REGULATION OF HOMOSERINE O-TRANSACETYLASE[1], FIRST STEP IN METHIONINE BIOSYNTHESIS IN SACCHAROMYCES CEREVISIAE[2]

H. de Robichon-Szulmajster and H. Cherest
Laboratoire d'Enzymologie, C.N.R.S.-91, Gif-sur-Yvette, FRANCE

Received June 16, 1967

The first step in methionine biosynthesis in E.coli consists in a transsuccinylation from succinyl-CoA to homoserine, leading to the formation of O-succinyl-homoserine (Rowbury, 1961 ; Rowbury and Woods, 1964). The same pathway operates in Salmonella (Flavin et al, 1964). In Neurospora, nutritional data indicate that the homoserine derivative formed is O-acetyl-homoserine, (O-AcHS) but the acetyl donor remains unknown (Nagai and Flavin, 1966). In plants (spinach), the two derivatives can be utilized, in vitro, for synthesis of cystathionine (Giovanelli and Mudd, 1966). In yeast, the first step in methionine biosynthesis had not yet been studied. The present report shows that this step is catalyzed by homoserine O-transacetylase (HS-Tase). The synthesis of this enzyme is repressed by exogenous methionine and its activity inhibited by S-adenosyl-methionine (S.A.M.).

EXPERIMENTAL : Two haploid strains of S.cerevisiae have been used : 4094-B (α, ad_2, ur_1) and D-6[3] (a, ur, met_a^1).

1) Even not knowing the acetyl donor, we assimilate the enzyme to a transacetylase by analogy with the homoserine O-transsuccinylase from bacteria. Until allelism test can be made with other "met" genes already known in S.cerevisiae, the present gene will be named "met_a".

2) This work has been supported by grants from the D.G.R.S.T. (66-00-140) and from the C.E.A., France.
3) The authors are thankful to Dr M.Grenson for the homocysteine requiring mutant D-6, to Dr M. Flavin for a generous gift of O-AcHS, to Dr M.Cherest who synthetized this compound for us and to Miss F.Eichler for her skillful assistance.

Cells are grown in a synthetic medium (Galzy and Slonimski, 1957) supplemented, when necessary with uracil, 10µg/ml, adenine, 20µg/ml, DL-methionine, 10µg/ml. Cells are harvested at the end of exponential phase by centrifugation, washed twice and resuspended in potassium phosphate buffer, pH 7.4, 10^{-1}M. Cell-free extracts are obtained in a Nossal highspeed shaker as described by de Robichon-Szulmajster (1961) and used immediately. Such extracts usually contain 7 to 15 mg of protein/ml.

Attempts to demonstrate enzymatic formation of acetyl- or succinyl- derivatives, using homoserine in the presence of succinate or acetate and CoA, acetyl-CoA or succinyl-CoA have been unsuccessful. We therefore estimate the rate of O-AcHS formation by using the following exchange reaction, as suggested by Nagai and Flavin (1966) :

O-acetyl-HS + ^{14}C-homoserine $\rightleftharpoons$ ^{14}C-acetyl-HS + homoserine

A complete reaction mixture contains, in .2ml : O-acetyl-DL-homoserine[3,4], 2.5 µmoles ; ^{14}C-DL-homoserine[4], .6 µmoles (6.6×10^6 cpm/µmole) ; potassium phosphate buffer, pH 7.4, 20 µmoles ; cell-free extract, 70 µl. Incubation is done at 37°C for 60 minutes and stopped by heating at 100°C for 2 minutes. Coagulated proteins are eliminated by centrifugation and the supernatants are kept in ice, if used immediately or frozen, if used the following day. For each extract, two types of controls are made : one is a complete reaction mixture stopped at 0 time, the other one contains no O-AcHS but is incubated for 60 minutes. When necessary, methionine, S.A.M., or both are added at concentrations given in table I. Separation of labeled O-AcHS from unreacted ^{14}C-HS is obtained by thin layer chromatography on cellulose powder plates using butanol/acetic acid/H_2O (120/30/50) as the solvent system. Exactly 10µl of each incubation supernatant are deposited in a band (2-3 cm wide) with an Hamilton seringe. Radioautograms of the plates are then taken (Kodirex film, 2-3 days exposure). The spots corresponding to O-AcHS and its deaminated product (see fig. 1) are scraped and the radioactivity measured in a scintillation counter (Packard) after immersion of the cellulose powder into Bray mixture. Proteins are estimated according to Lowry et al (1956).

RESULTS : ^{14}C-HS incorporation into synthetic O-AcHS can easily be shown by the technic we have described. It is dependant upon enzyme concentration from .5 to 5 mg protein/ml. pH dependance shows a sharp increase between 6 and 7 followed by a broad optimum between 7 and 8. Activity is proportional to incubation time until 90 minutes. Activity is also dependant upon substrate concentrations. K_m values have not been estimated precisely. We have

---

4)O-acetyl-DL-homoserine has been synthesized according to Sakami and Toennies (1942). DL-C_4-^{14}C-homoserine was purchased from the C.E.A., France, and S-adenosyl-methionine iodide from Calbiochem, U.S.A.

chosen the ratio O-AcHS, 2.5×10^{-2}M/HS, 3×10^{-3}M which gave the greatest labelling of O-AcHS in our experimental conditions.

