Transport of Sulfonium Compounds

CHARACTERIZATION OF THE S-ADENOSYLMETHIONINE AND S-METHYLMETHIONINE PERMEASES FROM THE YEAST SACCHAROMYCES CEREVISIAE*

Astrid Rouillon‡, Yolande Surdin-Kerjan, and Dominique Thomas§

From the Centre de Génétique Moléculaire, CNRS, 91198 Gif-sur-Yvette, France

We report here the characterization and the molecular analysis of the two high affinity permeases that mediate the transport of S-adenosylmethionine (AdoMet) and S-methylmethionine (SMM) across the plasma membrane of yeast cells. Mutant cells unable to use AdoMet as a sulfur source were first isolated and demonstrated to lack high affinity AdoMet transport capacities. Functional complementation cloning allowed us to identify the corresponding gene (SAM3), which encodes an integral membrane protein comprising 12 putative membrane spanning regions and belonging to the amino acid permease family. Among amino acid permease members, the closest relative of Sam3p is encoded by the YLL061w open reading frame. Disruption of YLL061w was shown to specifically lead to cells unable to use SMM as a sulfur source. Accordingly, transport assays demonstrated that YLL061w disruption mutation impaired the high affinity SMM permease, and YLL061w was therefore renamed MMP1. Further study of sam3Δ and mmp1Δ mutant cells showed that in addition to high affinity permeases, both sulfonium compounds are transported into yeast cells by low affinity transport systems that appear to be carrier-facilitated diffusion.

Among the wide diversity of naturally occurring metabolites that contain a sulfur atom, few are sulfuric salts. In fact, only four sulfonium metabolites have been reported to exist in living organisms. These are S-adenosylmethionine (AdoMet), S-methylmethionine (SMM), and their two derivatives, decarboxylated S-adenosylmethionine and dimethylsulfoniopropionate, respectively (Fig. 1A). The unique property that actually defines these sulfonium compounds is the presence of a trivalent sulfur atom. As a consequence of the electron deficiency at the sulfur pole, the carbon-sulfur bonds that are present in sulfonium compounds are highly susceptible to nucleophilic displacement reactions. The way by which such a chemical reactivity was recruited by living organisms to establish their intermediary metabolism is strikingly exemplified by AdoMet, which is involved in a large set of remarkably versatile reactions. Indeed, all the chemical moieties linked to the sulfur atom of AdoMet can be transferred, with or without modification, to a large number of acceptor molecules. AdoMet serves as donor of its methyl group in the majority of the transmethylation reactions resulting in protein, nucleic acid, lipid, and soluble metabolites modifications. AdoMet further functions as donor of an amino group during the biosynthesis of biotin (1), as donor of a carboxyaminopropyl group for nucleotide modifications, and also as donor of an adenosine group during the synthesis of queuine, a modified base present in some tRNAs from Escherichia coli (2). In addition, AdoMet is the immediate precursor of the sulfonium decarboxylated S-adenosylmethionine, which in turn constitutes the obligate precursor for the biosynthesis of polyamines, spermine, and spermidine (3). The metabolism of SMM was less extensively characterized than that of AdoMet. SMM was first identified as a constituent of cabbage leaves in 1954 (4). Since then, this sulfonium compound has been found in a large number of plants (see Ref. 5 and references therein). Biosynthesis of this compound in plants was shown to result from the S-methylation of methionine by AdoMet (6), and to date, only two enzymes have been found to utilize SMM in plants: SMM-homocysteine S-methyltransferase, which catalyzes the transfer of a methyl group to homocysteine, yielding two methionine molecules, and SMM hydrolase, which cleaves SMM to dimethylsulfide and homoserine (5). Despite these studies, no physiological role could be attributed to SMM until it was shown to be the precursor of the biosynthesis of dimethylsulfiniopropionate. This sulfonium compound is accumulated by many marine algae and by the angiosperma Wollastonia biflora and Spartina alterniflora (see Ref. 7 and references therein). Dimethylsulfiniopropionate has been shown to function as an osmoprotectant for bacteria (8, 9), and the accumulation of dimethylsulfiniopropionate to high levels in the cytoplasm of algal cells and in W. biflora chloroplasts supports the idea that it functions also in plants as an osmoprotectant (10–12). Despite the wide occurrence of these sulfonium compounds in metabolic processes, little attention has been paid to the mechanisms responsible for their transport across biological membranes. A mutation impairing AdoMet transport in the yeast Saccharomyces cerevisiae was described several years ago (13), but it was without subsequent molecular characterization. To address this point, we devised a specific genetic screen in S. cerevisiae aimed to specifically describe the genetic determinants of AdoMet transport in yeast. This allowed us to identify the high affinity AdoMet permease and, furthermore, led to the characterization of a second system capable of transporting AdoMet but with a low affinity. In addition, the work presented here led to the molecular identification of a new permease, capable of specifically transporting SMM, which we show to be used by S. cerevisiae as an efficient sulfur source.

