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Effects of Regulatory Mutations upon Methionine Biosynthesis in *Saccharomyces cerevisiae*: Loci *eth2-eth3-eth10*

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The effects of mutations occurring at three independent loci, *eth2*, *eth3*, and *eth10*, were studied on the basis of several criteria: level of resistance towards two methionine analogues (ethionine and selenomethionine), pool sizes of free methionine and *S*-adenosyl methionine (SAM) under different growth conditions, and susceptibility towards methionine-mediated repression and SAM-mediated repression of some enzymes involved in methionine biosynthesis (met group I enzymes). It was shown that: (i) the level of resistance towards both methionine analogues roughly correlates with the amount of methionine accumulated in the pool; (ii) the repressibility of met group I enzymes by exogenous methionine is either abolished or greatly lowered, depending upon the mutation studied; (iii) the repressibility of the same enzymes by exogenous SAM remains, in at least three mutants studied, close to that observed in a wild-type strain; (iv) the accumulation of SAM does not occur in the most extreme mutants either from endogenously overproduced or from exogenously supplied methionine; (v) the two methionine-activating enzymes, methionyl-transfer ribonucleic acid (tRNA) synthetase and methionine adenosyl transferase, do not seem modified in any of the mutants presented here; and (vi) the amount of $tRNA^{met}$ and its level of charging are alike in all strains. Thus, the three recessive mutations presented here affect methionine-mediated repression, both at the level of overall methionine biosynthesis which results in its accumulation in the pool, and at the level of the synthesis of met group I enzymes. The implications of these findings are discussed.

In *Saccharomyces cerevisiae*, methionine biosynthetic enzymes have been previously divided into two groups on the basis of their response towards methionine-mediated repression and of the pleiotropic effect of mutations occurring at the locus *eth2*. The synthesis of methionine enzymes from group I (met group I enzymes), namely, homoserine-*O*-transacetylase (17), homocysteine synthetase (24), adenosine triphosphate (ATP) sulfurylase (ATP: sulfate adenylyl transferase, EC 2.7.7.4), and sulfite reductase (hydrogen sulfide nicotinamide adenine dinucleotide phosphate oxidoreductase, EC 1.8.1.2), is strongly repressed by exogenous methionine in wild-type strains (3). In addition, it was found that the pattern of repression of these enzymes is greatly modified in strains carrying mutant alleles of genes *eth2*:*eth2-1* (3) and *eth2-2* (13). Results from the study of a mutant carrying an impaired methionyl-transfer ribonucleic acid (tRNA) synthetase (L-methionine: soluble RNA ligase [adenosine monophosphate], EC 6.1.1.10) and from the study of methionine auxotrophs in chemostats led to the implication of methionyl-tRNA rather than free methionine in this regulatory process (3, 22).

More recently, further studies of wild-type strains of *S. cerevisiae* have uncovered an important regulatory effect of *S*-adenosyl methionine (SAM) over the synthesis of met group I enzymes. This effect was shown to be due to SAM per se and not to its reconversion into free methionine (2).

It could be expected that the study of regulatory mutants would permit a better understanding of the molecular mechanism by which methionyl-tRNA and SAM are both involved in the regulatory process. The mutants selected for such a study were those showing a dual resistance towards two methionine analogues: ethio-

nine and selenomethionine. Since the structure of these two analogues is different, there is no a priori reason that they would exert the same biochemical effects. Therefore, it was expected that mutants resistant to both analogues would more likely show some disturbance in the regulation of methionine biosynthesis.

MATERIALS AND METHODS

Strains. The haploid wild-type strain used for this investigation was 4094-B (*α ade2 ura1*) from F. Sherman's collection. The other strains were mutant strains resistant towards at least two methionine analogues: ethionine and selenomethionine. They were the following: strains E10 (*α ade2 ura1 eth10-1*) and P1S4 (*α ade2 ura1 eth10-2*) were isolated as spontaneous mutants from strain 4094-B on the basis of resistance towards ethionine and selenomethionine, respectively; strain CJ21-11B (*α ade3 eth3-1*) was recovered from a cross between a wild-type strain and a spontaneous mutant from strain 4094-B isolated on ethionine-supplemented medium; strain 199M1-102 (*α arg4 his5 lys1 ade2 leu1 eth2-2*) was also selected as an ethionine-resistant mutant but after nitrous acid mutagenesis of a wild-type strain, 199M1 (from P. Meuris's collection); strain MM101-2B (*α met2 eth2-2*) was a segregant from a cross between strain 199M1-102 and a methionine auxotroph D6 (*α met2 ura*: gene number unknown) from M. Grenson's collection. Strains 199M1-102 and MM101-2B were obtained in our laboratory by M. Masselot. Genetic nomenclature is according to von Borstel (23).

