or *sam4*$^-$/*mht1*$^-$ *S. cerevisiae* extracts. Extracts (0.1 mg of protein) were prepared as described under "Experimental Procedures" and incubated with either (*R,S*)- or (*S,S*)-[$^3$H]AdoMet at a final concentration of 1 nM for 60 min at 30 °C. The reactions were buffered with 150 mM sodium phosphate (pH 7.0) in the presence or absence of 0.8 mM DL-homocysteine. The activity was quantified by the amount of [$^3$H]methionine produced as determined by cation-exchange chromatography as described under "Experimental Procedures." Assays were done in triplicate, and *error bars* represent S.D.

We thus proceeded to determine whether Mht1 is responsible for all or part of the Sam4-independent homocysteine methyltransferase activity. We first analyzed activity in an extract of an *mht1*$^-$ deletion strain. We detected little change in activity with either (*R,S*)- or (*S,S*)-[$^3$H]AdoMet, confirming the major role of the Sam4 enzyme. We then prepared a *sam4*$^-$/*mht1*$^-$ double knock-out strain. Extracts of this strain were found to be incapable of catalyzing homocysteine methylation with either (*R,S*)- or (*S,S*)-[$^3$H]AdoMet (Fig. 6). This experiment demonstrates that the utilization of (*R,S*)-AdoMet for methionine synthesis in the *sam4*$^-$ extract is due to the Mht1 protein. This protein was demonstrated previously to catalyze homocysteine methylation using *S*-methylmethionine as a methyl donor (29). These results show that it can also utilize (*R,S*)-AdoMet. Overall, our data suggest that Sam4 and Mht1 are responsible for the (*R,S*)-AdoMet-dependent homocysteine methyltransferase activity seen in *S. cerevisiae* and that Sam4 is responsible for the activity with (*S,S*)-AdoMet.

Because zinc has been shown to play a role in the homocysteine-binding site of other methionine-forming enzymes (38, 39), we investigated whether the activities of the yeast enzymes might be enhanced by the addition of zinc ion to the assay mixture. However, in experiments with *mht1*$^-$ and *sam4*$^-$ extracts, we found no effect of the addition of either 1 mM zinc chloride or 1 mM sodium EDTA on the homocysteine methyltransferase activities (data not shown).

*Kinetics of Mht1 and Sam4 AdoMet-dependent Homocysteine Methyltransferases*—To better understand the importance of these enzymes *in vivo*, we characterized the kinetics of the Mht1 and Sam4 methyltransferases *in vitro*. Using *sam4*$^-$ extracts as a source of the Mht1 enzyme, we confirmed that this enzyme is specific for the *R,S* form of AdoMet (Fig. 7*A*). Similarly, using *mht1*$^-$ extracts, we demonstrated the ability of Sam4 to use both forms of AdoMet (Fig. 7*B*). The maximal velocities of these enzymes and their $K_m$ values for homocysteine from these data are compared in Table 2. In these experiments, the presence of other components in the cell extracts may have influenced the observed $K_m$ and $V_{\max}$ values, but would be expected to reflect the situation in intact cells. We found that, although Sam4 is capable of using both (*S,S*)- and (*R,S*)-AdoMet as substrates, it has a 2.5-fold higher activity with the *R,S* form (Table 2). The $K_m$ for homocysteine is similar with both methyl donors, ranging from 32 to 44 $\mu$M, resulting in a catalytic efficiency ($V_{\max}/K_m$) with (*R,S*)-AdoMet that is 3.3-fold larger than that with (*S,S*)-AdoMet. These results suggest that the predominant physiological substrate of the Sam4 homocysteine methyltransferase is the racemized (*R,S*)-AdoMet form.