Further evidence for O-AcHS as an intermediate in methionine biosynthesis in S.cerevisiae derives from growth and enzymatic studies of a methionine-auxotroph. The mutant D-6 grows with the same efficiency and identical generation time on methionine, homocysteine or O-AcHS : yields are respectively 3×10^6 ; 2×10^7 ; and 5×10^7 cells/ml for 1,3 and 10 µg/ml of either one of these three compounds (starting with an inoculum 5×10^5 cells/ml). In the absence of any one of these compounds there is a residual growth, corresponding to exhaustion of endogenous methionine, which stops completely after 4 hours.

Extracts of this mutant are devoid of the HS-TAse activity found in wild-type extracts. Reproduction of a typical radioautogram is given in fig.1. It can be seen

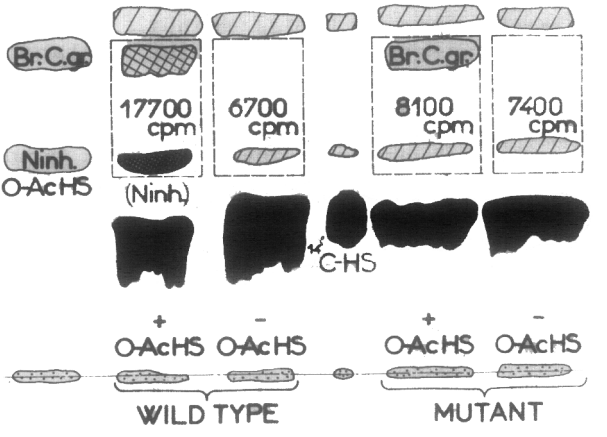

Figure 1 : Homoserine O-transacetylase activity in wild-type and mutant strains. O-AcHS reacts with ninhydrin. A ninhydrin-negative compound (R_f.72) is formed spontaneously during incubation and chromatography of O-AcHS (R_f.53), in standard conditions of assay, without enzyme. This compound can be visualized by bromocresol green spray (Br.C.gr.). Its R_f and properties are identical with the deaminated product of O-AcHS, 4-O-acetyl-2,4-dihydroxybutyrate found by Nagai and Flavin (1966), in studies with Neurospora. Upon incubation with ^{14}C-HS (R_f.34), both spots become labelled and therefore are counted together. There is an impurity in ^{14}C-HS which has the same R_f than O-AcHS but which is accounted for by the two controls.

that the O-AcHS spot plus the compound derived from it (see legend) become labelled after incubation when extract from the wild-type is used. Specific activity is comprised between 1.1 and 1.5 mμmoles of ^{14}C-HS incorporated into O-AcHS x min^{-1} x mg $protein^{-1}$. On the contrary, when extract of the mutant strain is used, there is no difference in radioactivity with or without O-AcHS.

Regulation : In wild-type, synthesis of HS-TAse appears to be dependant upon exogenous methionine concentration. Table I shows that repression was at least 63 % with DL-methionine $2x10^{-3}$M and attained 100 % in two experiments with higher methionine concentrations. DL-threonine $2x10^{-2}$M (concentration which repress yeast aspartokinase by at

Addition to minimal medium		Repression %
DL-methionine	$2x10^{-3}$M	65-70-63-70
"	$4x10^{-3}$M	100
"	$1x10^{-2}$M	75-100
DL-threonine	$2x10^{-2}$M	10

Addition to the reaction mixture		Inhibition %
DL-methionine	$1x10^{-3}$M	0
"	$2x10^{-2}$M	8
S.A.M.	$2.5x10^{-4}$M	34
"	$3x10^{-4}$M	38
"	$3x10^{-4}$M + DL-methionine $2x10^{-2}$M	46
S.A.M.	$5x10^{-4}$M	44
"	$5x10^{-4}$M + DL-methionine $2x10^{-2}$M	54
S.A.M.	$1x10^{-3}$M	51
"	$1x10^{-3}$M + DL-methionine $2x10^{-2}$M	62
S.A.M.	$1x10^{-2}$M	74
"	$1x10^{-2}$M + DL-methionine $2x10^{-2}$M	80

Table I : Regulation of Homoserine O-transacetylase synthesis and activity.
Standard conditions of culture, and assay have been used as described under "Experimental" and legend of fig.1. Repression and inhibition are calculated on the basis of the activity found in wild-type grown on minimal medium. Inhibition numbers represent the mean value of a few independant experiments.

least 70 %) (de Robichon-Szulmajster and Corrivaux,1963), has only a slight insignificant effect (10 %) upon HS-TAse synthesis.

Activity of the enzyme is strongly inhibited by S.A.M. as shown in Table 1. Methionine alone, at 10^{-2}M, gives only a slight inhibition. This inhibition is, at most, additive to the inhibition caused by different concentrations of S.A.M. The K_i for S.A.M. is approximately $5x10^{-4}$M. Homocysteine has also been tried. At $1x10^{-2}$M there is no detectable inhibition, and no increase of inhibition due to S.A.M.

