Homocysteine Methyltransferases Mht1 and Sam4 Prevent the Accumulation of Age-damaged (R,S)-AdoMet in the Yeast Saccharomyces cerevisiae*

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The biological methyl donor S-adenosyl-l-methionine (AdoMet) is spontaneously degraded by inversion of its sulfonium center to form the R,S diastereomer. Unlike its precursor, (S,S)-AdoMet, (R,S)-AdoMet has no known cellular function and may have some toxicity. Although the rate of (R,S)-AdoMet formation under physiological conditions is significant, it has not been detected at substantial levels in vivo in a wide range of organisms. These observations imply that there are mechanisms that either dispose of (R,S)-AdoMet or convert it back to (S,S)-AdoMet. Previously, we identified two homocysteine methyltransferases (Mht1 and Sam4) in yeast capable of recognizing and metabolizing (R,S)-AdoMet. We found similar activities in worms, plants, and flies. However, it was not established whether these activities could prevent R,S accumulation. In this work, we show that both the Mht1 and Sam4 enzymes are capable of preventing R,S accumulation in Saccharomyces cerevisiae grown to stationary phase; deletion of both genes results in significant (R,S)-AdoMet accumulation. To our knowledge, this is the first time that such an accumulation of (R,S)-AdoMet has been reported in any organism. We show that yeast cells can take up (R,S)-AdoMet from the medium using the same transporter (Sam3) used to import (S,S)-AdoMet. Our results suggest that yeast cells have evolved efficient mechanisms not only for dealing with the spontaneous intracellular generation of the (R,S)-AdoMet degradation product but for utilizing environmental sources as a nutrient.

Aging can be seen as the accumulation of damaged biomolecules over time (1–3). As such, understanding the mechanisms by which organisms can slow such accumulation, as well as how these mechanisms may themselves eventually break down and fail, is crucial to an understanding of the aging process. Repair pathways for damaged DNA have been well established (4); damaged proteins can be removed by a combination of proteolytic and repair pathways (3, 5–8). However, we only are begin-

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findings suggest that there are mechanisms responsible for keeping (R,S)-AdoMet at levels low in cells.

There are several possible ways that (R,S)-AdoMet can be depletred from cells. At least one radical AdoMet enzyme, the HemN coproporphyrin III oxidase in Escherichia coli, has been found to bind both (R,S)- and (S,S)-AdoMet (25). However, it is unclear whether the R,S form is converted to methionine and the deoxyadenosyl radical. Another depletion pathway involves homocysteine methylenetransferase activity that transfers methyl groups from a methyl donor to homocysteine to create methionine. We previously found that two homocysteine methyltransferases in the yeast Saccharomyces cerevisiae, Mht1 and Sam4, are capable of using (R,S)-AdoMet as the methyl donor, thus metabolizing this molecule (15). Sam4 was found to metabolize both the S,S and the R,S form of AdoMet, with a higher affinity for the R,S form, while Mht1 was found to metabolize only the R,S form. Homologs for Mht1 and Sam4, as well as (R,S)-AdoMet dependent homocysteine methyltransferase activities, were also found in worms, plants, and flies (15).

In this work, we now demonstrate that these enzymes are capable of limiting the accumulation of (R,S)-AdoMet in intact yeast cells. Furthermore, we show that yeast cells are capable of transporting (R,S)-AdoMet from the external environment, suggesting that yeast can metabolize both endogenously formed and environmental (R,S)-AdoMet.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth for Accumulation Assays**—Strains used are described in Table 1. Strains CVY1, -2, and -3 were prepared by mating the mht1Δ (BY4741) and sam4Δ (BY4742) strains followed by tetradd analysis as described previously (15). The presence or absence of the MHT1 and SAM4 genes was determined by PCR analysis and sensitivity to kanamycin as described previously (15). Cells were grown after an initial inoculation in a 6-ml YPD medium (1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose) starter culture and shaken overnight at 30°C. These cultures were then diluted in 250 ml of YPD to an optical density of 0.01 at 600 nm and then incubated with shaking at 225 rpm at 30°C. For the accumulation experiments, 25 ml of the culture was collected at various time points. A 100-μl aliquot was diluted with 900 μl of water to measure the optical density at 600 nm with a Beckman DU640B spectrophotometer. The rest of the 25 ml was centrifuged at 3,000 rpm for 5 min at 4°C in a Beckman Coulter Allegra X-15R swinging bucket centrifuge. The resulting pellets were washed once after resuspension in 5 ml of deionized water and centrifugation as before. The final pellet was then stored at −80°C until needed for extract preparation.