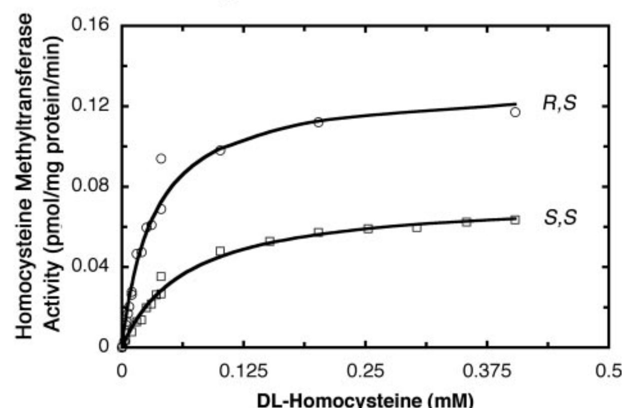




FIGURE 7. **Effect of DL-homocysteine concentration on the activities of the yeast Mht1 and Sam4 homocysteine methyltransferases using either (*R,S*)- or (*S,S*)-AdoMet.** Assays were performed as described under "Experimental Procedures" with 0.1 mg of extract protein in a final concentration of 1.7 nM (*S,S*)-[$^3$H]AdoMet or 1 nM (*R,S*)-[$^3$H]AdoMet in 150 mM sodium phosphate buffer (pH 7.0) with varying concentrations of DL-homocysteine. Incubations were done at 30 °C for 30 min for the *sam4*$^-$ extract (*A*) or for 10 min for the *mht1*$^-$ extract (*B*). ○, *R,S* form; □, *S,S* form. Data were obtained from three experiments using (*R,S*)-AdoMet and from two experiments using (*S,S*)-AdoMet.

Using (*R,S*)-AdoMet as the methyl donor, we found that the Mht1 enzyme has a $K_m$ for homocysteine of 45 $\mu$M, a value similar to those of the Sam4 enzyme with (*R,S*)- and (*S,S*)-AdoMet (Table 2). However, we found that the catalytic efficiency of Mht1with the *R,S* form is 27-fold less than that of

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**TABLE 2**

**Kinetic values of the Mht1 and Sam4 homocysteine methyltransferases with (*R,S*)- or (*S,S*)-AdoMet in cell extracts**

Kinetic values were determined by fitting the data shown in Fig. 7 to the Michaelis-Menten equation using the $K_m$ calculator of the BioMechanic program (www.biomechanic.org/). Values are the means ± S.D., calculated from individual activity measurements at DL-homocysteine concentrations from 0.004 to 0.4 mM. The S.D. values for $K_m$ values were calculated based on the fitted $V_{max}$ values and the S.D. values for $V_{max}$ values from the fitted $K_m$ values. In all cases, the concentration of AdoMet was 1 nM. Hcy, homocysteine.

| Enzyme | (*R,S*)-AdoMet | | | (*S,S*)-AdoMet | | |
|---|---|---|---|---|---|---|
| | $K_m$ for Hcy | $V_{max}$ | $V_{max}/K_m$ | $K_m$ for Hcy | $V_{max}$ | $V_{max}/K_m$ |
| | *mM* | *pmol/min/mg protein* | | *mM* | *pmol/min/mg protein* | |
| Mht1 | 0.045 ± 0.015 | 0.0065 ± 0.008 | 0.15 | | 0 | 0 |
| Sam4 | 0.032 ± 0.009 | 0.13 ± 0.02 | 4.0 | 0.044 ± 0.007 | 0.052 ± 0.003 | 1.2 |