EXPERIMENTAL PROCEDURES

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§ To whom correspondence should be addressed. Tel.: 33-1-69-82-32-32; Fax: 33-1-69-82-43-72; E-mail: thomas@cgm.cnrs-gif.fr.
1 The abbreviations used are: AdoMet, S-adenosylmethionine; AAP, amino acid permease; SMM, S-methylmethionine; ORF, open reading frame.

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[35S]methionine (1000 Ci/mmol) were obtained from Amersham Pharmacia Biotech.

To screen for strains unable to use AdoMet as sulfur source, the strain C112 (met25::HIS3) was mutagenized with nitrous acid to 30–40% survival. After mutagenesis, the cells were grown for 6 h in B medium in the presence of 0.5 mM AdoMet, followed by a 2-h incubation in the presence of nystatin as described by Fink (16). The nystatin treatment was used to counterselect cells capable of growing in the presence of AdoMet. This treatment led to 0.6% surviving cells, which were plated on B medium containing 0.2 mM L-methionine as sulfur source. The resulting colonies were then replicated and tested for their capacity to grow in the presence of AdoMet as sulfur source. Among 1000 colonies, 2 were found to be unable to use AdoMet as a sulfur source.

### Biochemical Methods

Biochemical Methods—L-[35S]SMM was synthesized by treating L-[35S]methionine with 250 mM methanol in 6 M HCl at 110 °C for 4 h (17). The reaction mixture was then evaporated under vacuum, and the resulting material was dissolved in 0.5 ml of distilled water and evaporated under vacuum. The resulting material was then dissolved in distilled water in order to obtain a 20 mM solution of L-[35S]SMM. The radiochemical purity was checked by TLC on cellulose developed in butanol:acetic acid:H2O (12:30:50). The SMM zone was located by autoradiography. The radiochemical purity of [35S]SMM was at least 95%, the main impurity being methionine. The radiochemical purity of [14CH3]AdoMet was checked also by TLC chromatography developed in the same solvent, and the AdoMet zone was located by autoradiography. No impurity could be detected. The purity of nonradioactive AdoMet was also checked. The main impurity revealed by ninhydrine was homoserine. In this case, the other impurity found in AdoMet is 5'-deoxy-5'-methylthioadenosine, which is not transported by yeast.

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**FIG. 1.** A, the natural sulfonium compounds. B, S-adenosylmethionine metabolism in *S. cerevisiae* and the met25Δ block.

**TABLE I**

Yeast strains

| Strain     | Genotype                  | Origin      |
|------------|---------------------------|-------------|
| W303–1A    | MATa, his3, leu2, ura3, ade2, trp1 | R. Rothstein |
| W303–1B    | MATa, his3, leu2, ura3, ade2, trp1 | R. Rothstein |
| CC821–14A  | MATa, leu2, ura3, sam3      | This work   |
| CC899–2D   | MATa, his3, leu2, ura3, trp1, sam3 | This work   |
| CD192      | MATa, his3, leu2, ura3, ade2, trp1, sam3::URA3 | This work   |
| CD203      | MATa, his3, leu2, ura3, ade2, trp1, sam3::URA3|mmp1::TRP1  | This work   |
| CD204      | MATa, his3, leu2, ura3, ade2, trp1, sam3::URA3|mmp1::TRP1  | This work   |

**FIG. 2.** The SAM3 region. A, physical map of the SAM3 region. B, Southern blot analysis of SAM3 gene disruption. Lane 1, SacI-EcoRV digest of genomic DNA extracted from strain W303–1A; lane 2, SacI-EcoRV digest of genomic DNA extracted from strain CD192. The filter was probed with the SacI-EcoRV fragment of SAM3. The arrows indicate the two fragments expected from EcoRV cleavage within the URA3 gene.

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Transport of Sulfonium Derivatives in \textit{S. cerevisiae}

The assay for AdoMet uptake was performed at 28 °C on exponential phase cells \((A_{600} = 1)\) grown in YNB minimal medium complemented to meet the auxotrophic requirements of the strain. Labeled AdoMet (about 2000 cpm/nmol) was added to a final concentration of 0.02–0.1 mM for the assays of the high affinity AdoMet permease and to a final concentration of 0.06–1 mM for the assays of the low affinity AdoMet permease. Samples were taken each minute for 4 min and filtered through a glass fiber filter. Each filter was washed three times with 10 ml of cold distilled water and counted in a scintillation counter. It was verified that uptake was linear during 4 min. The assay for SMM uptake was performed as AdoMet uptake. Labeled L-[35S]SMM (about 28098 cpm/nmol) was added to a final concentration of 0.01 mM for the assay of the high affinity permease and to 0.1 mM for the assay of the low affinity permease.

