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Biochemical and Regulatory Effects of Methionine Analogues in *Saccharomyces cerevisiae*

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The effect of three methionine analogues, ethionine, selenomethionine, and trifluoromethionine, on the biosynthesis of methionine in *Saccharomyces cerevisiae* has been investigated. We have found the following to be true. (i) A sharp decrease in the endogenous methionine concentration occurs after the addition of any one of these analogues to growing cells. (ii) All of them can be transferred to methionine transfer ribonucleic acid in vitro as well as in vivo with, as a consequence, their incorporation into proteins. In the absence of radioactive trifluoromethionine, this conclusion results from experiments of an indirect nature and must be taken as an indication rather than a direct demonstration. (iii) Ethionine and selenomethionine can be activated as homologues of *S*-adenosylmethionine, whereas trifluoromethionine cannot. (iv) All of them can act as repressors of the methionine biosynthetic pathway. This has been shown by measuring the de novo rate of synthesis of methionine in a culture grown in the presence of any one of the three analogues.

A biosynthetic pathway can be regulated through modulation of enzyme activity or through modulation of enzyme biosynthesis, or both. As far as the biosynthetic pathway leading to methionine and *S*-adenosylmethionine (SAM) is considered in *Saccharomyces cerevisiae*, results from in vivo isotopic experiments do not support the existence of an immediate inhibitory effect of either methionine or SAM on enzyme(s) activity but indicate the existence of a potent "repressive" effect of these compounds on their own biosynthesis (1; J. Antoniewski, Thèse de Docteur-Ingénieur, Université Paris-Sud, France, 1972). These results on the overall biosynthetic pathway are in agreement with previous studies showing that methionine and SAM exert a regulatory function on the synthesis of at least four enzymes that have been designated as methionine group I enzymes (3, 5). Results obtained with a thermosensitive mutant (11) bearing an impaired methionyl-transfer ribonucleic acid (tRNA) synthetase (L-methionine:soluble RNA [adenosine monophosphate], EC 6.1.1.10) and with a methionine auxotroph grown in methionine limitation have shown that the repressive effect exerted by methionine involves the formation of methionyl-$tRNA^{met}$ (5, 22). A modified pattern of repressibility by exogenous methionine was also found in mutant strains isolated in our laboratory on the basis of resistance towards ethionine, a toxic analogue of methionine.

However, it should be emphasized that these mutants, as well as the mutant carrying a modified methionyl-tRNA synthetase, remain as sensitive as a wild-type strain towards a SAM-mediated repression (4, 12; Cherest, Surdin-Kerjan, and de Robichon-Szulmajster, unpublished data).

Already reported data (3, 4), as well as unpublished data from this laboratory, suggest that the repressive effects of methionine and SAM are independent. So far, we have only selected strains with a methionine-modified pattern of repressibility, and therefore it was of interest to search for mutants with a modified pattern of repressibility by SAM. We thought that the isolation of strains resistant to toxic analogues of methionine other than ethionine, such as selenomethionine or trifluoromethyl-L-homocysteine (TFM), could lead to the selection of such mutants. Although in yeast the mechanism of ethionine toxicity has been well investigated (see 19 for review), little information was available on the modes of action of the other analogues; therefore we decided first to investigate their action. Among the various aspects of our study, one of the most important points resides in the ability of the analogues to replace methionine in the two activation processes in which this amino acid is involved, i.e., activation for incorporation into proteins and

[1] This paper is dedicated to the memory of Huguette de Robichon-Szulmajster, deceased in April 1974 while this manuscript was in preparation.

activation for transmethylation reactions with, as a consequence, a possible role as co-repressors.

MATERIALS AND METHODS

Strains. The two haploid strains of *S. cerevisiae* we used are the following: 4094-B (*α ade2 ura1*) from F. Sherman's collection and S.963-18C (*α met2 his*) from R. K. Mortimer's collection.

Media and cultures: (i) Synthetic minimal medium GO. Synthetic minimal medium GO (9) contained mineral salts, trace elements, vitamins, and glucose. When strain S.963-18C was grown, this medium was supplemented with L-histidine (100 mg/liter) and DL-methionine at the concentration given below. When strain 4094-B was grown, the medium was supplemented with adenine (20 mg/liter) and uracil (20 mg/liter) and was designated as G21 medium.

(ii) Low-sulfur-containing medium. Low-sulfur-containing medium was G21 medium in which the sulfate content has been lowered from 20 mM to 0.5 mM as reported previously (1). This medium was used when radioactive sulfate was added (0.5 to 1 mCi of $^{35}SO_4^{2-}$ per 100 ml of culture) to minimize isotopic dilution. All cultures were shaken at 28 C.

(iii) Growth curves. For the determination of growth rates, cells were incubated in 100-ml flasks with a side-arm optical tube which allowed direct optical density measurements in a Jouan spectrophotometer. Optical density was followed at 650 nm. Each flask received 10 ml of the appropriate medium and was inoculated with 5×10^6 cells from a 24-h culture grown in YPGA medium (yeast extract, 5 g/liter; peptone [Difco], 5 g/liter; glucose, 30 g/liter; and adenine, 0.02 g/liter). Incubations were carried out at 28 C and were vigorously shaken.

Pool determinations: (i) Free methionine. Extraction of free methionine pools was carried out as described previously (6). Measurement was based on $^{35}SO_4^{2-}$ incorporation into methionine according to Antoniewski and de Robichon-Szulmajster (1).