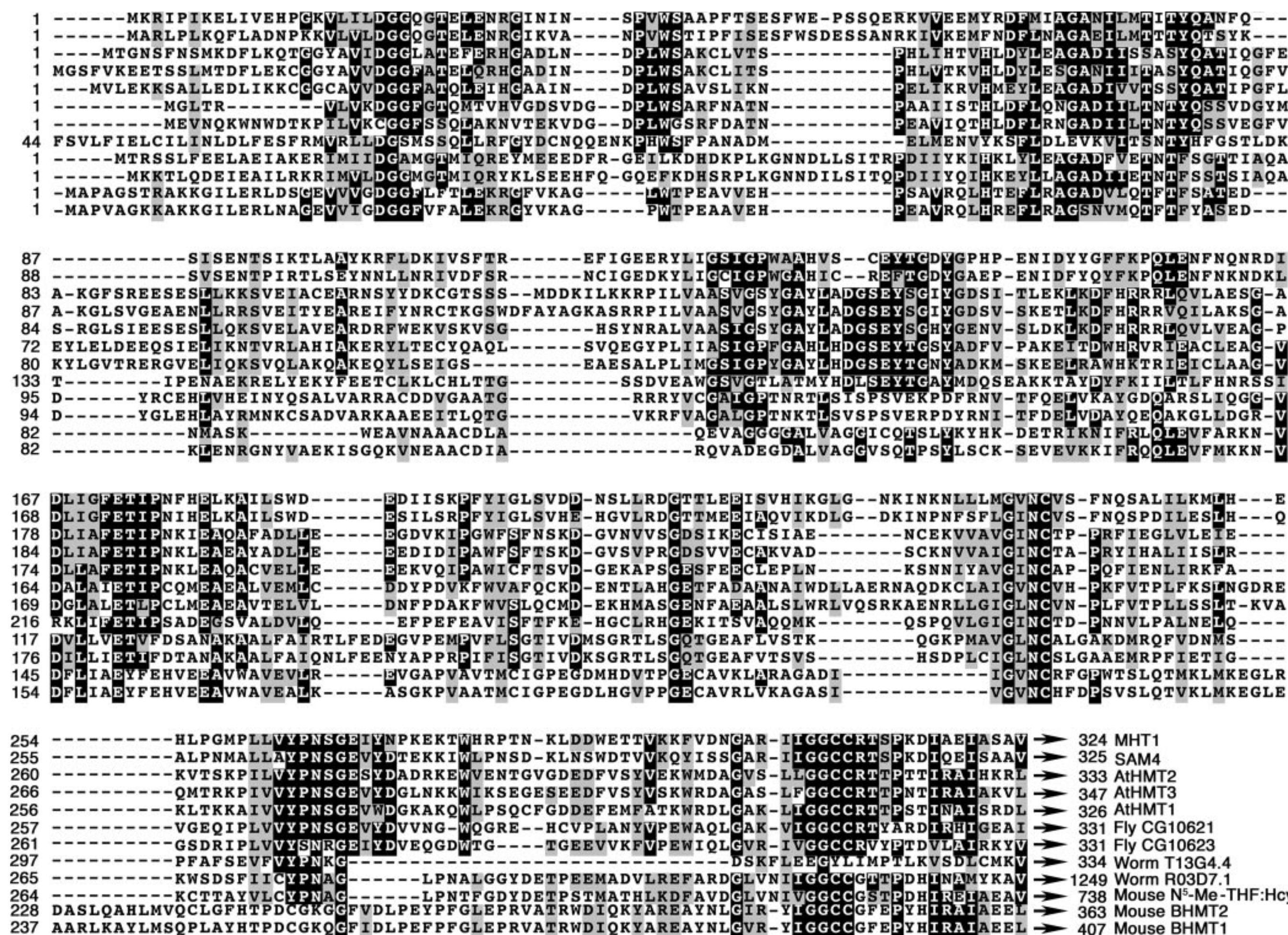


FIGURE 8. **Comparison of deduced amino acid sequences of the yeast *SAM4* and *MHT1* genes with those of homologs in *Drosophila*, mouse, *Arabidopsis*, and *C. elegans*.** Sequence alignments were made using the ClustalW (www.ebi.ac.uk/clustalw/) and BOXSHADE (www.ch.embnet.org/software/BOX_form.html) programs. We manually aligned the sequence in the region of the conserved GXNC sequence (29, 40). *Black boxes* indicate identical residues, and *gray boxes* indicate conservative replacements. Proteins are identified on the bottom right. NCBI accession numbers include NP_013038 (Mht1), NP_015050 (Sam4), AAF23822 (*A. thaliana* (*At*) HMT2), AAG10301 (*A. thaliana* HMT3), NP_189219 (*A. thaliana* HMT1), NP_609920 (fly CG10621), AAM50732 (Fly CG10623), AAK84573 (worm T13G4.4), CAA86855 (worm R03D7.1), BAE24997 (mouse $N^5$-methyltetrahydrofolate-homocysteine methyltransferase (*$N^5$-Me-THF:Hcy MT*)), AAH13515 (mouse BHMT2), and AAI10308 (mouse BHMT1).

Sam4 for the *R,S* form. Mht1 has little or no detectable activity for the *S,S* form.

*Homocysteine Methyltransferase Activities That Utilize the R,S Form of AdoMet Are Present in a Variety of Organisms*—Genes that are homologous to the yeast *SAM4* and *MHT1* genes are present in *Arabidopsis*, *Drosophila*, and *Escherichia coli* (29, 40). Using a series of BLAST searches, we determined that there are at least three homologous genes present in *Arabidopsis*, two in *Drosophila*, two in *C. elegans*, and three in mice (Fig. 8). We thus looked for *R,S*- and *S,S*-dependent AdoMet activity in extracts of these organisms. In embryonic *Drosophila* extract, a situation similar to that in yeast was observed in which both the *S,S* and *R,S* forms of AdoMet were used by the extract to methylate homocysteine, with a similar preference for the *R,S* form (Fig. 9) (*cf.* Fig. 6). Unlike the situation seen in yeast or *Drosophila*, *Arabidopsis* seed extract had little or no detectable homocysteine methyltransferase activity with (*S,S*)-AdoMet (Fig. 9). However, (*R,S*)-AdoMet was found to be an effective substrate (Fig. 9). Similar *R,S*-specific activity could be seen with the *C. elegans* extract.