For kinetic analysis of AdoMet transport, the concentration range of AdoMet was from 2 μM to 1 mM. The uptake was linear for at least 3 min even at the lowest AdoMet concentration (2 μM). For kinetic analysis of SMM transport, the concentration range was from 0.2 μM to 0.1 mM. It appears that the simple Michaelis-Menten equation can give an adequate description of transport processes. However, the uptake being measured on whole cells, the \(V_{\text{max}}\) and the \(K_m\) values cannot have the same meaning as in the case of an enzymatic reaction. We have thus referred to the apparent \(K_m\) and \(V_{\text{max}}\) calculated from double reciprocal plots as \(K_e\) and \(J_{\text{max}}\).

\textbf{RESULTS}

\textbf{Isolation of a Mutation Impairing AdoMet Uptake}—Unlike enteric bacteria or other fungi, such as \textit{Neurospora crassa} or \textit{Aspergillus nidulans}, the yeast \textit{S. cerevisiae} possesses a nearly complete set of enzyme activities, allowing its growth in the presence of a large number of both inorganic and organic sulfur sources. Accordingly, a mutant strain unable to assimilate sulfate is capable of growing in the presence of either methio-

\textbf{DNA Manipulations and Plasmid Constructions}—The shuttle vectors pEMBLYe23, pUC19, and pSK were used in subcloning and integrating experiments (19). The \textit{S. cerevisiae} genomic library that allowed the cloning of the \textit{SAM3} gene was constructed by inserting the product of a partial \textit{HindIII} digest of DNA from strain X2180–1A in the \textit{HindIII} site of plasmid pEMBLYe23.

The ORF called \textit{YLL061w} \((\textit{MMPI})\), see “Results”) has been identified on chromosome XII of \textit{S. cerevisiae}. To inactivate this gene, the \textit{YLL061w} region was synthesized by polymerase chain reaction from chromosomal DNA using synthetic primers. The N-terminal primer was complementary to the coding strand between positions −507 and −479 with respect to the ATG codon. The C-terminal primer was complementary to the noncoding strand between positions 523 and 546 with respect to the stop codon. Amplifications were performed using the Taq DNA polymerase (Appligène). The amplification products were digested by PstI (−469 before the ATG codon) and SmaI (459 after the stop codon) and cloned into plasmid pUC19 digested by SmaI and PstI, yielding plasmid pYLL061.

The construction of disrupted alleles followed the strategy of Rothstein (20). To disrupt \textit{SAM3}, the \textit{SacI-EcoRI} fragment of pSAM3–1 was inserted in plasmid pSK, yielding plasmid pSAM3–2. The \textit{BglII-HpaI} fragment of the \textit{SAM3} region of plasmid pSAM3–2 was removed and replaced by a 1.1-kilobase pair fragment bearing the \textit{URA3} gene. The resulting plasmid was digested by EcoRI and SacI and used to transform strains W303–1B to uracil prototrophy. One of the transformants was called CD192. The disruption was verified in strain CD192 by Southern blotting (see “Results”). To disrupt \textit{YLL061w}, the \textit{XbaI-HpaI} fragment of pYLL061 was removed and replaced by a fragment bearing the \textit{TRP1} gene. The resulting plasmid was digested by \textit{PstI}, then treated by the \textit{BalI} exonuclease for 5 min and then digested by \textit{PstI}. The resulting fragment was purified and used to transform strains W303–1A and CD192 to tryptophan prototrophy. The disruption was verified by Southern analysis (not shown).

![Fig. 3. Growth of different mutants on AdoMet.](image)

The strains were grown on minimal B medium containing 0.1 mM L-methionine or 0.1 or 1 mM AdoMet as the sole sulfur sources.

\(A\) strain W303–1A (\textit{SAM3}); \(B\) strain CD192 (\textit{sam3Δ}). AdoMet uptake was measured as described under “Experimental Procedures.” The incubations were for 3 min. The AdoMet concentrations were from 0.002 to 1 mM. The data were analyzed in two sets: for the high affinity, 0.002–0.1 mM, and for the low affinity, 0.06–1 mM.
nine, cysteine, or AdoMet (21). In the latter case, as shown in Fig. 1B, methionine synthesis from exogenous added AdoMet could be reached through either the methyl cycle (with the intermediary formation of adenosylhomocysteine and homocysteine) or the methythioadenosine recycling pathway (21). This metabolic redundancy thus suggested that the use of a met25Δ strain as a parent strain in a screen for mutants capable of growing in the presence of methionine but not in the presence of AdoMet should mainly identify the components of the AdoMet uptake system.