(ii) SAM and sulfonium derivatives of methionine analogues. Sulfonium compounds were extracted as described previously (3) and were separated from other compounds by chromatography on Dowex 50 in the Na^+ form as described by Shapiro and Ehninger (18).

The total amount of SAM and sulfonium derivatives was determined by measurement of their optical density at 256 nm, using E_M at 256 nm = 14,700 (18). According to Stekol (20), the chromatographic behavior of the sulfonium derivatives formed from ethionine, selenomethionine, and TFM was assumed to be similar to that of SAM. Determination of the amount of SAM alone was carried out by measurement of $^{35}SO_4^{2-}$ incorporation, with calculations made on the basis of the specific activity of the SO_4^{2-} present in the culture medium.

Determination of the amount of methionyl-tRNA^{met}. Extraction of tRNA's and determination of the amount of tRNA^{met} charged in vivo were as described previously (3, 22).

Determination of in vitro acylation of tRNA^{met} by methionine analogues. In vitro acylation of tRNA^{met} was carried out either with commercial yeast tRNA's (uncharged) or with tRNA's extracted from wild-type strain 4094-B as described previously (22). These tRNA's were stripped by a 2-h incubation period at 37 C in the presence of 1.8 M tris(hydroxymethyl)aminomethane-acetate buffer, pH 8.2. Uncharged tRNA's were then incubated for 30 min at 30 C in a 1-ml incubation mixture containing: adenosine 5'-triphosphate (sodium salt), 10 μmol; $MgCl_2$, 20 μmol; KCl, 10 μmol; dithiothreitol, 2 μmol; potassium phosphate buffer (pH 7.5), 100 μmol; tRNA, 2 mg; and either L-ethionine (10 μmol), L-TFM (10 μmol), or seleno-DL-methionine (0.2 μmol). Two kinds of control incubations were also carried out, one with 0.1 μmol of L-methionine instead of the analogues and the other without amino acid substrate. The second control was made to verify that the stripping of the tRNA's and the periodate oxidation were effective. In addition, each incubation mixture contained an excess of partially purified methionyl-tRNA synthetase from yeast provided by Y. Surdin-Kerjan. At the end of the incubation period and to isolate tRNA's, the reaction mixtures were deposited onto a diethylaminoethyl-cellulose column (DE52, Whatman; 1 by 4 cm), prepared according to Burgess (2), and equilibrated with a buffer containing (per liter): NaCl, 50 mmol; sodium acetate (pH 4.5), 10 mmol; $MgCl_2$, 10 mmol; ethylenedinitrotetraacetic acid (disodium salt), 1 mmol; 2-mercaptoethanol, 20 mmol; and isoamylacetate, 0.1 ml. The column was washed with the same buffer but containing 0.25 M NaCl until the optical density at 280 nm was zero, i.e., about five times the column volume. The tRNA's were then eluted by four column volumes of the same buffer containing 0.7 M NaCl. Fractions (2 ml) were collected. Those containing tRNA's, as determined by measurement of the optical density at 260 nm, were pooled (in general, fractions one to five). tRNA's were then precipitated with 2 volumes of cold ethanol, centrifuged for 10 min at 15,000 × *g*, and dissolved in 0.1 M potassium acetate buffer, pH 4.5. Resulting tRNA's were submitted to periodate oxidation (to destroy uncharged tRNA's) and then stripped as described above, and the determination of their charging capacity by methionine was carried out as described previously (5). At the end of these experiments, it was expected that, at least in the case of tRNA's isolated from the control incubation with methionine, the charging capacity would be near 100%, and that in the case of tRNA's provided from the control incubation without amino acid substrate all of these uncharged tRNA's would be destroyed during periodate oxidation, resulting in 0% charging capacity.

Incorporation of ^{75}selenomethionine into proteins. Wild-type strain 4094-B was allowed to grow for a few generations in 200 ml of G21 minimal medium. During the exponential phase of growth, 0.02 mM seleno-DL-methionine (labeled with ^{75}seleno-L-methionine, 0.2 mCi) was added for a period of 6 h, which corresponds to 1.5 generations in the presence of the analogue. Cells were then centrifuged and washed, and the quantity of selenomethionine incorporated into protein was determined on the basis of

radioactivity in the hot trichloroacetic acid-insoluble fraction.

Incorporation of methionine analogues into proteins. The determination of nonradioactive methionine analogue incorporation into proteins was carried out by the method of Stieglitz and Calvo (21). The methionine auxotrophic strain S.963-18C was grown in 0.6 mM DL-methionine, which leads to the formation of an endogenous pool of methionine of 9 to 10 μmol/g (dry weight) (Surdin-Kerjan, unpublished data). During the exponential phase of growth, cells were centrifuged as quickly as possible, washed, and suspended in the same quantity of fresh medium but without methionine to decrease the endogenous methionine pool. The time period of starvation was 40 min since under these conditions the endogenous methionine pool is about decreased 10-fold (Surdin-Kerjan, unpublished data). After this starvation, 100-ml volumes of culture were transferred to different flasks, each of them containing 0.1 mM L-[^{14}C]threonine (10 μCi per fraction). One of the flasks received no further addition and was used as a control to measure the limited rate of protein synthesis allowed by the presence of residual methionine or methionine arising from protein degradation. The other flasks received either 0.2 mM DL-methionine or one of the methionine analogues at concentrations given in the legend to Fig. 4. For 2 h, 1-ml samples were removed from each flask at 10-min intervals and mixed with 1 ml of 10% trichloroacetic acid. These mixtures were incubated for 30 min at 95 C, then filtered on GF/C Whatman glass-fiber circles, washed three times with 2 ml of 5% cold trichloroacetic acid, and dried with ethanol. Radioactivity of the insoluble fraction was determined in the presence of scintillation liquid with a Intertechnique counter.

