METHIONINE BIOSYNTHESIS FROM THE 4-CARBON SKELETON OF ETHIONINE IN SACCHAROMYCES CEREVISIAE

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In genetic studies on ethionine resistance in S.cerevisiae three genes have been characterized : AAP, eth_1, eth_2. The first gene is concerned with the active transport of amino acids, the allele aap giving resistance to various amino acid analogs (Surdin et al., 1965). The strains carrying mutant alleles of the two other genes (eth_1^r dominant and eth_2^r recessive) show specific resistance to ethionine (Cherest and de Robichon-Szulmajster, 1966 ; de Robichon-Szulmajster and Cherest, 1966).

Comparative in vivo studies have shown that methionine biosynthesis is completely suppressed either by exogenous methionine or ethionine in the wild-type strain 4094-B (eth_1^s, eth_2^s) but remains unaffected under these conditions in the double mutant strain CH82-7D (eth_1^r, eth_2^r) (Cherest and de Robichon-Szulmajster, unpublished results ; de Robichon-Szulmajster, 1967). These results led us to study regulatory aspects of methionine biosynthesis in S.cerevisiae.

We have previously reported that, as in Neurospora (Nagai and Flavin, 1967), the first step of methionine biosynthesis from homoserine in S.cerevisiae is catalyzed by homoserine-O-transacetylase (de Robichon-Szulmajster and Cherest, 1967). This conclusion was derived from two sets of data : 1) the existence of a methionine auxotroph, D6 (gene me_a**), devoid of homoserine-O-transacetylase activity ; 2) the regulatory properties of this enzyme, whose synthesis is repressed by methionine, and whose activity is inhibited by S-adenosyl-methionine (SAM). Further-

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** me_a, and ur, designate genes for which allelism test have not yet been carried out.

more, it was pointed out that the mutant allele eth$_2^r$ leads to non-repressibility of homoserine-O-transacetylase.

More recently, we have shown that the presence of the allele eth$_2^r$ also causes the simultaneous derepression of homocysteine synthetase (Cherest and de Robichon-Szulmajster, 1968), the enzyme that catalyzes the direct synthesis of homocysteine from O-acetyl-homoserine and H_2S (Wiebers and Garner, 1967).

The role of eth$_1$ is still under study, but does not seem to be implicated in the regulation of any of the four steps controlled by methionine that we have studied so far (Cherest and de Robichon-Szulmajster, 1968).

In a cross involving a methionine auxotroph (me$_a$) and a double mutant strain (eth$_1^r$, eth$_2^r$) we obtained two classes of methionine negative segregants, one class being identical to the parental strain me$_a$, the other being able to grow in the presence of ethionine instead of methionine. The present report deals with in vivo studies of this special recombinant strain which will be called "me$_a$ eth$^+$" until a more precise genotype can be determined.

Experimental : Haploid strains of *S. cerevisiae* have been used : 4094-B (α, ad$_2$, ur$_1$, eth$_1^s$, eth$_2^s$) ; CH82-7D (α, ad$_2$, ur$_1$, eth$_1^r$, eth$_2^r$) ; CH82-9C (a, ad$_2$, ur$_1$, eth$_1^r$, eth$_2^r$) ; D6 (a, ur**, me$_a$**) ; CC92-8D (α, ad$_2$, ur**). Cells were grown in a synthetic medium (Galzy and Slonimski, 1957), and supplemented, when necessary, with adenine, 10 μg/ml, uracil, 10 μg/ml and radioactive compounds (.4 to .6 mC/200ml cultures). Concentrations of DL-methionine, DL-ethionine and O-acetyl-DL-homoserine used are given in the text. Cells were harvested at the end of exponential phase, washed twice and resuspended in distilled water (2.5 ml H_2O per 1 ml of packed cells).

Soluble compounds were extracted by heating this suspension at 100°C for 10 minutes. Centrifugation gave a supernatant, called "boiled extract". The residue was washed twice with cold water, treated twice with 5 % TCA at 100°C for 30 minutes (30 ml TCA per 1 ml of initial packed cells) and then washed twice with cold 5 % TCA. The new residue was submitted to acid hydrolysis, (5 ml of 7N HCl per 1 ml of initial packed cells) at 120°C for 24 hours in sealed tubes. HCl was then eliminated under vacuum, the dry residue was resuspended in distilled water and dried again. This treatment was repeated three times. The last dry residue dissolved in 500 μl of distilled water constitutes the "protein hydrolyzate".

Aliquots of this protein hydrolyzate were submitted to thin layer cellulose chromatography with n-butanol-acetic acid water (120 : 30 : 50) as a solvent and radioautograms were made. Compounds were identified by submitting duplicate plates to coloration tests (ninhydrin and iodoplatinate) and comparing the R_f values of the different spots obtained (radioactive or not) with reference substances. Radioactivity contained in those spots was counted in scintillation vials. The amount of each compound (amino acid plus its corresponding sulfoxide) was calculated on the basis of specific radioactivity of the compound introduced at the beginning of the experiment.

Radioactive compounds have all been purchased from the C.E.A. France.