Growth conditions. The minimal medium used for all strains except strain 199M1-102 was GO medium (8) supplemented with uracil (10 mg per liter) and adenine (10 mg per liter). For strain 199M1-102, the minimal medium referred to in the text was the above medium supplemented with histidine, leucine, arginine (100 mg per liter), lysine (40 mg per liter), and tryptophan (20 mg per liter). When methionine or SAM was added, concentrations are specified in the tables.

The culture conditions were as described in Cherest et al. (1). Growth curves were performed according to de Robichon-Szulmajster and Cherest (5). The optical densities were measured in a Jouan spectrophotometer (1 unit of optical density at 650 nm [1 cm] corresponded to 10^7 cells/ml).

Extracts. The cell extracts were made as previously described (3). The crude extract was centrifuged for 90 min at 40,000 rpm in rotor 40 of a preparative Spinco centrifuge ("Spinco extract"), which results in a lowering of the blanks in the ATP sulfurylase assay. For homocysteine synthetase, ATP sulfurylase, and sulfite reductase, buffers used for cell extracts were as already reported (3). For methionine adenosyl transferase (ATP: L-methionine *S*-adenosyl transferase, EC 2.5.1.6), extracts (2 volumes of buffer for 1 volume of cells) were made in potassium phosphate buffer (pH 7.5, 100 mM) containing 25% (wt/wt) glycerol and 5 mM 2-mercaptoethanol. These extracts were then dialyzed for 16 h against potassium phosphate buffer (pH 7.5, 10 mM) containing 25% (wt/wt) glycerol and 5 mM 2-mercaptoethanol (1 liter of buffer for about 5 ml of extract). For methionyl-tRNA synthetase, extracts (2 volumes of buffer for 1 volume of cells) were made in potassium phosphate buffer (pH 7.5, 100 mM) and dialyzed for 16 h against potassium phosphate buffer (pH 7.5, 10 mM; 5 liters of buffer for 3 ml of extract).

Enzymatic assays. Homocysteine synthetase activity was assayed in the "Spinco extract" as described by Wiebers and Garner (24) at 30 C, and the homocysteine formed was estimated by the method of Kredich and Tomkins (10). ATP sulfurylase activity was measured in the "Spinco extract" by the method of Wilson and Bandurski (25) as described by de Vito and Dreyfuss (7). The sulfite reductase activity was assayed in the crude extract kept at room temperature, by the method of de Vito and Dreyfuss (7). The sulfide formed was estimated by the method of Siegel (21). The assay mixture for methionyl-tRNA synthetase contained, in a volume of 0.1 ml: 10 μmol of potassium phosphate buffer (pH 7.4), 1 μmol of KCl, 1 μmol of $MgCl_2$, 0.2 μmol of ATP (Na), 0.2 μmol of dithiothreitol, 0.0032 μmol of ^{14}C-L-methionine (2×10^5 counts per min per assay), 0.5 mg of commercial baker's yeast tRNA, and extract containing 150 μg of protein. After 10 min of incubation at 30 C, the reaction was stopped with 1 ml of a cold 0.1% trichloroacetic acid solution containing 5% DL-methionine, followed by addition of 2 ml of a cold 10% trichloroacetic acid solution containing 0.5% DL-methionine. After 30 min at 4 C, the resulting precipitates were filtered through Whatman glass-fiber circles (GF/C) and washed with a cold 5% trichloroacetic acid solution containing 0.5% DL-methionine. The radioactivity retained on the filters was counted in a scintillation counter (Intertechnique SL30). For K_m determinations, the range of substrate concentrations used was 0.00016 to 0.64 mM for L-methionine, 0.05 to 10 mM for ATP, and 0.05 to 10 mg/ml for tRNA. When one substrate was varied, the two others were kept constant and at the concentration given in the assay procedure.

The methionine adenosyl transferase activity was measured as follows. The incubation mixture contained, in 0.5 ml: tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.5), 50 μmol; KCl, 50 μmol; reduced glutathione, 10 μmol; $MgCl_2$, 22.5 μmol; ATP (Na), 15 μmol; and ^{14}C-L-methionine, 5 μmol containing about 2×10^6 counts/min. After 1 h of incubation at 37 C, the SAM formed was estimated on a 50-μliter sample of the incubation mixture by the method of Chou and Lombardini (5). For K_m determinations, the range of substrate concentrations used was 0.1 to 10 mM for L-methionine and 1.6 to 40 mM for ATP. When one substrate was varied, the other was kept constant and at the concentration given in the assay procedure.

Benzene-treated cells. The cells were grown as previously described (1) but in 20 ml of the corresponding minimal medium with SAM added or not at the concentrations given in the text. When the optical density at 650 nm (1 cm) was about 2.0, the cells were harvested by centrifugation and resuspended in 2 ml

of potassium phosphate buffer (pH 7.2, 10 mM). They were rendered more permeable by treatment with benzene as described by Magee and de Robichon-Szulmajster (12). Homocysteine synthetase was assayed directly on 0.1 or 0.3 ml of this suspension.