Enzymatic activities: (i) Methionyl-tRNA synthetase. Conditions of extraction and of assay of methionyl-tRNA synthetase were as described previously (4). For K_m determination, all substrates were in excess except for L-methionine, the concentration of which was varied from 0.00016 to 0.26 mM. For the determination of inhibition constants of this enzyme by methionine analogues, the methionine concentration used was 0.003 and 0.006 mM, whereas the range of analogue concentrations was as follows: 0.0001 to 0.04 mM for seleno-DL-methionine, 0.1 to 4 mM for L-TFM, and 0.06 to 3 mM for L-ethionine.

(ii) Methionine adenosyl transferase. Conditions of extraction and of assay of methionine adenosyl transferase (adenosine triphosphate:L-methionine-*S*-adenosyl transferase (EC 2.5.1.6) were as described previously (4). *S*-adenosylmethionine formed during the assay was determined by the method of Chou and Lombardini (7). For determination of the K_m for methionine, the concentration of this amino acid was varied from 0.1 to 10 mM, whereas the other substrates were in excess. For the determination of inhibition constants by methionine analogues, the methionine concentration was 2 to 4 mM in the case of seleno-DL-methionine and 0.02 and 0.05 mM in the case of L-TFM and L-ethionine. The ranges of methionine analogue concentrations were 0.2 to 15 mM for seleno-DL-methionine and 1 to 3.5 mM for L-TFM and L-ethionine.

Compounds. TFM was purchased from Cyclo-Chemical Co., seleno-DL-methionine was from Sigma Chemical Co., DL-ethionine and DL-methionine were from A.E.C. (France), L-ethionine was from Calbiochem, L-methionine was from Fluka, and L-threonine was from Koch Light. Commercial yeast tRNA was obtained form Schwarz/Mann, and Dowex 50W X8 was from Lambert-Rivière (France).

^{14}COOH-L-methionine (49 mCi/mmol), L-[U-^{14}C]threonine (30 mCi/mmol), and $^{35}SO_4Na_2$ were purchased from C.E.A., France; ^{75}seleno-L-methionine (200 mCi/mmol) was provided by Radiochemicals Centre, Amersham, England.

RESULTS

Effect of methionine analogues on growth of wild-type strains. The inhibitory effect of TFM on growth of various microorganisms, including *S. cerevisiae*, was first observed by Zygmunt and Tavormina (23). These workers reported that in *S. cerevisiae* 5×10^{-5} and 2×10^{-4} M TFM produced 50 and 100% growth inhibition, respectively. Similar growth experiments performed with the wild-type strain used in our laboratory, 4094-B, have shown that this strain displays a greater sensitivity towards TFM. In Table 1 it can be seen that complete

TABLE 1. *Effect of different methionine analogues on the growth of a wild-type strain[a] of S. cerevisiae*

Addition to G21 medium[b] (M)		Lag period[c] (h)	Mean generation time[d] (h)
None		0	3
L-TFM,	2×10^{-7}	0	3
	5×10^{-7}	8	3
	1×10^{-6}	10	3
	5×10^{-6}	30	4.5
	1×10^{-5}	∞	∞
Seleno-DL-methionine,	2×10^{-7}	0	3
	5×10^{-7}	8	3
	2×10^{-6}	12	3.5
	3×10^{-6}	14	3.5
	5×10^{-6}	∞	∞
DL-Ethionine,	1×10^{-6}	0	3
	5×10^{-6}	4	3.5
	1×10^{-5}	8	6
	2×10^{-5}	15	9
	2×10^{-4}	∞	∞

[a] Strain 4094-B.

[b] Compounds were present from the beginning of the cultures.

[c] The "lag period" is taken as the period of time during which no detectable variation of optical density was observed under our measurement conditions.

[d] Mean generation times were deduced from the exponential part of the growth curves.

growth inhibition was obtained with 10^{-5} M TFM. In addition, for a range of concentrations varying from 7×10^{-7} to 10^{-5} M, the growth of strain 4094-B occurred after a lag period that depended on the concentration. We verified that cells grown after a 30-h lag period were still wild-type cells and were not the result of the selection of some TFM-resistant mutants. This lag period could be attributed to the disappearance of TFM from the culture medium after it had been concentrated by the cells and perhaps partially degradated. If one assumes that there is some interference between this analogue and the metabolism of methionine, one can expect that the endogenous production of methionine becomes sufficient, after a certain period of time, to antagonize the toxic effect of TFM.

The inhibitory effect of selenomethionine on growth is somewhat comparable to that of TFM (Table 1). We can observe, too, the existence of a lag period in the presence of low concentrations of selenomethionine, but these periods were shorter than those observed in the presence of TFM. Complete cessation of growth occurred in the presence of 5×10^{-6} M seleno-DL-methionine. In the case of ethionine, our results are in good agreement with those previously reported (8). In fact, this analogue leads essentially to an increase of the mean generation time depending on the concentration used.