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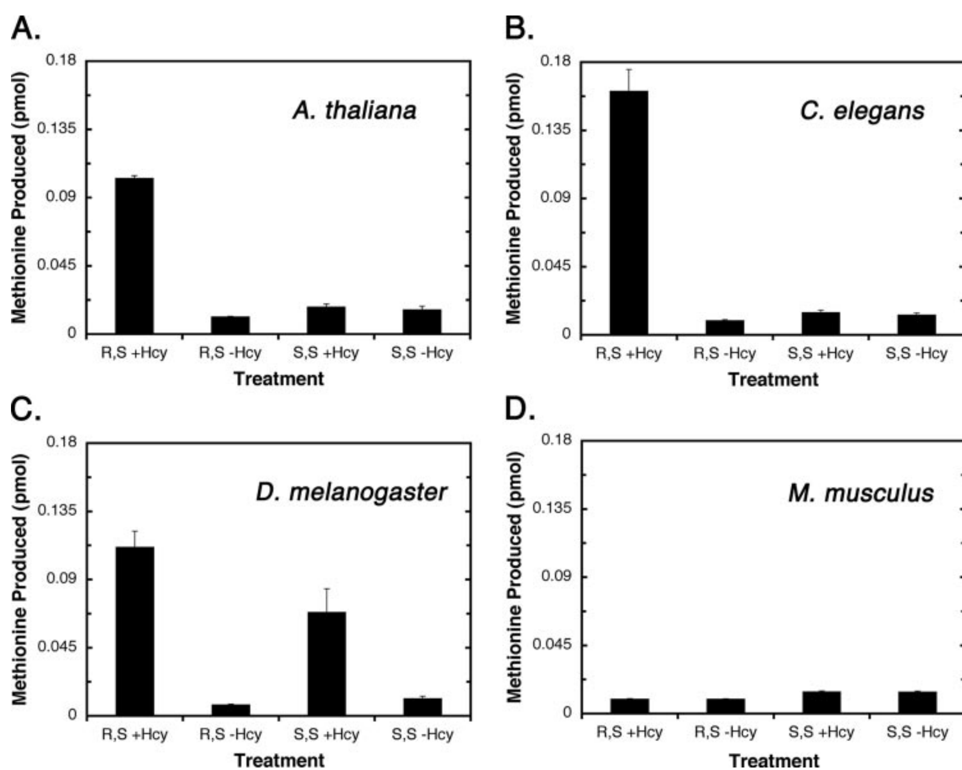


FIGURE 9. ***R,S*- and *S,S*-specific homocysteine methyltransferase activities in extracts of *A. thaliana*, *C. elegans*, *D. melanogaster*, and *M. musculus*.** Extracts were prepared as described under "Experimental Procedures." Extracts (0.1 mg of protein) were incubated for 60 min with 1 nM (*R,S*)- or (*S,S*)-[$^3$H]AdoMet in the presence or absence of 0.8 mM DL-homocysteine. Homocysteine methyltransferase activity was determined as described under "Experimental Procedures." *A*, *A. thaliana* seed extract at 40 °C; *B*, *C. elegans* extract at 30 °C; *C*, *D. melanogaster* embryonic extract at 30 °C; *D*, *M. musculus* brain extract at 37 °C. Assays were done in duplicate, and *error bars* represent S.D.

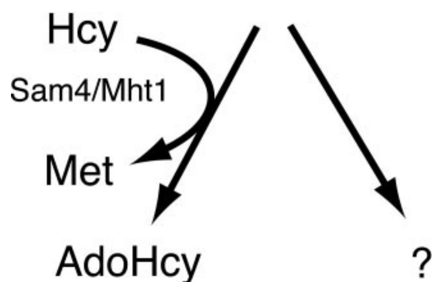


FIGURE 10. **Metabolism of (*R,S*)-AdoMet in yeast.** The Sam4 and Mht1 homocysteine (*Hcy*) methyltransferases can selectively utilize (*R,S*)-AdoMet that forms because of the spontaneous degradation of (*S,S*)-AdoMet.