Protein concentration. The protein estimation was performed by the biuret method (9) with bovine serum albumin as reference.

Specific activities. Specific activities of the enzymes were expressed in nanomoles per minute per milligram of protein, i.e., 10^{-3} international units, or in nanomoles per minute per milligram of cells in the case of benzene-treated cells.

Determination of the amount of methionyl-tRNA^{met} and tRNA^{met}. The extraction of tRNAs and the determination of the amount of tRNA^{met} and of the tRNA^{met} charged in vivo were as previously described (2, 22).

Determination of the intracellular SAM pool. Cells from 1 liter of culture for the wild-type strain and from 2 liters for each of the mutant strains were harvested by centrifugation, washed once with cold water, and resuspended in 8 ml of 1.5 N perchloric acid. This suspension was shaken for 1 h at 4 C. It was then centrifuged (12,000 × *g*, 10 min), and the clear supernatant fluid was neutralized to pH 6 to 7 with a 2 M $KHCO_3$ solution. The mixture was cleared by centrifugation, and the SAM concentration was estimated on a sample of the supernatant fluid as described by Shapiro and Ehninger (20) by chromatography on Dowex 50 in the Na^+ form. The SAM concentration was calculated by its absorption, taking E_M (256 nm) = 14,700 (20).

Determination of the intracellular methionine pool. The cells from 1 liter of culture were harvested by centrifugation and washed three times with cold water. After that, 10 ml of sterile water was added to the packed cells, and the resulting suspension was boiled for 10 min in a water bath and then centrifuged. The concentration of L-methionine in the supernatant fluid was determined with *Leuconostoc mesenteroides* P-60 ATCC8052 by use of the Difco methionine assay medium as described in the Difco manual. A standard curve was constructed with a known solution of L-methionine and was repeated for each series of assays. Suitable samples of the above supernatant fluid were used so that their methionine content fell into the linear part of the standard curve.

Chemicals. SAM (*S*-adenosyl-L-methionine) was purchased from Sigma Chemical Co. Dowex 50W X8 (100 to 200 mesh) was purchased from Lambert-Rivière (France) and washed prior to use as described by Shapiro and Ehninger (20). L-Methionine-*1*-^{14}C (49.1 mCi/mmol) was obtained from the Commissariat à l'Energie Atomique, France.

All values reported in the tables are mean values of at least two, and more often five, independent experiments.

RESULTS

Physiological and genetic characterization of the mutant strains. Some mutants selected on the basis of resistance towards either ethionine or selenomethionine were tested for their ability to grow in the presence of different concentrations of both methionine analogues. Figure 1 shows the mean generation time observed for cultures in the presence of analogue concentrations that still allow some growth to occur without any lag period. It can be seen in Fig. 1 that selenomethionine was much more inhibitory than ethionine, since 10^{-6} M seleno-DL-methionine stopped the growth of a wild-type strain, whereas 2×10^{-5} M DL-ethionine only led to a twofold increase of the mean generation time. This difference in sensitivity towards the two analogues is also demonstrated by the ability of methionine to overcome their inhibitory effects. As previously reported (5), at a given concentration of ethionine, a 10-fold

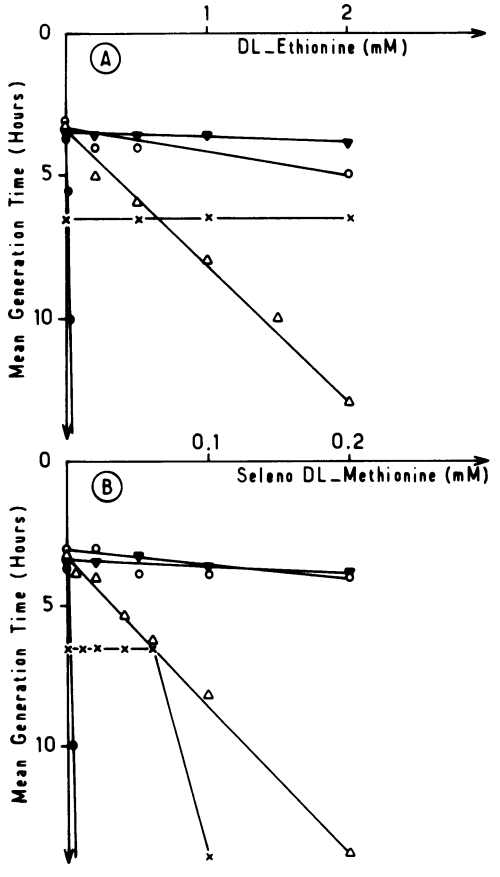

FIG. 1. *Mean generation time of various strains in the presence of different concentrations of methionine analogues. (A) Growth in the presence of ethionine. (B) Growth in the presence of selenomethionine. Symbols:* ●, *4094-B (WT);* ×, *199M1-102 (eth2-2);* ○, *P1S4 (eth10-2);* ▼, *E10 (eth10-1);* △, *CJ21-11B (eth3-1).*

lower concentration of methionine is already sufficient to restore some growth, and an equivalent concentration of this amino acid completely overcomes the toxic effects of ethionine. In the case of selenomethionine, an equal concentration of methionine had only a slight stimulatory effect on growth and, to restore a normal growth rate, a 10-fold excess of methionine over selenomethionine was required.