Reversal of the growth inhibitory effect caused by the methionine analogues. The addition of methionine to the culture medium supplemented with any of the analogues permitted restoration of a mean generation time identical to that observed in minimal medium (Table 2). However, the ratio of methionine concentration to analogue concentration, required to obtained such a normal rate, depends on the analogue studied. If we consider successively ethionine, selenomethionine, and TFM, it appears that the methionine concentration needed to completely suppress the growth inhibition is, respectively, equal, 10-fold greater, and 20-fold greater than the analogue concentration. As far as ethionine is considered, these results confirm previous reports (8, 13). If one considers now the efficiency of SAM to antagonize the toxic effects of the analogues on growth, it appears that SAM completely overcomes the inhibition of growth resulting from the presence of selenomethionine in the culture medium by a concentration 10 times greater than the analogue concentration. In the case of TFM, SAM only leads to a decrease of the lag period, but even for a SAM concentration 100-fold greater than the analogue one this lag period remains near 12 h. In the case of ethionine, whatever the SAM concentration used, the mean generation time remains three- to fourfold greater than the one observed in minimal medium.

Effects of the analogues on the intracellular level of methionine. It was previously shown in our laboratory that, in a wild-type strain, the intracellular pool of free methionine remained constant during the exponential part of growth at approximately 1 μmol/g (dry weight) (1). By using the same technique, i.e., the growth of a wild-type strain in a low-sulfur-containing medium supplemented with $^{35}SO_4^{2-}$, we determined the variation of the intracellular free-[^{35}S]methionine pool after the addition during the exponential phase of growth of one of the methionine analogues. Accord-

TABLE 2. *Reversal by methionine or SAM of the growth inhibition induced by three methionine analogues*

Addition to G21 medium[a] (M)		Lag period[b] (h)	Mean generation time[b] (h)
None		0	3
L-TFM (1×10^{-5})		∞	∞
+ DL-Methionine,	2×10^{-6}	24	5
	2×10^{-5}	24	4
	1×10^{-4}	8	3
	2×10^{-4}	0	3
+ L-SAM,	1×10^{-6}	≥30	6.5
	1×10^{-5}	20	6.5
	1×10^{-4}	12	5
	2×10^{-4}	12	5
	1×10^{-3}	12	5
Seleno-DL-methionine (5×10^{-6})		∞	∞
+ DL-Methionine,	1×10^{-6}	23	7
	5×10^{-6}	23	6
	1×10^{-5}	16	6
	2×10^{-5}	4	3
	5×10^{-5}	0	3
1 L-SAM,	2.5×10^{-7}	∞	∞
	2.5×10^{-6}	0	6
	2.5×10^{-5}	0	4
	1×10^{-3}	0	3
DL-Ethionine (2×10^{-4})		∞	∞
+ DL-Methionine,	2×10^{-6}	∞	∞
	2×10^{-5}	0	Low yield
	2×10^{-4}	0	3.5
	2×10^{-3}	0	3.5
+ L-SAM,	1×10^{-6}		
	1×10^{-5}	0	12
	1×10^{-4}	0	10
	1×10^{-3}	0	12

[a] All compounds were present from the beginning of the cultures.

[b] See legend of Table 1.

ing to the rate of renewal of the free-methionine intracellular pool (1) at the beginning of the experiment (time zero) all of the intracellular free methionine was radioactive. Under these conditions and with whatever analogue used (TFM, selenomethionine, or ethionine), we observed a sharp decrease of the intracellular free-methionine pool. Such a decrease is well supported by the results shown in Fig. 1, where it can be seen that about 10 min after the addition of any one of the methionine analogues the endogenous methionine pool was decreased to about 50% of its initial value, while during the same period of time only little growth occurred. After this sharp decrease, the endogenous content of free methionine seemed to reach a steady state corresponding to about 30% of the initial methionine content. In Fig. 2 are reported the results, obtained from an experiment carried out with TFM, confirming both the sharp decrease and the existence of a "residual" methionine pool. Curve A corresponds to the kinetics of methionine biosynthesis as followed by the rate of incorporation of $^{35}SO_4^{2-}$ into free methionine; the initial velocity was of the order of magnitude of 0.1 μmol/g (dry weight) per min. Curve B represents what happened when $^{35}SO_4^{2-}$ and TFM were added together to growing cells. It appears that some [^{35}S]methionine biosynthesis occurred during the first 5 min, followed by a partial utilization before a residual pool was established. Curve C corresponds to the addition of TFM when about all the methionine biosynthesized was radioactive. We notice again the sharp decrease of the intracellular methionine content and the formation of a methionine residual pool.

It can be asked whether the sharp decrease of the free-methionine pool is accompanied by a similar decrease in the amount of methionyl-tRNA^{met}. In fact, we have found that the level of in vivo charging of tRNA^{met}, determined after periodate oxidation, was maximal and near

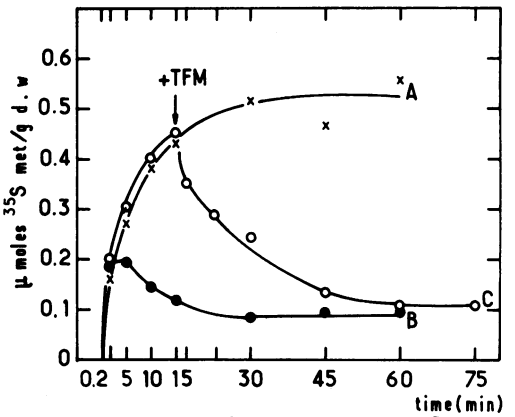

FIG. 2. *Kinetics of $^{35}SO_4^{2-}$ incorporation, into free intracellular methionine, of a wild-type strain in the presence or absence of TFM. The wild-type strain used was strain 4094-B. For these experiments we used a low-sulfate containing medium. (A) Addition at time zero of $^{35}SO_4^{2-}$; (B) simultaneous addition at time zero of both $^{35}SO_4^{2-}$ and L-TFM (10^{-5} M); (C) addition at time zero of $^{35}SO_4^{2-}$, then addition at 15 min of L-TFM (10^{-5} M). At time zero cells were in the exponential phase of growth (optical density at 650 nm [1-cm light path] equals 1) and the intracellular methionine was not radioactive. At various times indicated, samples were removed to determine the intracellular pool of radioactive methionine.*