The parent met25Δ was mutagenized by nitrous acid, and a nystatin enrichment-based protocol was used to counterselect strains capable of growing in the presence of AdoMet as a methionine source (see under “Experimental Procedures”). After 1000 survival cells were plated on methionine-containing medium, they were replicated and tested for their capacity to grow in the presence of AdoMet. This genetic screen allowed the isolation of two strains unable to grow in the presence of AdoMet but able to grow in the presence of either methionine, homocysteine, or cysteine. The two mutant strains (D162 and D201) were then crossed with a met25 mutants. The resulting diploids were sporulated, and the analysis of the progeny revealed that in both cases, the incapacity to grow in the presence of AdoMet segregates as a monogenic trait. Complementation assays, furthermore, showed that the mutations present in the two mutant strains D162 and D201 affected the same genetic locus. According to the genetic nomenclature of yeast, this locus was called sam3 rather than smp3, the original term used by Spence (22).

To further substantiate the hypothesis that the isolated mutation really impaired the AdoMet transport system, the D201 mutant strain was crossed to a wild-type strain, and the phenotype of a MET25, sam3 resulting spore was studied. Growth assays were performed using a specific medium (B medium) devoid of sulfur atoms. In contrast to congenic MET25, SAM3 cells, the MET25, sam3 mutants were unable to use AdoMet as a sulfur source when this compound was added to a final concentration of 0.1 mM. As expected, sam3 cells are fully competent for growing in the presence of methionine as a sulfur source (not shown).

**Cloning of the SAM3 Gene**—To clone the SAM3 gene, we used the inability of sam3 mutants to grow in the presence of 0.1 mM AdoMet on B medium. Strain CC821-14A (ura3, sam3) was transformed by a pEMBLYe23-based yeast genomic library, and transformed cells capable of growing in the absence of uracil and in the presence of AdoMet were directly selected on B medium. Among 8 × 10^5 transformants, four strains were selected. Plasmid DNA was recovered from these colonies and used to retransform the strain CC821-14A. Only one plasmid led to uracil prototroph transformants being able to use AdoMet as sulfur source. This plasmid harbored a 2.8-kilobase pair insert (Fig. 2A), and determination of the sequence of its extremities revealed that it corresponds to a fragment of the left arm of chromosome XVI. Sequence analysis revealed that this fragment contains only one long open reading frame (YPL274w) with the potential to encode a protein of 587 amino acids.

To confirm that the YPL274w ORF indeed corresponds to the

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**TABLE II**

The presence of plasmid pSAM3–1 increases the activity of the high affinity AdoMet permease

| Strain       | Relevant genotype | Plasmid present | Transformant number | High affinity transport specific activity |
|--------------|-------------------|-----------------|---------------------|----------------------------------------|
| W303–1A      | SAM3              |                 | 1                   | 3.6                                    |
| W303–1A      | SAM3 pSAM3–1      | 1               | 12.9                |
| CC899–2D     | sam3              |                 | 1                   | 0.56                                   |
| CC899–2D     | sam3 pSAM3–1      | 1               | 9.1                 |
|              |                   |                 | 2                   | 10.5                                   |

*Expressed in nmol of transported AdoMet/min/mg of dry weight.*

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**Fig. 5. Alignment of the Sam3 and Yll061 proteins.** The two proteins were aligned using the Clustal V program (34). =, identical amino acids; –, conservative replacements. The 12 putative membrane spanning domains are boxed.
SAM3 gene, the isolated fragment was cloned in the integrative vector pEMBLY22 bearing the URA3 marker and targeted to its corresponding genomic locus within chromosome XVI of the W303-1B strain (ura3). A strain containing the integrated construct was then crossed with a sam3, ura3 double mutant strain, and the resulting diploid was sporulated. Analysis of the progeny (24 tetrads) showed that in all tetrads, the two uracil auxotroph spores were unable to use AdoMet as sulfur source, in contrast to the uracil prototroph spores that were able to use AdoMet. This experiment thus demonstrated that the YPL274w ORF does correspond to the SAM3 gene.

To gain further information on the function of its encoded product, the SAM3 gene was disrupted by the one-step gene disruption method as described under “Experimental Procedures.” Correct integration events were verified by Southern analysis (Fig. 2B). Growth characteristics of the sam3Δ mutant strain were determined. As expected, this strain is unable to grow in the presence of 0.1 mM AdoMet used as the sulfur source, whereas it is still capable of growing in the presence of 0.1 mM L-Met. However, when a higher amount of AdoMet (1 mM) was added to the B medium, the sam3Δ mutant strain was able to grow (Fig. 3). This result prompted us to determine the kinetic parameters of AdoMet uptake in yeast cells.