Results and discussion

From the cross CC92 (D6 x CH82-9C), a "me_a eth^+" segregant, CC92-8D, was selected for biochemical studies. As already pointed out, such a methionine auxotroph is able to grow when ethionine replaces methionine in the growth medium. Growth studies (table 1) indicate that, as expected in the case of homoserine-O-transacetylase deficient mutant, this strain is able to grow at the expense of the product of the reaction, O-acetyl-homoserine. Moreover, it can be seen that generation times and yields are almost identical in methionine and ethionine supplemented media.

	Generation time			Yield		
Concentration	Compounds added to minimal medium					
	AcHS	Meth	Eth	AcHS	Meth	Eth
$1x10^{-5}M$	12.5	12.5	10.5	50	103	93
$1x10^{-4}M$	5.5	5.0	5.2	290	392	306
$1x10^{-3}M$	3.7	3.7	4.2	490	485	485

Table 1 : Growth parameters for the haploid strain CC92-8D.

AcHS : O-acetyl-DL-homoserine ; Meth : DL-methionine ; Eth : DL-ethionine. Generation times are expressed in hours. Yields are taken after 32 hours growth and expressed in µg dry weight per ml of culture.

These results favor the hypothesis of a utilization of at least part of the ethionine molecule, rather than a hypothesis based upon induction by ethionine of an alternate methionine biosynthetic pathway. Nevertheless, an experiment has been undertaken to check the second hypothesis. Cells (CC92-8D) harvested after growth in the presence of methionine or ethionine, were washed and inoculated into fresh minimal, or minimal supplemented with methionine or ethionine, media. The finding that, in all cases, growth occured only in supplemented media permit to exclude the induction hypothesis.

It then appeared necessary to undertake a study of the origin of the different parts of methionine molecules synthesized in ethionine grown cells. As the only known deficiency in the mutant CC92-8D is the inability to acetylate homoserine it can be assumed that sulfate utilization, at least until sulfide formation, is not impaired. Amounts of methionine recovered have been determined comparatively in protein hydrolyzates of methionine prototrophs and of the two mutant types (CC92-8D, "$me_a eth^+$" and D6 "$me_a eth^-$"), when grown in minimal medium supplemented with ^{35}S sulfate. It can be seen in table 2 that there is no noticeable difference between the amounts of methionine or cysteine formed from ^{35}S sulfate by the four strains studied.

Strains	4094-B	CH82-7D	CC92-8D	D6
Genotypes	α, ad_2, ur_1	α, ad_2, ur_1	α, ad_2, ur, me_a eth^+	a, ur, me_a eth^-
	µmoles of ^{35}S/g dry weight			
Methionine	11.7	11.3	9.5	10.5
Cysteine	4.6	4.8	3.0	4.4

Table 2 : Sulfate utilization in methionine biosynthesis with methionine prototrophic and auxotrophic strains.

Minimal medium contains adenine and uracil, and in addition, O-acetyl-DL-homoserine $2x10^{-3}M$ for D6 and DL-ethionine $2x10^{-3}M$ for CC92-8D.

It can be concluded that the sulfur of methionine synthesized by the mutant strain CC92-8D originates from sulfate, even in the presence of ethionine. Confirmation of these findings was given by a similar experiment in which ^{35}S-ethionine was used. The results in table 3 show that 1.5 %, at most, of the methionine sulfur can have originated from the sulfur of ethionine.

These results prompted us to investigate, in turn, the origin of the carbon atoms of methionine formed in ethionine grown mutant cells.

In a first experiment we attempted to verify whether the normal 4-carbon precursor, homoserine, could be used even in the absence of the acetylation reaction. Although ^{14}C-bicarbonate or ^{14}C-glucose grown cells of CC92-8D have shown no accumulation of ^{14}C-homoserine in boiled extracts, the culture of this strain was made in the presence of $2x10^{-3}M$ DL-ethionine and $1x10^{-2}M$ DL-threonine. The latter is known to repress and inhibit yeast aspartokinase (de Robichon-Szulmajster and Corrivaux, 1963) and so minimizes the eventual participation of endogenously produced homoserine. ^{14}C-homoserine was added for one generation at $1.5x10^{-4}M$. Only traces of radioactivity were found in the methionine spot (see first column in table 3).

These results show that homoserine cannot be the precursor of the 4-carbon skeleton of methionine formed in the presence of ethionine. It then seemed unavoidable to postulate the participation of the carbon atoms of ethionine itself in this synthesis. Two sets of experiments were carried out using ^{14}C-ethionine labeled in the ethyl- or in the carboxyl-groups. It can be seen in table 3 that the 2 carbon atoms of the ethyl group do not participate to methionine formation. On the contrary, a massive utilization of the carboxyl group is observed.

The amount of methionine synthesized when calculated on the basis of the carboxyl group (10.7 μmoles), is identical to the amount synthesized when calculated on the basis of sulfate incorporation (9.5 μmoles - see table 2). These results clearly indicate that the carboxyl group of ethionine is incorporated without dilution into the methionine molecule by the "me_a eth^+" strain.