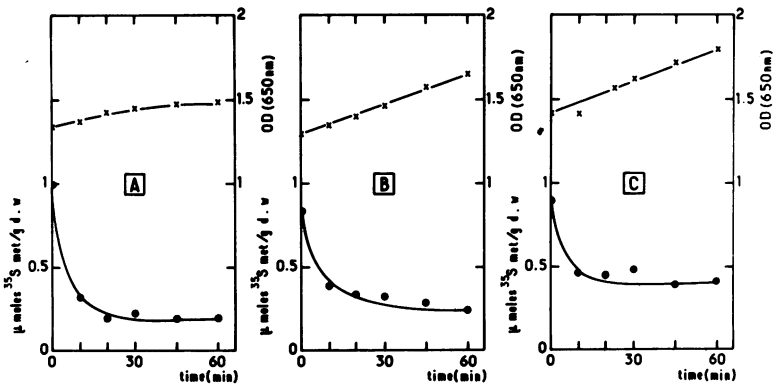

FIG. 1. *Evolution of the intracellular content of free methionine in a wild-type strain after the addition to the culture medium of one of the methionine analogues. Before the beginning of the experiment, cells of strain 4094-B were grown for a few generations in low-sulfate-containing medium supplemented with $^{35}SO_4^{2-}$. At time zero without eliminating the $^{35}SO_4^{2-}$ from the medium, the experiment started by the addition to the culture medium of the methionine analogues (A) L-TFM, 10^{-5} M; (B) seleno-DL-methionine, 4×10^{-5} M; (C) DL-ethionine, 2×10^{-4} M. Samples were taken at time intervals to follow the evolution of radioactive intracellular methionine. This evolution is reported in the lower part of the figure; the upper part is the growth curve of the corresponding cultures.*

100% in the wild-type strain grown in minimal medium supplemented with any one of the analogues for 30 min. This period of time was chosen because we have shown previously that it corresponds to the maximal decrease of the free-methionine pool. However, it remains possible that the methionine utilization that stops after 10 min does not lead to an apparent decrease in the level of in vivo charging of $tRNA^{met}$ because this $tRNA^{met}$ could be charged by the analogues. This possibility was supported by the fact that the incorporation of ethionine into proteins of *S. cerevisiae* has already been reported (6, 14; G. A. Maw, Biochem. J. **98**:28P, 1966). Then, we decided to determine whether the same could be true for the two other analogues.

Incorporation of methionine analogues into proteins: (i) Action of the analogues on methionyl-tRNA synthetase activity. In *S. cerevisiae*, a detailed study of methionyl-tRNA synthetase had not been undertaken so far; therefore, the kinetic constants of this enzyme were determined in our laboratory and it was also shown that its affinity for methionine corresponds to an apparent K_m of 2×10^{-6} M (Surdin-Kerjan, unpublished data). We found that each analogue is able to inhibit the methionyl-tRNA synthetase activity, but with apparent K_i values that differ depending on the analogue considered (Table 3). In fact, it appears (Table 3) that methionyl-tRNA synthetase exhibits a greater affinity for selenomethionine than for methionine; on the contrary, in the case of TFM and ethionine, the affinity of the enzyme for these two analogues is 200- and 1,000-fold lower, respectively, than for the natural amino acid. In all cases, we found that the inhibition of methionyl-tRNA synthetase by the analogues is a competitive one (Fig. 3).

(ii) Determination of in vitro acylation of $tRNA^{met}$ by methionine analogues. Whatever the origin of the tRNAs used during these experiments (commercial yeast tRNA or tRNA extracted from a wild-type strain), the degree of $tRNA^{met}$ charging determined in vitro was identical with TFM, selenomethionine, ethionine, or methionine as control (Table 4). The other control, i.e., incubation mixture without amino acid substrate, led as expected to a total absence of tRNA's charging, showing that both stripping and periodate oxidation have been effective.

TABLE 3. *Inhibition constants of methionyl-tRNA synthetase for three methionine analogues*[a]

Analogues	K_i[b] (M)
Seleno-DL-methionine	2×10^{-6}
L-TFM	6×10^{-4}
L-Ethionine	2×10^{-3}

[a] The wild-type strain used was strain 4094-B.

[b] These determinations have been made in the presence of an L-[^{14}C]methionine concentration equal to or twofold above the K_m value.