AdoMet Transport Is Mediated by Two Permeases in S. cerevisiae—The first genetic and kinetic data concerning AdoMet transport in S. cerevisiae were mainly obtained by Spence and co-workers (23, 24). These authors reported that AdoMet is transported across the plasma membrane by a single transport system. However, when we examined the kinetics of AdoMet uptake in a wild-type strain (W303-1A) using a large range of radioactive AdoMet concentrations, we obtained results that appeared not to be compatible with a model involving a single transport component. As shown in Fig. 4A, the double reciprocal plot of AdoMet uptake in the wild-type W303-1A strain is clearly biphasic, therefore suggesting that AdoMet uptake is mediated by more than one permease. The results fit if we assume the existence of two permeases exhibiting different kinetic parameters, one being a high affinity AdoMet permease ($K_T = 3.3 \times 10^{-8}$ M; $J_{max} = 5.3$ nmol of AdoMet transported/min/mg of dry weight) (see under “Experimental Procedures”) and the second, a low affinity AdoMet permease ($K_T = 1.6 \times 10^{-4}$ M; $J_{max} = 15$ nmol of AdoMet transported/min/mg of dry weight) (in this second case, it must be noted that the kinetic parameters are not accurately determined due to the presence of the high affinity permease).

The values obtained for the high affinity AdoMet permease are close to those reported for AdoMet uptake by Spence et al. (13) in their first analysis of AdoMet transport in yeast. Next, we determined the kinetic parameters of AdoMet uptake in cells that do not express the Sam3 protein. In contrast to what was observed with its congenic parental strain, the kinetic data of AdoMet uptake in the sam3Δ mutant cells led to a linear double reciprocal plot showing that in these cells, the AdoMet uptake is mediated by a unique transport system. The calculated kinetic parameters ($K_T = 2.5 \times 10^{-4}$ M; $J_{max} = 21$ nmol of AdoMet transported/min/mg of dry weight) (Fig. 4B) are very close to those determined for the low affinity component of the wild-type cells. These results therefore confirmed that in yeast, AdoMet uptake is actually mediated by two different transport system of high and low affinity, the former being encoded by the SAM3 gene.

To obtain further evidence that Sam3p is indeed the high affinity AdoMet permease, the two strains CC899-2D (sam3) and W303-1A (SAM3) were transformed by a multicopy plasmid bearing the SAM3 gene. For each strain, two resulting transformants were assayed for AdoMet permease activity, using AdoMet at 0.05 mM to specifically measure the activity of the high affinity permease (Table II). In both cases, the two transformants exhibited the same specific activity, which was 2–3-fold higher than that of the untransformed parental strain W303-1A. As expected, under these assay conditions, the untransformed strain CC899-2D (sam3) displayed no AdoMet permease activity. All these results therefore proved that the SAM3 gene does encode the high affinity AdoMet permease in S. cerevisiae cells.

The High Affinity AdoMet Permease Belongs to the Amino Acid Permease Family—The Sam3 protein deduced from the nucleotide sequence of its corresponding gene has a molecular weight of 64,350 and a calculated isoelectric point of 8.1. The hydropathy profile analysis was performed using programs in the TopPred2 software. The results suggested that the Sam3 protein contains 12 transmembrane spanning regions. A search against the protein data bases using the BLAST program revealed that, surprisingly, the Sam3 protein is related to the amino acid permease (AAP) family of yeast. The AAP family is composed, in addition to Sam3p, of 17 transmembrane proteins that are all closely related proteins and that all seem to contain 12 transmembrane spanning regions. Owing to both biochemical and genetic studies, the function of 11 members of this family has been established (for review, see Ref. 25). In each case, these proteins were shown to transport amino acids, across the plasma membrane. It was therefore postulated that the remaining 7 proteins with unknown function of this family

| Strain | Relevant genotype | High affinity transport | Low affinity transport |
|--------|------------------|------------------------|-----------------------|
|        | $K_T$ | $J_{max}$ | $K_T$ | $J_{max}$ |
| W303-1A | 3.3 | 5.3 | 0.16 | 15 |
| CD203 | 3.3 | 5.3 | 0.16 | 15 |
| CD192 | 2.6 | 6.1 | 0.17 | 41 |
| CD204 | 2.6 | 6.1 | 0.17 | 41 |

$^a$ Expressed in nmol/min/mg of dry weight.

$^b$ —, not detectable.

Fig. 6. Disruption of YLL061w impairs use of S-methylmethionine as sole sulfur source. The strains were grown on minimal B medium containing 0.1 mM L-methionine or 0.1 mM D-SMM as the sole sulfur sources.
should be also amino acid permeases. The identification of the Sam3 protein reported here clearly demonstrates that it is not the case.