As far as the mutants studied here are concerned, the one selected on a rather low ethionine concentration, strain CJ21-11B (*eth3-1*), displayed intermediate levels of resistance towards ethionine; only strain E10 (*eth10-1*) selected for their high level of resistance towards ethionine showed a high level of resistance towards selenomethionine. Strain 199M1-102, which carries the nonsense mutation *eth2-2* (13), displayed a peculiar pattern with increasing selenomethionine concentrations. Its growth rate remained unaffected until 0.06 mM but decreased sharply in the presence of higher concentrations of this analogue. The mutant selected for its high level of resistance towards selenomethionine (P1S4) also exhibited a high level of resistance towards ethionine.

Mutations borne by the various strains described here (199M1-102, CJ21-11B, E10, and P1S4) are all monogenic and recessive. No linkage has been found between any of them and the loci *mes1* and *mes2*, encoding, respectively, for methionyl-tRNA synthetase (11) and for homoserine-*O*-transacetylase (6). From crosses between the different mutants, it appears that mutations borne by strains E10 and P1S4 are allelic; therefore, they will be designated as *eth10-1* and *eth10-2*, respectively. Biochemical characterization of the strains carrying these two mutations (see below) has shown that they are very much alike, although the two mutants were selected independently on different methionine analogues (ethionine for strain E10 and selenomethionine for strain P1S4). The loci *eth2*, *eth3*, and *eth10* segregated independently of one another.

Evidence that mutations at the loci eth2, eth3, and eth10 lead to an impaired methionine-mediated repression. Since the mutants described here display resistance to both methionine analogues, ethionine and selenomethionine, the hypothesis could be made that this dual resistance results from methionine overproduction. Therefore, the pool size of methionine was determined in wild-type and mutant strains.

It can be seen in Table 1 that the presence of the mutation *eth2-2*, *eth10-1*, or *eth10-2* led to a considerable increase of the methionine endogenous production in cells grown in minimal medium. With the *eth3-1* mutation, the enhancement of the methionine pool after growth in minimal medium, although significant, was not as elevated as in the other regulatory mutants. It has been shown with strains carrying the *eth2-2* or the *eth10-1* mutation that this high pool size of methionine is due to an increased rate of methionine biosynthesis (J. Antoniewski and H. de Robichon-Szulmajster, Biochimie, in press).

On the other hand, it was previously found

TABLE 1. *Methionine pool sizes and specific activities of three met group I enzymes in different strains after culture in the presence of various exogenous concentrations of methionine*[a]

Genetic lesion	Exogenous DL-methionine added to MM[b] (mM)	Methionine pool[c]	Specific activity[d]		
			Homocysteine synthetase	ATP sulfurylase	Sulfite reductase
—	0	1.5	340	105	1.7
	0.2	—	150	30	0.56
	2	55	75	7	0.07
	20	80	80	7	—
eth2-2	0	14	304	147	—
	0.2	—	300	150	—
	2	60	337	139	—
	20	62	304	138	—
eth10-1	0	16	350	153	1.5
	0.2	—	348	150	—
	2	50	342	143	1.1
	20	87	306	108	0.87
eth10-2	0	17	383	147	1.75
	0.2	—	—	—	—
	2	41	328	115	1.3
	20	71	268	67	0.98
eth3-1	0	4	228[e]	95[e]	—
	0.2	—	230	108	—
	2	64	135	10	—
	20	125	135	10	—

[a] Strains used were 4094-B (WT), 199M1-102 (*eth2-2*), E10 (*eth10-1*), P1S4 (*eth10-2*), and CJ21-11B (*eth3-1*).

[b] MM: minimal medium (see Materials and Methods).

[c] Pools were extracted and determined as described in Materials and Methods. They are expressed in micromoles per gram (dry weight).

[d] Specific activities are expressed in nanomoles per minute per milligram of protein.