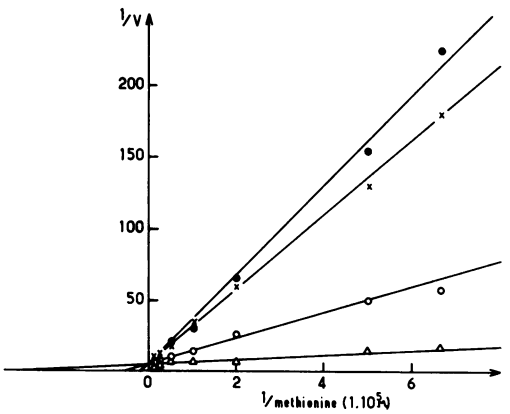

FIG. 3. *Kinetic studies of methionyl-tRNA synthetase from S. cerevisiae. Variation of enzyme activity with methionine concentration:* Δ, *in the absence of analogue;* ●, *in the presence of* 5×10^{-5} *M seleno-*DL*-methionine;* ×, *in the presence of* 2.5×10^{-3} *M* L*-TFM;* ○, *in the presence of* 2.5×10^{-3} *M* L*-ethionine.*

(ii) Direct determination of methionine analogue incorporation into proteins. Ethionine incorporation into proteins of yeast has been reported previously (6, 14; Maw, Biochem. J. **98**:28P, 1966). To demonstrate selenomethionine incorporation, we cultivated a wild-type strain in minimal medium and added 10^{-5} M [^{75}Se]selenomethionine during the exponential phase of growth. After 6 h of growth (1.5 gen-

TABLE 4. *In vitro charging of tRNAs by two methionine*[a] *analogues*

Substrates[b]	Commercial yeast tRNA[c]	tRNA from 4094-B[c]
Control	0	5
L-Methionine, 10^{-4} M	74	88
Seleno-DL-methionine, 2×10^{-4} M	81	70
L-Ethionine, 10^{-2} M	74	89
L-TFM, 10^{-2} M	86	83

[a] These determinations were carried out by using methionyl-tRNA synthetase from *S. cerevisiae*, purified 10 times by Y. Surdin-Kerjan.

[b] Different concentrations were used for the analogues and for methionine to take into account the different affinities of these compounds for methionyl-tRNA synthetase.

[c] Percentage of $tRNA^{met}$ charged in vivo.

erations) in the presence of the analogue, cells were collected and treated as described previously. Under these conditions, we found about 10% of the methionine residues replaced by selenomethionine.

We did not have TFM in a radioactive form. So, TFM incorporation into proteins was determined by measuring the stimulation of protein synthesis using a methionine auxotrophic strain starved for this amino acid and incubated in the presence of TFM. Similar experiments were performed in the presence of ethionine, selenomethionine, and methionine. An experiment was carried out without any addition, as a control. In all cases, we obtained an incorporation of [^{14}C]threonine greater than the one observed in the control culture (Fig. 4). In the case of TFM, the maximal incorporation measured was only a little higher than the level found in the control culture, but it must be noted that this result was very reproducible. In addition, it can be seen that the initial velocity of [^{14}C]threonine incorporation into proteins was identical for TFM and ethionine and significantly different from that of the control culture. It must be noted that some contamination of TFM by methionine could lead to a misinterpretation of the results, the observed growth being due to this methionine content. However, two concentrations of TFM were used in these experiments, 5×10^{-5} and 2×10^{-3} M, and in both cases we obtained exactly the same curve of [^{14}C]threonine incorporation, i.e., the same initial velocity and the same maximal incorporation. If the growth was due to some methionine contamination, it appears obvious that the [^{14}C]threonine incorporation would be greater in the presence of 2×10^{-3} M TFM than in the presence of 5×10^{-5} M TFM. Thus, it seemed very likely that TFM itself can be incorporated into proteins.

Effects of methionine analogues at the level of SAM formation. In yeast, it was previously reported that the analogues ethionine, selenomethionine, and TFM are able to inhibit methionine adenosyl transferase activity. In addition, it was shown that ethionine and selenomethionine can be used as substrates for this enzyme, leading to the formation of *S*-adenosylethionine and of seleno-adenosylmethionine (15–17). On the contrary, it was reported that, in vitro, TFM cannot be activated (10). Since these studies were performed with commercial yeast, we decided to verify the effect of the methionine analogues on methionyl adenosyl transferase in our wild-type strain of *S. cerevisiae*. Using a crude extract of this strain, we found that seleno-DL-methionine, L-TFM, and L-ethionine inhibit methionine adenosyl transferase activity with respective K_i values of 2, 26, and 45 mM, while the apparent K_m for methionine was about 1 mM under our assay conditions. In all cases, we found that the inhibition was competitive towards methionine. We then searched for the existence in vivo of activated compounds corresponding to each analogue (Table 5). If one considers the SAM pool itself as measured by ^{35}S incorporation, no significant differences can be observed in its values after growth in the absence or in the presence of any one of the methionine analogues. Thus, it can be concluded that the presence of the analogues in the culture