**Preparation of Yeast Extracts for AdoMet Analysis**—Cell pellets for each time point prepared as described above were melted on ice. 100 μl of each resulting wet cell pellet was then combined with 200 μl of deionized water and 100 μl of glass beads (0.55 mm soda lime; BioSpec Products, Bartlesville, OK) in a 1.6-ml S̄ low retention polymer microcentrifuge tube. The resuspended cells were then alternately vortexed and iced for 1 min for seven cycles as described previously (15). Broken cells were then transferred to new tubes and centrifuged for 10 min at 20,800 g at 4°C. Supernatants were then transferred to new tubes and combined with 200 μl of 20% (w/v) trichloroacetic acid. After vortexing, the tubes were incubated on ice for 10 min and then centrifuged for 10 min as above. The supernatants were then stored at −80°C until needed for HPLC analysis.

**Measurement of (R,S)- and (S,S)-AdoMet in Yeast Exports**—50 μl of each extract was injected on a Partisil SCX column eluted at 1 ml/min with a 60/40 ratio of buffers A and B as described previously (15). Elution times for (R,S)- and (S,S)-AdoMet were determined using a 50/50 (R,S)/(S,S)-AdoMet standard prepared as described previously (15) and injected prior to each set of experiments.

**Measurement of AdoMet Uptake in Intact Yeast**—Single colonies of the different yeast strains were inoculated in 6 ml of YPD medium. They were grown overnight with shaking at 30°C. Samples were then diluted 10-fold in 5 ml of fresh medium and grown to an optical density at 600 nm of 1.0. Depending on the experiment, a specified amount of radiolabeled (R,S)- or (S,S)-[3H]AdoMet, prepared as described previously (15), was combined with an aliquot from each culture for a total volume of 1 ml each and was incubated with shaking at 30°C for the specified amount of time. At each time point, the tubes were removed from shaking and spun at 3,000 × g for 5 min at 4°C. The resulting pellets were separated from the supernatants, and extracts were prepared by adding an equal volume of glass beads and two volumes of water and lyzed as described above. Radioactivity was measured for the supernatants and extracts by combining each with 5 ml of fluor (Fluorosolve, Research Products International, Mount Prospect, IL) and counting them on a Beckman LS6500 counter. The configuration of the internalized (R,S)-AdoMet 9 h after uptake by BY4742 yeast was determined by injection of the extract onto the Partisil SCX column as described above along with the 50/50 R,S/S,S standard.

**NMR Analysis of AdoMet Racemization**—AdoMet (chloride salt; purity ≥70% with 1 mol/mol H2O and 4.6% methanol; Sigma) was dissolved in 0.1 M HCl at a concentration of 30 mg/ml and incubated at 30°C and 37°C. At specified time points, 100 μl aliquots were collected, dried, and dissolved in D2O to final concentrations of 6 mg/ml. The 1H NMR spectrum for 500 μl of each aliquot was determined using a Bruker

**TABLE 1**

**Yeast strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>BY4741</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</td>
<td>SGDp&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>mht1&lt;sup&gt;Δ&lt;/sup&gt; (BY4741)</td>
<td>MATa his3Δ1 leu2Δ0lys2Δ0ura3Δ0</td>
<td>SGDp&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>BY4742</td>
<td>MATa his3Δ1 leu2Δ0 lys2Δ0ura3Δ0</td>
<td>SGDp&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>sam4Δ (BY4742)</td>
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<td>SGDp&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>mht1&lt;sup&gt;Δ&lt;/sup&gt; (BY4742)</td>
<td>BY4742, Δygl062c::Kan&lt;sup&gt;1&lt;/sup&gt;</td>
<td>SGDp&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>CVY1 mht1&lt;sup&gt;Δ&lt;/sup&gt; sam4Δ</td>
<td>MATa Δygl273w::Kan&lt;sup&gt;1&lt;/sup&gt; Δygl062c::Kan&lt;sup&gt;1&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>CVY2 mht1&lt;sup&gt;Δ&lt;/sup&gt; sam4Δ</td>
<td>MATa Δygl273w::Kan&lt;sup&gt;1&lt;/sup&gt; Δygl062c::Kan&lt;sup&gt;1&lt;/sup&gt;</td>
<td>This study</td>
</tr>
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<td>mmp1&lt;sup&gt;Δ&lt;/sup&gt; (BY4742)</td>
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<td>BY4742, Lyn&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>1</sup> Strains were prepared by the Saccharomyces Genome Deletion Project and purchased from Invitrogen.
ARX400 spectrometer operating at 400.13 MHz as described previously (15, 26). Relative levels of \((R,S)\) and \((S,S)\)-AdoMet were determined using the integrals of NMR peaks at 2.89 and 2.93 ppm, respectively.