[e] It is commonly observed that strains of different origins show some difference in their level of enzymes.

that mutations at the *eth2* locus result in modified methionine-mediated repression. It was shown that the mutation *eth2-1* leads to a partial loss of repressibility (3), and the *eth2-2* nonsense mutation leads to a complete loss of repressibility (13) of met group I enzymes in the presence of exogenous methionine. The methionine-mediated repression of the other mutants, which has not previously been reported, is shown in Table 1. As a basis for comparison, we have included results obtained for the wild-type strain and for the mutant carrying the nonsense mutation *eth2-2*. It can be seen that even the lowest methionine concentration used (0.2 mM) led to a significant decrease of specific activities of met group I enzymes in the wild-type strain, whereas those in the various mutant strains remained constant. As expected, the strain carrying the mutation *eth2-2* remained nonrepressible even at the highest methionine concentration used (20 mM). Under the same growth conditions, two of the other regulatory mutants, those carrying the mutations *eth10-1* and *eth10-2*, showed a very reduced repressibility of the met group I enzymes. For these two mutants, the decrease in specific activity of ATP sulfurylase and sulfite reductase was more pronounced than for homocysteine synthetase but remained only partial for the three enzymes, even at the highest methionine concentration used. When the mutant carrying the mutation *eth3-1* was grown in the presence of 2 mM DL-methionine, the repression of ATP sulfurylase synthesis was complete whereas homocysteine synthetase showed only a 50% decrease in specific activity. In addition, repressibility of the latter enzyme was not enhanced by using 20 mM DL-methionine.

The modification in repressibility of met group I enzymes in the mutant strains cannot be attributed to a decrease in the methionine uptake since, as shown in Table 1, methionine was concentrated from the medium in the mutant strains as efficiently as in the wild-type strain. Therefore, all of these results agree with the hypothesis that the products of the loci *eth2*, *eth3*, and *eth10* are involved in the regulation of methionine biosynthesis.

Since a mutation at the *mes1* locus in *S. cerevisiae* has already been shown to lead to a decreased $tRNA^{met}$ in vivo charging and, concomitantly, to a modified repressibility of met group I enzymes (3), it was conceivable that the product of one of the regulatory genes studied here could also participate in methionyl-tRNA synthetase activity. Genetic data mentioned above showed the absence of linkage between the *mes1* locus (structural gene for methionyl-tRNA synthetase) and the different regulatory mutations studied here. However, there is evidence for multigenic control of glutamyl-tRNA synthetase of *E. coli* (15, 16). Therefore, a study of the kinetic parameters of this enzyme was undertaken in the various strains described in the present report. Results obtained have shown no significant difference between the wild type and the various mutant strains with regard to either specific activities or K_m values for the three substrates, methionine, ATP, and tRNA. In addition, we have found that the amount of total and acylated $tRNA^{met}$ is identical in a wild type and in the mutant strains.

All of these findings render it unlikely that any of the effects observed in strains carrying the mutation *eth2-2*, *eth10-1*, *eth10-2*, or *eth3-1* could be the result of a modified methionyl-tRNA synthetase activity.

SAM-mediated repression in strains carrying mutations at the eth2, eth3, and eth10 loci. It was previously shown that, in a wild-type strain, SAM per se is able to promote complete repression of the synthesis of methionine group I enzymes. Such a complete repression can be achieved by using 0.1 to 0.15 mM SAM (2). Results in Table 2 show that such a concentration also leads to a complete repressibility of met group I enzymes in the *eth2-2* mutant. SAM also exerts a marked repressive effect on the other regulatory mutants. However, it is striking that for these mutants, and especially in the case of homocysteine synthetase, the sensitivity towards SAM concentration is lowered, as compared with the wild-type strain.

To determine the ability of SAM at some concentration to promote complete repression of met group I enzymes in all of the mutant strains studied, we had to use a large range of SAM concentrations. To minimize the use of this expensive compound, we developed a miniaturized technique for culture and enzyme assay. In preliminary assays, it appeared that homocysteine synthetase could be readily measured on benzene-treated cells (see Materials and Methods). Results obtained under such conditions are reported in Table 3. It can be seen that, for strains carrying the mutation *eth10-1* or *eth10-2*, a slight increase in the exogenous SAM concentration (0.4 mM) led to complete repression of homocysteine synthetase.

However, in the case of the strain carrying the *eth3-1* mutation, the highest SAM concentration used (2 mM) did not promote a higher repression of this enzyme than that obtained in methionine-grown cells. It should be pointed out that such an incomplete SAM-mediated

TABLE 2. *Effects of SAM on both the synthesis of two met group I enzymes and the pool size of methionine*[a]

Genetic lesion	Addition to minimal medium	Repression (%)[b]		SAM pool[c]	Methionine pool[c]
		Homocysteine synthetase	ATP sulfurylase		
—	—	—	—	1.1	1.5
	DL-Methionine, 20 mM	76.5	93.5	11	80
	L-SAM, 0.15 mM	82.5	94.5	23	2.4
eth2-2	—	—	—	0.25[d]	14
	DL-Methionine, 20 mM	0	6	0.3	62
	L-SAM, 0.15 mM	90	100	21	3
eth10-1	—	—	—	0.80	16
	DL-Methionine, 20 mM	12	30	1.4	87
	L-SAM 0.15 mM	48	80	30	2.5
eth10-2	—	—	—	0.8	17
	DL-Methionine, 20 mM	30	55	1.9	71
	LSAM, 0.15 mM	50	67	15	4
eth3-1	—	—	—	3.8	4
	DL-Methionine, 20 mM	41	90	25	125
	L-SAM, 0.15 mM	45	80	22	2.7

[a] Strains used were the same as in Table 1.