Among the members of the AAP family, the protein exhibiting the closest resemblance to Sam3p is the product of the \textit{YLLO61} ORF, a gene discovered on chromosome XII by the systematic sequencing of the yeast genome. To date, a function has not been associated to this gene. Sam3p and the product of the \textit{YLLO61} ORF are highly related proteins. As shown in Fig. 5, the two proteins share 419 identical residues (71%) and 77 conservative replacements (13%). Hydropathy analysis of the \textit{YLLO61}-encoded product suggests that this protein contains 12 transmembrane domains that are superimposable on those of Sam3p.

Identification of the High Affinity S-Methylmethionine Permease—Given the high degree of similarities exhibited by the Sam3- and \textit{YLLO61}-encoded products, it was tempting to postulate that the low affinity AdoMet permease would be specified by the latter gene. To directly assess such a hypothesis, the \textit{YLLO61} ORF was cloned by polymerase chain reaction amplification and the corresponding chromosomal locus was disrupted in wild-type cells as well as in \textit{sam3} cells (see under "Experimental Procedures"). The growth behavior of the two resulting strains, CD203 and CD204, was analyzed. As shown in Fig. 3, the presence of AdoMet used as sulfur source, the growth of both strains did not differ from that of their respective parental strains. In a second approach, we measured the kinetic parameters of AdoMet uptake in both mutant cells. As reported in Table III, the disruption of the \textit{YLLO61} ORF was without effect on AdoMet transport: the kinetics of AdoMet uptake was the same in the absence or in the presence of the \textit{yll061::TRP1} mutation, in both the \textit{SAM3} and the \textit{sam3::URA3} cells. Therefore, despite the high sequence similarity displayed by \textit{YLLO61}-encoded product and the high affinity AdoMet permease, the former protein appears not to be involved in AdoMet uptake and thus does not correspond to the low affinity AdoMet permease.

We next reasoned that such a resemblance between two proteins could possibly reflect a specificity for closely related compounds. As stated in the introduction section, among AdoMet analogs, one of the closest is the other natural sulfonium compound, SMM. To our knowledge, it has never been reported that yeast cells are able to grow in the presence of SMM used as a sole sulfur source, although the presence of an SMM-homocysteine methyl transferase activity was attested in yeast extracts (26). We first demonstrated that wild-type yeast cells are indeed capable of growing when 0.1 mM of DL-SMM was added as sole sulfur source (Fig. 6). Next, we examined the growth of cells disrupted for the \textit{yll061} locus on the same medium. As shown in Fig. 6, disruption of the \textit{YLLO61} ORF severely impairs the growth of the cells in the presence of 0.1 mM of DL-SMM as sulfur source. This effect was specific for SMM, as we have already demonstrated (Fig. 3) that the presence of the \textit{yll061::TRP1} disruption mutation is without effect on the growth of yeast cells in the presence of either L-methionine or AdoMet used as sulfur sources. In addition, it was verified that yeast cells bearing the \textit{yll061::TRP1} deletion could also use sulfate, homocysteine, and cysteine as sulfur sources (not shown). Taken together, the phenotype of the \textit{yll061}
TABLE V
Specificity of AdoMet and SMM uptake by the high affinity permeases

Strain W303–1A was used. Radioactive AdoMet was added at 0.05 mM for the assay of the high affinity AdoMet permease, and radioactive SMM was added at 0.02 mM for the assay of the high affinity SMM permease.

| Unlabeled metabolite added | High affinity AdoMet permease | High affinity SMM permease |
|---------------------------|------------------------------|---------------------------|
|                           | % inhibition                 |                           |
| S-Methylmethionine        | 14                           | ND                        |
| S-Adenosylmethionine      | ND                           | 11                        |
| S-Adenosyllethionine      | 67                           | ND                        |
| S-Adenosylhomocysteine    | 27                           | 0                         |
| Sinefungine               | 24                           | ND                        |
| Tryptophan                | 78                           | 9                         |
| Tyrosine                  | 35                           | 0                         |
| Cysteine                  | 0                             | 12                        |
| Methionine                | 0                             | 21                        |
| Aspartate                 | 0                             | 7                         |
| Glutamine                 | 0                             | 8                         |
| Glutamate                 | 15                           | 0                         |
| Isoleucine                | 0                             | 6                         |
| Leucine                   | 0                             | 2                         |
| Histidine                 | 0                             | 0                         |
| Arginine                  | 3                             | 13                        |
| Lysine                    | 11                           | 5                         |
| Glycine                   | 0                             | 4                         |
| Alanine                   | 0                             | 4                         |
| Serine                    | 0                             | 0                         |
| Threonine                 | 0                             | 0                         |
| Valine                    | 0                             | 4                         |
| Phenylalanine             | 4                             | 18                        |

* The unlabeled inhibitors were added at a concentration 20-fold higher than the substrate, 1 mM for the AdoMet permease and 0.4 mM for the SMM permease.
* ND, not determined.

 disrupted cells and the close resemblance exhibited by the high affinity AdoMet permease and the YLL061w-encoded product strongly suggested that this gene could specify an SMM permease of yeast.