**RESULTS**

\((R,S)\)-AdoMet Accumulates in \(mht1^−/sam4^−\) Yeast Grown in Stationary Phase—We first measured the relative levels of \((R,S)\)-AdoMet and \((S,S)\)-AdoMet after HPLC separation from extracts of yeast in stationary phase. For the BY4742 wild type parent strain, we found that almost all of AdoMet is in the \(S,S\) configuration at both the 72- and 152-h incubation points (Fig. 1). However, for the CVY1 mutant strain deleted in both the \(MHT1\) and \(SAM4\) genes, we found an accumulation of \((R,S)\)-AdoMet at 72 h in stationary phase and an even greater accumulation at 152 h (Fig. 1). Analysis of a similar experiment with an independently prepared double mutant (CVY2) and wild type parent strain (CVY3) gave similar results (data not shown). When we measured \((S,S)\) and \((R,S)\)-AdoMet levels in single mutant strains of either the \(MHT1\) or \(SAM4\) genes in stationary phase, we found that the presence of either gene was sufficient to reduce \(R,S\) levels to nearly those of wild type cells (Fig. 2).

To determine the kinetics of \(R,S\) accumulation, we measured the concentration of \((R,S)\)-AdoMet and \((S,S)\)-AdoMet as a function of incubation time for the BY4742 wild type strain and the CVY1 \(mht1^−/sam4^−\) double mutant strain (Fig. 3). We found that the total levels of AdoMet measured at times up to 72 h for both strains were similar to those found previously in other strains (27). For the wild type cells shown in Fig. 3A, the level of \((S,S)\)-AdoMet peaked at 72 h and then declined as cells continue in stationary phase. A similar rise and fall was found for the level of \((R,S)\)-AdoMet that was present at 0 to 7% of the total level of AdoMet (Fig. 3C). For the mutant strain, the levels of \((S,S)\)-AdoMet were similar to those found for the wild type strain (Fig. 3B). However, the level of \((R,S)\)-AdoMet in the mutant cells continued to rise with incubation time in stationary phase, resulting in its net accumulation (Fig. 3B). These data are summarized in Fig. 3C, which shows how the percent of AdoMet in the \(R,S\) form increases with time in both the wild type and mutant strains but much more extensively in the mutant strain.

To compare the rate of accumulation of \((R,S)\)-AdoMet in the mutant strain with the rate of spontaneous chemical formation, we measured the racemization rate of AdoMet in solution at the growth temperature of 30 °C (Fig. 3D). We found that the accumulation of \((R,S)\)-AdoMet in yeast cells and in chemical solution was similar (compare Fig. 3, C and D). The in vivo formation of \((R,S)\)-AdoMet in the \(mht1^−/sam4^−\) double knockout strain was somewhat less that that of chemical formation, possibly due to the continuous production of \((S,S)\)-AdoMet that occurs up until the 72-h incubation point (Fig. 3B). In both cases, the \(R:S:S:S\) ratio approached a 50:50 equilibrium.

In these experiments, we noted no difference in the growth of the parent, the \(mht1^−\) or \(sam4^−\) knock-out strains or the double knock-out \(mht1^−/sam4^−\) strain as determined by viable cell counts (data not shown).

\((R,S)\)-AdoMet Is Transported from the Medium into the Cell via Sam3—It was shown previously that a racemic mixture of AdoMet could be taken up by yeast as readily as \((S,S)\)-AdoMet (28). We now have measured directly the uptake of \((R,S)\)-AdoMet in both the BY4742 parent and the \(mht1^−/sam4^−\) double knock-out strains (Fig. 4). We showed that the uptake of...
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(R,S)-AdoMet was similar to that of (S,S)-AdoMet (Fig. 4A). We then asked whether this transport was facilitated by one or both of the known transporters Mmp1 (specific for S-methylmethionine (29)) and Sam3 (specific for AdoMet (30)). We found that the transport of both (S,S)- and (R,S)-AdoMet was blocked when the SAM3 gene was knocked out, whereas transport continued when the MMP1 gene was knocked out (Fig. 4B). These results indicate that Sam3 is a transporter for both (R,S)- and
AdoMet is taken up as readily as the racemate. It has been shown that the racemic mixture of (R,S)-[3H]AdoMet for 9 h at 30 °C in YPD medium. The configuration of the incorporated AdoMet was determined by injecting the extract along with unlabeled R,S,S standard on a cation exchange column as described in Fig. 1. The unlabeled AdoMet standard was determined via absorbance at 258 nm. The incorporated AdoMet (squares) was determined via radioactive counts of 500-μl fractions.

(R,S)-AdoMet. To compare the efficiency of transport of each stereoisomer, the initial rate of uptake was measured at low concentrations, where differences in either the $K_m$ or $V_{\text{max}}$ of transport would be apparent (Fig. 4C). We found that the rate was similar for each isomer, supporting the previous study showing similar rates of uptake of racemic and (S,S)-AdoMet (28).