[b] The percent repression was calculated from the specific activities of the corresponding strain in minimal medium (see Table 1).

[c] Pools were extracted and determined as described in Materials and Methods. They are expressed in micromoles per gram (dry weight).

[d] The SAM pool of 199M1, the parental strain of 199M1-102, is 0.25.

repression in the latter mutant strain cannot be attributed to an impaired SAM uptake since, as shown in Table 2, the pool size of SAM in this mutant grown in the presence of exogenous SAM is comparable to that found in the other strains studied.

Therefore, it seems that in the strain carrying the *eth3-1* mutation SAM- and methionine-mediated repression have been simultaneously modified. In contrast, in strains carrying mutations at the loci *eth2* and *eth10*, methionine-mediated repression was impaired whereas SAM-mediated repression remained comparable to that observed in a wild-type strain. As expected, in the various regulatory mutants that are partially or completely repressed in SAM-supplemented media, the overproduction of methionine was entirely suppressed in such supplemented cultures.

Since, as shown by previous workers, exogenous methionine is efficiently converted into SAM in wild-type cells of *S. cerevisiae* (19), one could assume that such a transformation would occur from endogenously overproduced methionine. Therefore, it was of interest to investigate the biosynthesis of SAM in our strains.

Relationship between methionine and SAM pool sizes. It appears in Table 2 that pool sizes of methionine and SAM are of the same order of magnitude in the wild-type strain grown in minimal medium and that the pool size of SAM is increased 10-fold after growth of this strain in the presence of exogenous methionine.

In the strain carrying the *eth3-1* mutation, it can be seen that, after growth in minimal medium, the presence of a higher methionine pool than that in the wild-type strain was accompanied by an equivalent increase in SAM pool size. In addition, after growth of this strain in the presence of exogenous methionine, a large accumulation of SAM occurred. Thus, the biosynthesis of SAM seems to be undisturbed in the *eth3-1* mutant.

In *eth2* and *eth10* mutant strains, although (as shown above) the endogenous production of methionine was greatly enhanced after growth in minimal medium, the pool size of SAM remained at a level comparable to that found in their respective wild-type strains. Moreover, after growth in the presence of methionine-supplemented medium, the amount of SAM in their pool increased not at all or only slightly. It was conceivable that SAM accumulation was masked by further biochemical events (degradation, excretion, etc.). Results obtained from

TABLE 3. *Repressive effect of increasing SAM concentrations on synthesis of homocysteine synthetase in various strains*[a]

Genetic lesion	Exogeneous *S*-adenosyl-L-methionine added to MM[b] (mM)	Homocysteine synthetase	
		Units/mg (dry wt)[c]	Repression (%)
—	0	81	
	0.1	7	91.5
	0.4	6	92.5
	2	—	—
eth2-2	0	88	
	0.1	1	99
	0.4	2	97.5
	2	—	—
eth10-1	0	106	
	0.1	50	53
	0.4	5	95
	2	6	94
eth10-2	0	128	
	0.1	49	62
	0.4	7	94.5
	2	8	93.5
eth3-1	0	120	
	0.1	—	—
	0.4	—	—
	2	44	63.5

[a] Strains used were the same as in Table 1.

[b] MM: minimal medium (see Materials and Methods).

[c] Units: nanomoles per minute. Activities were determined in benzene-treated cells (see Materials and Methods) and thus related to dry weight instead of protein content.

short-term experiments (see Table 4) show that a high pool of SAM was rapidly formed from exogenous methionine in the wild-type cells and reached its maximal value after 3 h, as observed by Pigg et al. (18). However, even in this short-term experiment, the pool of SAM remained low and constant in mutant E10. Thus, there is no evidence for even a transient accumulation of SAM from free methionine in this class of mutant.

The peculiar behavior of the class of mutant strains which, unlike the wild-type strain, seem unable to accumulate large amounts of SAM, could be interpreted in many ways, such as compartmentation, production of an inhibitor, or presence of a modified methionine adenosyl transferase.

Methionine adenosyl transferase in the regulatory mutants. Although the same effect was observed for mutations in two independent loci, *eth2* and *eth10*, it was not unreasonable to think that they both could have affected the synthesis of methionine adenosyl transferase.

Consequently, a study of this enzyme was undertaken in wild-type and mutant strains. As shown in Table 5, an almost identical level of methionine adenosyl transferase was observed in all strains, with the exception of strain 199M1-102, bearing the *eth2-2* allele, in which a rather low specific activity was found. To provide a possible explanation for this observation, we first have to introduce some results dealing with the regulation of methionine adenosyl transferase synthesis in the various strains.