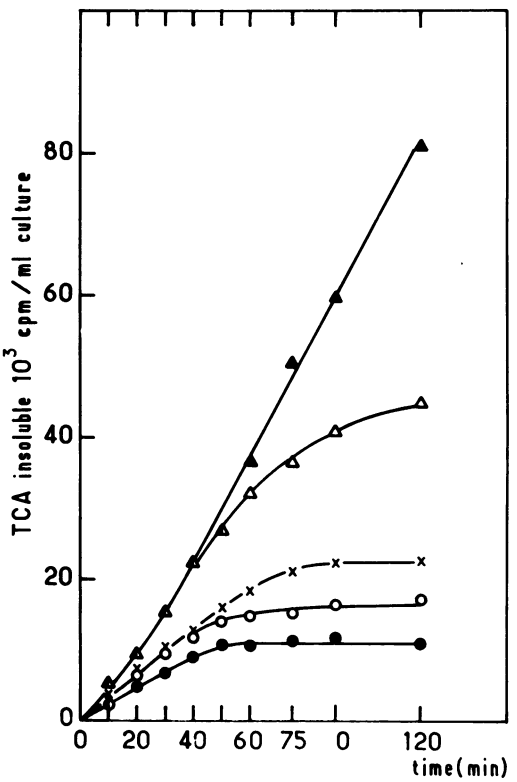

FIG. 4. *Effect of methionine analogues on [^{14}C]threonine incorporation into protein in a methionine auxotrophic strain of S. cerevisiae. The strain used was strain S.963-18C. The determination of [^{14}C]threonine incorporation into proteins of strain S.963-18C was performed:* ●, *in the absence of any addition;* ○, *in the presence of* 5×10^{-5} *M* L-*TFM;* ×, *in the presence of* 4×10^{-4} *M* L-*ethionine;* △, *in the presence of* 2×10^{-5} *M seleno-*DL-*methionine;* ▲, *in the presence of* 2×10^{-4} *M* DL-*methionine. In the case of TFM we also used other concentrations such as* 10^{-4} *M,* 2×10^{-4} *M, and* 2×10^{-3} *M; in all cases we obtained exactly the same curve of [^{14}C]threonine incorporation.*

TABLE 5. *Formation of sulfonium derivatives from methionine analogues in wild-type strain 4094-B*

Addition to medium[a]	Sulfonium derivative formation[b]	
	^{35}S	Ultraviolet light
None	1	1
L-TFM, 5×10^{-4} M	0.6	1
L-Ethionine, 5×10^{-4} M	0.5	8.6
Seleno-DL-methionine, 1×10^{-3} M	1	6

[a] Cells were grown for a few generations in low-sulfur-containing medium and supplemented with $^{35}SO_4^{2-}$. The analogues were added during the exponential phase of growth, and the cells were harvested 1 h after the addition.

[b] The radioactive method of measurement corresponds to the SAM pool itself, whereas the ultraviolet light method of determination corresponds both to the SAM pool and to the sulfonium derivative pools arising from each analogue studied. In the case of a culture made in absence of analogue, the two methods of measurement correspond both to the SAM accumulated. Values (micromoles of sulfonium compounds formed per gram dry weight) were normalized to a value of 1 for each method of detection to clarify the results. In fact, the normalized value of 1 given in the case of ultraviolet light detection corresponds to an absolute value threefold greater than the one found by ^{35}S incorporation. This is not too surprising since the radioactive method of measurement is much more precise than the ultraviolet light method of detection, especially when the pool size of SAM is low as it is the case after growth in minimal medium.

medium does not lead to any enhancement of the pool size of biosynthesized SAM. If one considers now the total sulfonium compounds as measured by ultraviolet light absorption, it appears that the amount of these compounds accumulated in the presence of exogenously added TFM was similar to that found in the unsupplemented culture, leading to the conclusion that no sulfonium derivative of TFM was made. On the contrary, the amount of sulfonium compounds found in cultures made in the presence of ethionine or selenomethionine is significantly higher than in the absence of these analogues. Since we have shown that the pool size of SAM is the same under all culture conditions used, it appears that ethionine and selenomethionine can be activated into homologues of SAM and that activation of TFM does not occur.

Methionine analogues considered as possible repressors. Antoniewski and de Robichon-Szulmajster (1) have reported that the overall endogenous synthesis of methionine, measured by following the ^{35}S incorporation from the medium into the free-methionine pool, is decreased in the presence of 0.8 mM exogenous DL-methionine, with kinetics compatible with that of repression as the major regulatory mechanism for this biosynthetic pathway in *S. cerevisiae*. These authors have not found any additional effect that could be attributed to inhibition. It can be seen in Table 6 that the percent dilution of [^{35}S]methionine observed 5 h after the addition of 0.8 mM DL-methionine is identical to that theoretically expected if one considers that complete cessation of methionine synthesis occurs from the moment of addition of the exogenous repressor.

The same kind of experiments cannot be carried out directly to study the ability of the methionine analogues to act as repressors since we have shown above that the addition of methionine analogues to growing cells leads to a sharp decrease of the intracellular methionine content and to cessation of growth. However, preliminary experiments have shown that the addition of 0.1 mM DL-methionine to cultures supplemented with any one of the analogues (at a given concentration) is sufficient to ensure growth and prevent the rapid decrease in the endogenous methionine concentration. In addition, we know that 0.1 mM DL-methionine is unable to promote repression of methionine group I biosynthetic enzymes (5). Results (Table 6) show that the same effect was observed at the level of the overall methionine biosynthesis, since 5 h after the addition of 0.1 mM DL-methionine no dilution of the free [^{35}S]methionine pool was observed, indicating that the biosynthesis of methionine was not stopped by such an exogenous concentration.

It appears then that the capacity of the different analogues to act as repressors can be examined in cultures supplemented with both the analogues and 0.1 mM DL-methionine. It can be seen (Table 6) that for each analogue studied the percent dilution of [^{35}S]methionine obtained is very similar to the expected one. Thus, it seems likely that the three analogues, ethionine, selenomethionine, and TFM, can act as repressors.