Utilization of Exogenous (R,S)-AdoMet in Yeast—We then attempted to trace the metabolism of radiolabeled (R,S)-AdoMet when added to intact yeast cells in log phase for 9 h (Fig. 5). We were surprised to find it remained in the $R,S$ configuration. This suggests that either the in vivo homocysteine methylation reaction is slow or that (R,S)-AdoMet is stored, possibly into the vacuole. Previous studies have provided evidence for the transport of both (R,S)- and (S,S)-AdoMet into this organelle (28). This result suggests that yeast cells may store exogenous (R,S)-AdoMet as a reserve and only utilize it in during stationary phase when nutrients are more scarce, presumably metabolizing it with the Mht1 and Sam4 enzymes once released into the cytosol.

DISCUSSION

This study demonstrates that (R,S)-AdoMet can form in yeast from (S,S)-AdoMet and that both the Mht1 and Sam4 enzymes are capable of metabolizing it. The finding that (R,S)-AdoMet accumulates when both the SAM4 and MHT1 genes are deleted suggests that these are the major enzymes involved in limiting its buildup. It thus appears that YMR321C, encoding a protein that is 99% identical to Sam4 over the last 103 amino acids, may not be involved in (R,S)-AdoMet metabolism (31–33). These results also suggest that (R,S)-AdoMet may not be simply excreted, or converted back to the $S,S$ form via radical SAM enzymes, or spontaneously degraded. Thus, Mht1 and Sam4 are the major factors that maintain low levels of cellular (R,S)-AdoMet in yeast.

It was shown previously that yeast are capable of taking up exogenous (S,S)-AdoMet (34) and that a single protein Sam3 is the transporter responsible for this uptake (29, 30, 35). It has also been shown that the racemic mixture of (R,S)/(S,S)-AdoMet is taken up as readily as the $S,S$ form, suggesting that the $R,S$ form can be transported as well (28). Our study confirms that the Sam3 transporter can take up (R,S)- as well as (S,S)-AdoMet. The fact that that $R,S$ is taken up by yeast suggests that these cells may be capable of not only utilizing (R,S)-AdoMet that breaks down from (S,S)-AdoMet intracellularly, but also that R,S-AdoMet that may be available extracellularly. It is likely that AdoMet present in the environment from decaying organisms may be largely racemized, and the ability of yeast cells to use R,S-AdoMet may give them a significant advantage in obtaining carbon, sulfur, and nitrogen. The capability to use the $R,S$ as well as the $S,S$ form of AdoMet as a nutrient source may, in fact, provide an evolutionary advantage to the yeast, as well as to other lower organisms containing (R,S)-AdoMet specific homocysteine methyltransferases (36). Interestingly, we have not detected any such activity in mammalian cells; how these cells limit (R,S)-AdoMet accumulation is still unknown (36).

In yeast cells, AdoMet can be transported and stored in the vacuole (34). The identity of these transporters remains elusive (37). Both the $R,S$ and $S,S$ forms of AdoMet are apparently transported into the vacuoles (28). These results provide a rationale for our observation here that exogenously supplied (R,S)-AdoMet remains in the $R,S$ configuration for at least 9 h after uptake from the medium; this material may be rapidly taken up into the vacuole and sequestered from the Mht1 and Sam4 enzymes localized in the cytosol (38, 39). For cells growing in log phase, the ready availability of nutrients may not require the utilization of vacuolar stores of AdoMet. However, when cells are in stationary phase, they may draw upon the stored (R,S)-AdoMet and use the Mht1 and Sam4 proteins present in the cytoplasm to metabolize it to methionine. We summarize the metabolism of (R,S)-AdoMet in Fig. 6.

Several recent studies have suggested that Mht1 and Sam4 may play a role in the ability of yeast to survive under stressful conditions. For example, a 2-fold amplification at the left
end of chromosome 16 that contains the SAM3 and SAM4 genes was found in the JAY270 strain of yeast that demonstrates unusual tolerance to high temperature and oxidative stress (40). It has been speculated that these genes may play a role in this tolerance (40). Additionally, Sam4 protein levels were found to be increased under menadione-induced oxidative stress (41). Finally, both MHT1 and Sam4 have been linked to the arsenic response network (42), and MHT1 was found to be up-regulated 2-fold in response to methanol (43). SAM4 mRNA has been shown to decrease in aging yeast (44). Although the specific mechanisms by which Mht1 and Sam4 protect cells have not been determined, their role in preventing (R,S)-AdoMet accumulation may be important, especially under conditions such as heat stress where the spontaneous formation of this material would be enhanced.

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REFERENCES