Unexpectedly, no AdoMet-dependent homocysteine methyltransferase activity was observed in mouse brain extract (Fig. 9) or in extracts of liver, kidney, and heart tissue (data not shown). The only gene products in mouse with apparent homology to the two yeast gene products are the $N^5$-methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase), the betaine-specific homocysteine methyltransferase (BHMT1), and the BHMT2 product (Fig. 8). These latter enzymes appear to be more evolutionarily distant and appear to have lost the ability to utilize (*R,S*)-AdoMet as a methyl donor. We performed control experiments to show that both methionine synthase and BHMT2 were active in our preparation of mouse liver extracts. Methionine synthase was assayed as described (41); BHMT2 was assayed using *S*-methylmethionine as a substrate (42) in the presence of the BHMT1-specific homocysteine methyltransferase inhibitor *S*-(δ-carboxybutyl)-L-homocysteine (43, 44).[3] Because the conserved C-terminal cysteine residues of the enzyme homologs (Fig. 8) are associated with bound zinc ions in the active site in some of these enzymes (38–40), we performed controls in which reaction mixtures of (*R,S*)-AdoMet and mouse liver extracts were supplemented with 1 mM zinc. However, no activity was observed (data not shown). If there are enzymes in mice that can use (*R,S*)-AdoMet or (*S,S*)-AdoMet to methylate homocysteine, we could not detect their activity under our conditions.

## DISCUSSION

In this work, we have provided evidence that at least two enzymes in yeast are capable of recognizing the age-damaged *R,S* form of AdoMet. These enzymes catalyze the formation of methionine from homocysteine utilizing the methyl group of (*R,S*)-AdoMet in a reaction that converts it to *S*-adenosyl-L-homocysteine (AdoHcy) for further metabolism to adenosine and homocysteine. The combined activities of the Mht1 and Sam4 enzymes, along with AdoMet synthetase and AdoHcy hydrolase, would be expected to catalyze the ATP-dependent conversion of (*R,S*)-AdoMet to (*S,S*)-AdoMet (Figs. 2 and 10). It is not clear at this point how effective these reactions are at preventing the accumulation of damaged AdoMet and whether these methyltransferases are the only activities in cells that can recognize (*R,S*)-AdoMet (Fig. 10).

In *E. coli*, the HemN coproporphyrin III oxidase has been crystallized with either (*R,S*)- or (*S,S*)-AdoMet in the active site adjacent to a [4Fe-4S] cluster (45). It has been speculated that this enzyme may be able to interconvert the *R,S* and *S,S* forms (45), although it is unclear what type of mechanism may drive

[3] T. A. Garrow, personal communication.

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**TABLE 3**

**Substrate specificities for homocysteine methyltransferases in nature utilizing $N^5$-methyltetrahydrofolate, S-methylmethionine, betaine, and AdoMet as methyl donors**

$N^5$-MeTHF, $N^5$-methyltetrahydrofolate; $N^5$-MeTHF-Hcy MT, $N^5$-methyltetrahydrofolate-homocysteine methyltransferase; +, experimental evidence exists for methyl donor utilization; −, experimental evidence exists for the inability to serve as a methyl donor.

| Enzyme | EC No. | Methyl donor | | | | | Ref. |
|---|---|---|---|---|---|---|---|
| | | $N^5$-MeTHF | *S*-MeMet | Betaine | (*R*,*S*)-AdoMet | (*S*,*S*)-AdoMet | |
| Yeast Mht1 | 2.1.1.10 | | + | | + | − | 29, this study |
| Yeast Sam4 | 2.1.1.10 | | | | + | + | 29, this study |
| *A. thaliana* HMT1 | 2.1.1.10 | | + | | | + | 40 |
| *A. thaliana* HMT2 | 2.1.1.10 | | + | | | + | 40 |
| *A. thaliana* HMT3 | 2.1.1.10 | | + | | | + | 40, 47 |
| Mammalian $N^5$-MeTHF-Hcy MT | 2.1.1.13 | + | | | | | 48 |
| Mammalian BHMT1 | 2.1.1.5 | | | + | | | 49 |
| Mammalian BHMT2 | | | + | − | | | 42 |

the formation of (*S*,*S*)-AdoMet and whether such an activity is present in other organisms.