It was shown previously in wild-type cells that the synthesis of this enzyme is repressed in the presence of exogenously added methionine (2, 14) and derepressed during methionine limitation, although not coordinately with met group I enzymes (2). Data in Table 5 confirm these results for the wild-type strain and for the mutants, again with the exception of strain 199M1-102. It can be seen that, for the strain 199M1-102, the maximal attainable repression in the presence of 10 mM DL-methionine was about 30%. For the other strains, this methionine concentration promoted a 70 to 100% repression, and a lower concentration (2 mM) promoted a repression ranging from 48 to 70%.

In view of these results and of the fact that strain 199M1-102 overproduces large quantities of methionine, one could ask whether the low activity and the almost total absence of repressibility of methionine adenosyl transferase in this strain could not be due to repression of the enzyme by endogenously formed SAM. To test this assumption, we have undertaken the study of a double mutant strain, MM101-2B, carrying the *eth2-2* allele and a mutation in the structural gene for homoserine-*O*-transacetylase (*met2*) which prevents methionine accumulation. In agreement with the

TABLE 4. *Evolution of methionine and SAM pools from exogenous methionine in wild type and one mutant strain as a function of time*[a]

Time (h)[b]	Methionine pool[c]		SAM pool[c]	
	4094-B	E10	4094-B	E10
0	1.5	8	0.9	1
1	5	12	3.8	1.45
2	5.4	12	5	1.41
3	8.5	9	8	1.4

[a] Strains: 4094-B (WT) and E10 (*eth10-1*).

[b] Time after addition to minimal medium of 2 mM DL-methionine.

[c] See footnotes of Tables 2 and 3.

above assumption, it can be seen in Table 5 that in strain MM101-2B both the specific activity of methionine adenosyl transferase and the extent of repression exerted by exogenous methionine on the synthesis of this enzyme were comparable to those observed in a wild-type strain. It seems, therefore, unlikely that the low specific activity and the nonrepressibility of methionine adenosyl transferase found in strain 199M1-102 might be a direct consequence of the mutation *eth2-2*. Moreover, the K_m values for the two substrates of the reaction catalyzed by methionine adenosyl transferase, i.e., methionine and ATP, showed no significant differences in any of the strains studied. It is, therefore, very unlikely that any of the regulatory mutations studied and any of their phenotypic expressions could be due to a genetically impaired methionine adenosyl transferase.

TABLE 5. *Specific activities and extent of repression of methionine adenosyl transferase in wild-type and mutant strains grown under various conditions*[a]

Genetic lesion	Addition to minimal medium	Specific activity[b]	Repression (%)
—	—	1.17	
	DL-Methionine, 0.2 mM	1.25	0
	DL-Methionine, 2 mM	0.33	72
	DL-Methionine, 20 mM	0.35	71
	L-SAM, 0.15 mM	0.25	78
eth10-1	—	1.1	
	DL-Methionine, 0.2 mM	0.85	23
	DL-Methionine, 2 mM	0.55	50
	DL-Methionine, 20 mM	0.36	70
eth10-2	—	0.75	
	DLMethionine, 0.2 mM	0.77	0
	DL-Methionine, 2 mM	0.39	48
	DL-Methionine, 20 mM	0	100
eth3-1	—	1.1	
	DL-Methionine, 0.2 mM	1.5	0
	DL-Methionine, 2 mM	0.44	60
	DL-Methionine, 20 mM	0.3	73
eth2-2	—	0.23	
	DL-Methionine, 0.2 mM	0.19	17
	DL-Methionine, 2 mM	0.17	26
	DL-Methionine, 20 mM	0.16	30
eth2-2 met2	OAcHS, 1 mM	0.87	
	DL-Methionine, 0.2 mM	0.79	9
	DL-Methionine, 2 mM	0.15	83
	DL-Methionine, 20 mM	0	100

[a] Strains used were the same as in Table 1; *eth2-2 met2* corresponds to strain MM101-2B carrying a mutation at the locus encoding for homoserine-*O*-transacetylase (*met2*). *O*-acetyl-DL-homoserine (OAcHS), 0.5 mM, was added to minimal medium to ensure growth of this strain.

[b] Specific activities are expressed in nanomoles per minute per milligram of protein.

In any case, it could be remarked that the low specific activity of methionine adenosyl transferase (probably due to internal repression) found in a strain carrying the *eth2-2* mutation (199M1-102) is comparable to that observed in a wild-type strain grown in the presence of repressive concentrations of methionine. Since in the latter case potent accumulation of SAM in vivo was found to occur, the lack of SAM accumulation in some mutant strains cannot be attributed to the low activity of methionine adenosyl transferase. The two other alternatives, i.e., compartmentation and/or presence of an inhibitor, could then be considered as explanations of this phenomenon. Other experiments should be devised to check these hypotheses.