To ascertain this hypothesis, we next wanted to examine the kinetics of SMM transport in wild-type as well as in various mutant strains. To do that, [[35S]SMM was synthesized according to Gage et al. (17), and the purity of the resulting radioactive compound was checked by thin layer chromatography (see under "Experimental Procedures"). SMM transport in wild-type yeast cells was assayed with SMM concentrations ranging from 0.4 mM to 0.1 mM. The results obtained (Fig. 7A) suggested that, as for AdoMet, SMM uptake is mediated by two permeases with different kinetic characteristics, one exhibiting a high affinity for SMM and the other being a low affinity permease ($K_p = 2.5 \times 10^{-8}$ and $8 \times 10^{-3}$ mM and $J_{max} = 1.5$ and 6.6 nmol/min/mg of dry weight, respectively). Moreover, when SMM uptake was assayed in cells that bear a disruption mutation of the yll061w locus (strains CD203 and CD204), the resulting kinetic data clearly led to a linear double reciprocal plot showing that in mutant cells, SMM uptake is mediated by a unique transport system that corresponds to the low affinity SMM permease seen in wild-type cells (Fig. 7B and Table IV).

This was sustained by the fact that yll061w mutants cells are capable of growing, although more slowly than their parental strains, when the concentration of DL-SMM used as sulfur source was raised to 2 mM (not shown). Taken together, all these results both confirm the existence of two SMM permeases in yeast and demonstrate that YLL061w encodes the high affinity SMM permease. Therefore, the YLL061w ORF was named MMP1 (for methylmethionine permease) according to the standard yeast genetic nomenclature.

Substrate Specificity of the High Affinity AdoMet and SMM Permeases—First analyses of AdoMet transport in S. cerevisiae had underscored the narrow substrate specificity of this system (23, 24). As we demonstrated that yeast cells actually possess two AdoMet permeases, these results could be questioned. The analysis of the specificity of the AdoMet high affinity permease appeared to be further necessary because the SAM3 as well as the MMP1-encoded high affinity permeases belong to the family of amino acid permeases, several members of which have been shown to display large substrate specificity.

We thus assayed AdoMet and SMM uptake specificity in wild-type cells in the presence of various sulfur compounds and amino acids using substrate concentrations such that we could specifically measure the activity of the high affinity permeases only. As reported in Table V, the obtained results demonstrate that the high affinity AdoMet permease appears to be a rather specific enzyme. Among AdoMet analogs, S-adenosyllethionine (which contains an ethyl group in place of the methyl group of AdoMet) is the most potent inhibitor of the high affinity AdoMet permease, whereas S-adenosylhomocysteine (the de-methylated AdoMet analogue), sinefungin (a synthetic analogue of S-adenosylhomocysteine), and SMM are less effective inhibitors. In contrast, the presence of other organic sulfur compounds, such as methionine, homocysteine, or cysteine, was without effect on the high affinity AdoMet uptake. Because Sam3p belongs to the AAP family of transporters, we tested AdoMet transport in the presence of each amino acid. These assays revealed that the two aromatic amino acids, tryptophan and tyrosine, inhibit the high affinity AdoMet permease, the latter less efficiently. As also reported in Table V, the high affinity SMM permease is very specific, being only slightly inhibited by methionine and phenylalanine and to a lesser extent by cysteine, AdoMet, and arginine.

Low Affinity Transport of AdoMet and SMM Is a Facilitated Diffusion Mechanism—Results reported above demonstrated that AdoMet and SMM transport in cells devoid of the high affinity AdoMet permease (sam3Δ mutant) and the high affinity SMM permease (mmp1Δ mutant), respectively, are saturable processes with respect to substrate concentration (see the determination of kinetic constants of AdoMet uptake (Fig. 4) and SMM uptake (Fig. 7)). However, the growth of the sam3Δ mutant cells on a high concentration of AdoMet (1 mM) appears to be poor (Fig. 3), although the concentration used was expected to satisfy the kinetic requirements of the low affinity permease. This was a first indication that the intracellular concentration reached is not sufficient for optimal growth, probably due to the inability of the low affinity permeases to concentrate AdoMet intracellularly. In addition, at the concentrations used to assay the low affinity transport in strains lacking the high affinity permeases, AdoMet and SMM uptake is rapid, reaching equilibrium in less than 1 min. These properties have been described for uptake by facilitated diffusion (27).