DISCUSSION

The study of the effect of some methionine analogues on growth of a wild-type strain of *S. cerevisiae* showed that TFM is a potent inhibitor, since complete cessation of growth already occurs in the presence of very low concentrations of this analogue, such as 5×10^{-6} M. Whereas the inhibition of growth observed in the presence of ethionine corresponds to an increase of the mean generation time, the inhib-

TABLE 6. *Comparative effect of methionine analogues and methionine exogenously added upon the de novo synthesis of methionine in wild-type strain 4094-B*

Addition to culture medium[a]	Time (h)	Optical density[b]	Experimental results		Expected results[c]	
			[^{35}S]methionine obtained[d] (μmol/g dry weight)	[^{35}S]methionine dilution (%)	[^{35}S]methionine expected (μmol/g dry weight)	[^{35}S]methionine dilution (%)
DL-Methionine, 0.8 mM	0	0.6	1		1	
	5	2.1	0.24	76	0.29	71
DL-Methionine, 0.1 mM	0	0.3	0.55		0.55	
	5	1.8	0.70	0	0.09	84
DL-Methionine, 0.1 mM, + DL-ethionine, 0.2 mM	0	0.4	0.53		0.53	
	5	2.1	0.15	72	0.10	81
DL-Methionine, 0.1 mM, + seleno-DL-methionine, 0.02 mM	0	0.46	1		1	
	5	1.65	0.31	69	0.28	72
DL-Methionine, 0.1 mM, + L-TFM, 0.01 mM	0	0.95	0.71		0.71	
	5	2.25	0.28	60.5	0.3	58

[a] Before the beginning of the experiment, cells were grown for a few generations in low-sulfate-containing medium and supplemented with $^{35}SO_4^{2-}$. Under these conditions, at the beginning of the experiment (time zero) all of the intracellular methionine pool was radioactive. At time zero, without eliminating the $^{35}SO_4^{2-}$ from the medium, various compounds were added. Then samples were taken at time zero and at 5 h, and their content in free [^{35}S]methionine were determined in boiled extracts. It should be emphasized that under these conditions all the methionine synthesized de novo was radioactive.

[b] 650 nm; 1-cm light path.

[c] Results theoretically expected have been calculated as followed: expected value at 5 h = experimental value at time zero × (optical density at time zero/optical density at 5 h).

[d] The values reported here are those obtained from one typical experiment. The variations observed in the values given for time zero (0.5 to 1 μmol of [^{35}S]methionine/g dry weight) are in the range of experimental error. For each experiment performed, the percent dilution of [^{35}S]methionine at 5 h remains about 70% when repression occurs and near 0% when there is no repression.

itory effect of TFM and, to a smaller extent, of selenomethionine on growth is better characterized by the existence of long lag periods: the higher the analogue concentrations used, the longer the lag periods are. We have found that in all cases methionine is able to overcome the inhibitory effect of each analogue, whereas *S*-adenosylmethionine only overcomes the inhibitory effect of selenomethionine. This could suggest the existence of some differences between the behavior of selenomethionine and that of the two other analogues studied here. However, such a situation was not apparent. In fact, the only (major) difference between the analogues was found in their capacity to be activated as *S*-adenosylmethionine homologues, but in this case selenomethionine displays the same behavior as ethionine.

We found that the addition of each analogue to growing cells immediately leads to a sharp decrease in the endogenous content of free methionine. From these results, two points should be made. First, it should be emphasized that such a decrease in the de novo biosynthesized methionine does not occur after the addition of either exogenous methionine or exogenous SAM (1). It appears then that the lowering of the intracellular methionine pool is a specific effect of the analogues. Several interpretations can be given to this phenomenon. For example, it could be the result of the inhibition of some enzymatic activities implied in the methionine biosynthetic pathway and which would be insensitive to the natural amino acid, or more probably it could correspond to an exit of the intracellular methionine to the medium. Second, it can be recalled that the sharp decrease of the intracellular methionine pool is stopped after some time since there remains a pool of residual methionine corresponding to 30% of the initial one. It may be asked whether the nonutilization of the remaining methionine is due to a problem of localization.

As far as the other aspects of our study are considered, we have shown that each of the analogues used displays quite a similar behavior towards $tRNA^{met}$, since each of them can be charged in vitro as well as in vivo. Considering that methionyl-$tRNA^{met}$ is required not only for methionine incorporation into proteins and taking into account the regulation of the methionine biosynthetic pathway (5, 22), we tried to determine whether the $tRNA^{met}$ charged by the analogues is able to perform its two functions.

From our results, it appears that each of the methionine analogues studied here can act as a co-repressor. Thus, no distinction could be made between the ability of the analogues to be charged on the $tRNA^{met}$ and their ability to act as co-repressors. However, since we found that the other activated product of methionine, SAM, is able to exert a repressive effect independently of methionine, it may be asked whether the role of the analogues in regulation could be the result of their activation in *S*-adenosylmethionine homologues. In the case of TFM, this hypothesis can be completely excluded, since this compound can act as a co-repressor but cannot be activated by methionine adenosyl transferase. With respect to ethionine and selenomethionine, a clear-cut statement cannot be made yet, but the fact that methionine itself does not require its transformation into SAM does not favor this hypothesis (3; Surdin-Kerjan, submitted for publication). As mentioned above, only TFM is unable to give rise to a homologue of SAM; this fact confirms previous in vitro results showing that, in yeast, TFM can play a role as an inhibitor but not as a substrate of methionine adenosyl transferase (10). Then, it is conceivable that some mutants resistant to TFM can be the result of a mutation leading to the existence of a modified methionine adenosyl transferase which, in turn, could provide some modification in SAM-mediated repression. Selection and study of such mutants are under investigation.

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