We have shown here that (*R*,*S*)-AdoMet-specific homocysteine methyltransferase activities exist in a variety of species in addition to yeast, including plants and invertebrates. However, we found no evidence for such activities in several mouse tissues. It is thus unclear how mammalian cells metabolize (*R*,*S*)-AdoMet. In bacterial cells, the YagD protein can utilize not only *S*-methylmethionine as a methyl donor to either homocysteine or selenohomocysteine, but also a fraction of an AdoMet preparation that has been suggested to represent the *R*,*S* form (28, 29). Similar results have been found with a selenocysteine methyltransferase from the legume *Astragalus bisulcatus* that is homologous to the *yagD* gene of *E. coli*, suggesting that both enzymes may preferentially use (*R*,*S*)-AdoMet (28). It is interesting to note that *S*-methylmethionine-specific homocysteine methyltransferases from *Arabidopsis* transfer the pro-*R*-methyl group from *S*-methylmethionine that corresponds to the sulfonium-bound methyl group from (*R*,*S*)-AdoMet (40, 46).

Biochemical and sequence studies have shown that homologous homocysteine methyltransferases in nature can utilize a variety of methyl donors (Table 3). These enzymes do not have sequences similar to those of the seven-stranded β-sheet methyltransferase enzymes or other known types of methyltransferases (50) and appear to represent a new class of methyltransferases based on binding the methyl-accepting substrate and not the specific methyl-donating substrate. Although it has been reported that the three *Arabidopsis* enzymes use (*S*,*S*)-AdoMet (40, 47), the fact that we observed *R*,*S*-specific, and not *S*,*S*-specific, AdoMet-dependent homocysteine methyltransferase activity in *Arabidopsis* extracts suggests that these three enzymes might actually be more specific for (*R*,*S*)-AdoMet. It is also of interest that the closest mammalian homologs of the yeast Sam4 and Mht1 enzymes in mammals use $N^5$-methyltetrahydrofolate (48), betaine (49), or *S*-methylmethionine (42) as a methyl-donating substrate (Table 3). The fact that (*R*,*S*)-AdoMet-dependent methyltransferase activity was observed in yeast, *Drosophila*, nematodes, and plants but not in mammals is puzzling, but suggests that the mammalian enzymes may have enhanced specificity for the methyl donor. Interestingly, it has been reported that the human BHMT2 enzyme has a trace activity with AdoMet (42); further investigation is warranted here.

The utilization by the yeast Sam4 enzyme of both (*S*,*S*)- and (*R*,*S*)-AdoMet is of interest because Sam4 expression levels increase when yeast is grown in the presence of high levels of methionine (29). Because most enzymes involved in methionine synthesis, including Mht1, are down-regulated under this condition, Sam4 may have an additional role of regulating AdoMet when it is present in excess (29). If this is indeed the case, it would be an added benefit for this enzyme to deplete cells of both the *R*,*S* and *S*,*S* forms.

Although much is known about the biological pathways in which the *S*,*S* form of AdoMet participates, the cellular interactions and metabolism of its *R*,*S* degradation product are only beginning to be understood. We still need to know how "toxic" (*R*,*S*)-AdoMet is. It has been reported that (*R*,*S*)-AdoMet inhibits the (*S*,*S*)-AdoMet-dependent activities of catechol *O*-methyltransferase, histamine *N*-methyltransferase, hydroxyindole *O*-methyltransferase, and phenylethanolamine *N*-methyltransferase (24). However, another study demonstrated no effect of (*R*,*S*)-AdoMet on the activity of the latter enzyme (25). (*R*,*S*)-AdoMet may also be responsible for the generation of other types of cellular inhibitors. For example, the enzyme 1-aminocyclopropane-1-carboxylate synthase usually reacts with (*S*,*S*)-AdoMet to produce 1-aminocyclopropane 1-carboxylate and 5′-methylthioadenosine. However, with (*R*,*S*)-AdoMet, it can form L-vinylglycine as a suicide substrate inhibitor (51, 52). Although we are only in the initial stages of understanding how (*R*,*S*)-AdoMet may affect cellular function, the results presented in this study suggest that a variety of cell types contain enzymes that can specifically recognize it and convert it to molecules that can be readily metabolized. As such, these (*R*,*S*)-AdoMet-dependent homocysteine methyltransferases can be seen as a line of defense against the spontaneous accumulation of a potentially harmful molecule.

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