At this point, we formulated the hypothesis that ethionine participates in methionine biosynthesis not only by its carboxyl but by its 4-carbon skeleton. A biosynthetic pathway completely different from the usual one then had to be postulated.

Compounds	Homoserine	Ethionine			Cysteine
Label	^{14}C-C_4	^{35}S	^{14}C Ethyl	^{14}C Carboxyl	^{35}S
		μmoles per g dry weight			
Methionine	0.02	0.14	0.01	10.7	0.42
Ethionine	-	0.40	0.33	0.5	-
Cysteine	-	-	-	-	2.03

Table 3 : Utilization of different labeled compounds postulated as possible precursors for methionine biosynthesis by the mutant strain CC92-8D.

Growth was performed in minimal medium supplemented with adenine, uracil and 2x10^{-3}M DL-ethionine, or 4x10^{-4}M DL-ethionine in the case of the ^{14}C-carboxyl-ethionine experiment. Conditions used for homoserine and cysteine grown cells are described in the text.

We first tried to determine whether the other sulfur amino acid, cysteine, participated in this alternative pathway. Experiments with ^{35}S-cysteine (6.5x10^{-4} M) showed a small incorporation of cysteine sulfur into methionine synthesized under these conditions (see table 3). However, a 2-fold isotopic dilution is observed in cysteine recovery (4 μmoles formed from ^{35}S-sulfate (table 2) and 2 μmoles formed from ^{35}S-cysteine and ^{32}S-sulfate (table 3)). As a consequence, if cysteine were the only sulfur compound used as a precursor for the sulfur of methionine, 5 μmoles of labeled methionine would be expected to be found in this experiment. However, our results show a participation of cysteine amounting to only 8-10 % of that expected. These results favor a breakdown of cysteine and re-utilization of its sulfur rather than a direct participation of cysteine in the postulated pathway. Other sulfur intermediates then had to be postulated.

S-Methyl-cysteine (SMC) is a good candidate on the basis of studies from other laboratories. SMC was isolated as a naturally occuring metabolite in turnip roots (Morris and Thompson, 1955-1956) and Neurospora (Ragland and Liverman, 1956). In addition, the latter authors have shown that in Neurospora SMC can be used by wild type strains in sulfur-deficient medium and by certain methionine auxotrophs. They postulated a mechanism for condensation of the thiomethyl group with a 4-carbon unit. The findings of Wiebers and Garner (1964) reinforced this hypothesis by showing that sulfur and the methyl group from SMC are incorporated into methionine.

In yeast, SMC, but not cysteine, overcomes ethionine inhibition (Maw, 1961). Furthermore, an enzyme has been purified from this organism that is able to synthesize SMC by condensation of methylmercaptan and L-serine (Wolff et al., 1956). More recently, SMC synthesis in vitro has been shown to occur from O-acetyl-serine and methylmercaptan in extracts from spinach (Giovanelli and Mudd, 1968), Neurospora, S.cerevisiae, and Brassica rapa (Thompson and Moore, 1968).

In vivo studies in yeast have shown that methylmercaptan acts as a precursor for thiomethyladenosine synthesis, the latter compound being derived from methionine through SAM (Schlenk and Tillotson, 1954). We carried out such an experiment using ^{35}S-methylmercaptan (generated into the culture flask from S-methylthiouronium sulfate) in the mutant strain CC92-8D. In agreement with the previous authors, ^{35}S-thiomethyladenosine was found to be abundant in the boiled extract. Moreover, considerable quantity of ^{35}S-methionine was found in the protein hydrolyzate*. This last finding favors methylmercaptan as the thiomethyl precursor for the alternative pathway of methionine biosynthesis occurring in the mutant strain we are studying. Therefore, methionine could be formed directly from CH_3SH and the 4-carbon skeleton of ethionine, or SMC could be implicated as a possible intermediate.

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* This experiment will be published later in details.

It has not yet been elucidated if de novo methylmercaptan synthesis can occur in yeast. It can be recalled that production of this compound has been demonstrated in Schizophyllum commune (Birkinshaw et al., 1942).

So far, this new pathway for de novo biosynthesis of methionine has been found to occur only in strains simultaneously carrying the mutated alleles me_a (deficiency in homoserine-O-transacetylase) and eth^r_1,eth^r_2 (the combination of which confers resistance to $1x10^{-2}M$ ethionine in prototrophic strains). Genetic data already obtained do not allow us to determine whether this trigenic combination is the only one giving rise to such a metabolism. As far as the resistance genes are concerned, the data available are compatible with the participation of both. It is possible that an additional character may prove to be necessary. As for the metabolic block in methionine biosynthesis, genetic results recently obtained show that recombinants carrying me_8 (homocysteine synthetase deficiency) and the resistant alleles eth^r_1 eth^r_2 do not lead to expression of the alternative pathway of methionine biosynthesis (Cherest and de Robichon-Szulmajster, unpublished results).

These results imply that homocysteine synthetase activity may be involved in the alternative methionine pathway, whereas on the contrary, homoserine-O-transacetylase activity is not.

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