It is striking to note here that two of the characteristics of the *eth2-2* mutation, lack of SAM accumulation and low specific activity of methionine adenosyl transferase, were also recently reported by Mertz and Spence (14) for a mutant of *S. cerevisiae* resistant to rather low ethionine concentrations. One can wonder whether the same explanation provided here for the mutation *eth2-2* could also apply to the mutation described by these authors.

Some additional data are provided here dealing with the repressibility of methionine adenosyl transferase in a wild-type strain. Since a low concentration of SAM (0.15 mM) was shown to promote full repression of methionine biosynthetic enzymes (2, and present paper), it was thought of interest to determine whether this compound can also regulate the synthesis of methionine adenosyl transferase. As shown in Table 5, this was found to be the case, at least for the wild-type strain (regulatory mutants have not been checked yet). These findings agree with the suggestion made by Mertz and Spence (14) from the study of their ethionine-resistant mutant. Thus, SAM synthetase can be added to the group of enzymes already shown to be susceptible to SAM-mediated repression.

DISCUSSION

All of the mutant strains studied here have a growth rate in minimal medium identical to that of their parental wild-type strain. This renders it likely that the product of each locus involved is dispensable for the general metabolism.

These mutants, which show a dual resistance to both ethionine and selenomethionine, were found to overproduce methionine after growth

in minimal medium. One may ask if this overproduction can account for the resistance of the mutant strains towards methionine analogues. As far as ethionine is concerned, this seems to be the case because the higher the overproduction, the higher the level of resistance observed. For selenomethionine, such a relation seems to apply to *eth10* and *eth3* mutations but not entirely to the *eth2-2* mutation. There is so far no explanation for the peculiar behavior of this mutant in the presence of selenomethionine.

Both the unmodified growth behavior and the methionine overproduction of these mutants already suggest that they carry regulatory mutations. This suggestion is confirmed by the study of methionine-mediated repression, since in all of the mutants a modified pattern of repressibility of met group I enzymes by methionine was observed. However, a quantitative difference in the response to this amino acid could be noticed between the mutant strains. These differences could be due to the peculiar heteroallele for each locus we have studied, rather than to the nature of each gene product. In fact, it should be recalled that the first mutation we obtained at the locus *eth2* (*eth2-1*) leads only to a decreased sensitivity towards methionine-mediated repression (3), whereas further mutations at the same locus have effects which can range from complete insensitivity towards methionine to almost unmodified repressibility (13; Masselot and de Robichon-Szulmajster, unpublished data). In any case, the pleiotropic effects first reported from strains carrying *eth2* mutations are also evidenced in strains carrying a mutation at the loci *eth10* or *eth3*.

Modified methionine-mediated repression of met group I enzymes was also observed in a strain with an impaired methionyl-tRNA synthetase (3). Therefore, we have checked that none of the mutations presented here leads to a modification of this enzyme.

As far as SAM repressive effects are considered, results obtained with the mutants confirm those obtained with a wild-type strain (2) in that SAM participates in the overall regulatory process of methionine biosynthesis in *S. cerevisiae*. The most striking effect in the study of the mutants was observed with the strain carrying the *eth2-2* allele which, being completely insensitive towards methionine-mediated repression (13) as expected from a nonsense mutation, remains nevertheless as sensitive as a wild-type strain towards SAM-mediated repression.

One of the questions to be asked concerns the molecular mechanism underlying methionine- and SAM-mediated repression. Previous studies of a wild-type strain showed that SAM-mediated repression is due to SAM itself and not to its transformation into free methionine (2). If one now considers methionine-mediated repression, two hypotheses can be made. In the first hypothesis, the methionine repressive effect could result from its transformation into SAM. The behavior of our mutant strains is not contradictory with this assumption. No modification of methionine adenosyl transferase, the only enzyme involved in the conversion of methionine into SAM, was observed in the different mutant strains. Thus, the genes *eth2*, *eth3*, and *eth10* cannot encode for this enzyme. However, conditions that lead to potent accumulation of SAM in the wild type and in the *eth3-1* mutant do not lead to such an accumulation in the other mutant strains. This lack of SAM accumulation is not due to a direct defect or modification of the methionine adenosyl transferase molecule itself, but could be an indirect metabolic consequence, shared by mutations *eth2-2*, *eth10-1*, and *eth10-2*, which impairs the function of the enzyme in vivo.

The second hypothesis would be that methionine exerts its regulatory control independently of that exerted by SAM. As discussed in a previous publication (2), one could assume that regulation occurs at two levels: transcription and translation. The existence of two regulatory signals, methionyl-tRNA^{met} and SAM, would permit the two regulatory processes to occur independently.

For the time being, we have mutations which lead to modification of methionine-mediated repression. If the second hypothesis is correct, one would expect to find mutants in which only SAM-mediated repression would be modified.

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