DISCUSSION : The first evidence for regulation of the overall synthesis of methionine by exogenous methionine, in E.coli, has been provided by Cohn et al (1953). In this organism, repression and inhibition of the first step, homoserine O-transsuccinylase,by methionine has been demonstrated by Rowbury (1962) and Rowbury and Woods (1964) Recently, S.A.M. has been shown to cooperate with methionine, at low concentrations, to the inhibition of the enzyme (Lee et al, 1966).

The present results demonstrate that like in Neurospora, (Nagai and Flavin, 1966), S.cerevisiae, another eukaryotic organism, activates homoserine in its O-acetyl-derivative instead of the O-succinyl- derivative formed in bacteria. Two sets of results show that the exchange reaction measured corresponds to the biosynthetic enzyme and that HS-TAse constitutes the first step in methionine biosynthesis in yeast :

1) The enzyme is absent in a methionine-less mutant,able to grow at the expend of O-AcHS as well as on methionine. Growth experiments have shown that the absence of HS-TAse lead to absolute methionine auxotrophy. This finding indicates by itself that there is no other way to synthesize methionine efficiently in this mutant. A direct formation of homocysteine from homoserine and H_2S has been shown to occur in Neurospora. However, the specific activity given for this system (Wiebers and Garner, 1967) is much lower than the one we have found in yeast for HS-TAse. It seems then unlikely that such a system would be operative in

yeast. In addition, the specific activity we have found for HS-TAse in S.cerevisiae (1.1-1.5 mμmoles x min^{-1} x mg $protein^{-1}$) is comparable to the specific activity for HS-transsuccinylase found in E.coli (Giovanelli and Mudd, 1966).

2) The enzyme is susceptible to end-product regulation : We have found that the inhibitor of the reaction is S.A.M. and not methionine. This result can be related to the fact that S.A.M. is known to accumulate in yeast from exogenous methionine (Mudd, 1959). In addition, S-adenosyl-transfer from S.A.M. to homocysteine permits to regenerate free methionine when necessary (Shapiro et al, 1965). These facts may lead to consider S.A.M. as the real end-product of the methionine biosynthesis pathway. Furthermore, the finding that S.A.M. rather than methionine is the inhibitor of the first enzyme of this pathway reinforce such an idea. Inhibition of HS-TAse by S.A.M. has also been found in Neurospora (M. Flavin, personal communication). We have shown that synthesis of HS-TAse is dependent upon exogenous methionine concentration. However, in view of our results on regulation of the activity of the enzyme, it is conceivable that the co-repressor, acting in vivo, is not methionine itself, but, again, S.A.M.

The use of ethionine, a very potent analog of methionine for S.cerevisiae, has lead us to identify two genes implicated in ethionine resistance. One of them, recessive in nature, was supposed to be concerned with regulation of methionine biosynthesis (de Robichon-Szulmajster and Cherest ; Cherest and de Robichon-Szulmajster, 1966). Results will be published in a separate paper showing that the strains carrying the allele eth_2^r are unable to use methionine to repress HS-TAse synthesis.

REFERENCES

Cherest,H., and de Robichon-Szulmajster,H., Genetics, 54, 981 (1966).

Cohn,M., Cohen,G., and Monod,J., C.R.Acad.Sc.,236,746(1953)

Flavin,M., Delavier-Klutchko,C.,and Slaughter,C., Science, 143, 50, (1964)

Galzy,P. and Slonimski,P.P., C.R.Acad.Sc.,245, 2556 (1957)

Giovanelli,J. and Mudd,S.H., Biochem.Biophys.Res.Comm., 25, 366 (1966)

Lee,L.W., Ravel,J.M. and Shive,W., J.Biol.Chem., 193, 265, (1966)

Lowry,O., Rosebrough,M.J.,Farr,A.L., and Randall,R.J., J.Biol.Chem., 193, 265 (1956)

Mudd,S.H., J.Biol.Chem., 234, 87 (1959)

Nagai,S., and Flavin,M., J.Biol.Chem., 241, 3861 (1966)

de Robichon-Szulmajster,Ann.Tech.Agr., 10, 80 (1961)

de Robichon-Szulmajster,H., and Corrivaux,D., Biochim. Biophys.Acta, 73, 248 (1963)

de Robichon-Szulmajster,H., and Cherest,H., Genetics, 54, 993, (1966)

Rowbury,R.J., Biochem.J., 81, 42P (1961)

Rowbury,R.J., Biochem.J., 82, 24P (1962)

Rowbury,R.J., and Woods,D.D., J.Gen.Microbiol., 36,341 (1964)

Sakami,W., and Toennies,G., J.Gen.Microbiol., 37,171 (1964)

Shapiro,S.K., Almenas,A., and Thomson,J.F., J.Biol.Chem., 240,2512 (1965)

Wiebers,J.L., and Garner,H.R., J.Biol.Chem., 242, 12 (1966)