Carrier-mediated facilitated diffusion mechanism has been shown to mediate hexose uptake in yeast and was also reported to be responsible for the low affinity transport of urea (28–30). Because this type of transport is expected to be insensitive to...
metabolic inhibitors (27), AdoMet and SMM uptake was assayed in wild-type and mutant cells in the presence of sodium azide. As shown in Table VI, transport by the low affinity AdoMet and SMM permeases is insensitive to 1 mM sodium azide. In addition, we examined the kinetics of SMM transport in wild-type cells (strain W303-1A) in the presence of 1 mM sodium azide (Fig. 8A). The results show that under these conditions, only the low affinity permease is detected, the high affinity SMM permease activity being completely inhibited by the metabolic inhibitor. These results suggested that both AdoMet and SMM high affinity permeases are active transport components, as expected from their homology to the AAP family members, whereas, in contrast, both low affinity AdoMet and SMM transport could be carrier-mediated facilitated diffusion. The next question we wanted to address is the possibility that the low affinity AdoMet transport and the low affinity SMM transport could be mediated by the same uptake system.

In the absence of the genetic characterization of both AdoMet and SMM low affinity permeases, a clear response could not be given. However, cross-inhibition experiments were performed that showed that inhibition of the low affinity AdoMet permease by SMM and of the low affinity SMM permease by AdoMet appear to be competitive (Fig. 8, B and C). This could be an indication that the two low affinity permeases are identical.

**DISCUSSION**

A highly specific genetic screen allowed us to isolate, in *S. cerevisiae*, a mutation impairing AdoMet transport across the plasma membrane. The isolated mutation defined a new genetic locus, which was called SAM3. The kinetic study of AdoMet uptake by wild-type as well as *sam3*-disrupted cells led to the conclusion that the AdoMet transport system was dual. Indeed, contrary to what was originally reported, kinetics of
FIG. 9. Phylogenic tree of the yeast amino acid, AdoMet, and purine permeases as obtained using the Darwin program (35).

Gap1, general amino acid permease; Can1, arginine permease; Hip1, histidine permease; Put4, proline permease; Lyp1, lysine permease; Gnp1, glutamine permease; Dip5, glutamate and aspartate permease; Bap2, leucine, valine, and isoleucine permease; Put1, low affinity branched amino acid permease; Tat1, tyrosine and tryptophan permease; Tat2, tryptophan permease; Mup1, high affinity methionine permease; Mup3, very low affinity methionine permease; Fcy2, purine and cytosine permease (25); Sam3, high affinity AdoMet permease; mmp1, high affinity SMM permease.

does not transport one of the 20 common amino acids. This result underscores once more the absolute necessity of assessing, by the means of biochemistry and genetic studies, the enzymatic functions that have been predicted on the basis of sequence homologies uncovered by alignment algorithms. It must be noted that in the case of the yeast proteins embedded within the plasma membrane, such a methodological requirement was already demonstrated by the example of the inorganic phosphate transporter (encoded by the PHO84 gene), the sequence of which is very closely related to those of the hexose transporters (31).

We were able to identify as a SMM permease the transmembrane protein encoded by the YL061w ORF, a close sequence homolog of Sam3p, which shares with sam3p an overall sequence similarity greater than 85%. Up to now, SMM uptake and fate in \textit{S. cerevisiae} had not been studied, although the presence of a SMM-homocysteine methyltransferase activity was evidenced in yeast lysates (26). In the present work, we have brought evidence that SMM is a sulfur source able to promote growth of \textit{S. cerevisiae} cells and that it is transported in the yeast cells by two permeases, one with a high affinity and the other exhibiting a low affinity for SMM. It appears that the high affinity SMM permease is a specific permease, being inhibited only by methionine, AdoMet, cysteine, and phenylalanine. Therefore, our study points out that the three aromatic amino acids are able to interfere with the uptake of the two sulfonium metabolites: phenylalanine inhibiting SMM uptake, tryptophan and tyrosine inhibiting the AdoMet uptake.

The low affinity AdoMet and SMM permeases identified by our kinetic studies do not display the characteristics of active transports, both being insensitive to a metabolic inhibitor. However, they are saturable processes, so the participation of carrier proteins is probable. This identifies the two low affinity transport systems described here as facilitated diffusion. Whether AdoMet and SMM low affinity permeases are the same transport system is unknown. Competitive cross inhibitions could be an indication of the presence of only one facilitated diffusion system, but genetic evidence is needed to ascertain this point.

Recent work on sulfate and methionine uptake has shown that these sulfur compounds are transported by two and three transport systems (32, 33), respectively; the systems differ in their affinity for the substrate, in their transport capacity, and in their specificity. The results reported here show that AdoMet and SMM are two other sulfur compounds, each of which is also transported by two transport systems with different characteristics. This is probably a means for yeast cells to satisfy their growth requirements by the ability to capture various sulfur compounds whatever the external conditions.

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