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Methionine-Mediated Repression in *Saccharomyces cerevisiae*: a Pleiotropic Regulatory System Involving Methionyl Transfer Ribonucleic Acid and the Product of Gene *eth2*

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Detailed study of methionine-mediated repression of enzymes involved in methionine biosynthesis in *Saccharomyces cerevisiae* led to classification of these enzymes into two distinct regulatory groups. Group I comprises four enzymes specifically involved in different parts of methionine biosynthesis, namely, homoserine-*O*-transacetylase, homocysteine synthetase, adenosine triphosphate sulfurylase, and sulfite reductase. Repressibility of these enzymes is greatly decreased in strains carrying a genetically impaired methionyl-transfer ribonucleic acid (tRNA) synthetase (mutation *ts⁻296*). Conditions leading to absence of repression in the mutant strain have been correlated with a sharp decrease in bulk $tRNA^{met}$ charging, whereas conditions which restore repressibility of group I enzymes also restore $tRNA^{met}$ charging. These findings implicate methionyl-tRNA in the regulatory process. However, the absence of a correlation in the wild type between methionyl-tRNA charging and the levels of methionine group I enzymes suggests that only a minor iso accepting species of $tRNA^{met}$ may be devoted with a regulatory function. Repressibility of the same four enzymes (group I) was also decreased in strains carrying the regulatory mutation *eth2*^{r}. Although structural genes coding for two of these enzymes, as well as mutations *ts⁻296* and *eth2*^{r} segregate independently to each other, synthesis of group I enzymes is coordinated. The pleiotropic regulatory system involved seems then to comprise beside a "regulatory methionyl $tRNA^{met}$," another element, product of gene *eth2*, which might correspond either to an aporepressor protein or to the "regulatory $tRNA^{met}$" itself. Regulation of group II enzymes is defined by response to exogenous methionine, absence of response to either mutations *ts⁻296* and *eth2*^{r}, and absence of coordinacy with group I enzymes. However, the two enzymes which belong to this group and are both involved in threonine and methionine biosynthesis undergo distinct regulatory patterns. One, aspartokinase, is subject to a bivalent repression exerted by threonine and methionine, and the other, homoserine dehydrogenase, is subject only to methionine-mediated repression. Participation of at least another aporepressor and another corepressor, different from the ones involved in regulation of group I enzymes, is discussed.

In *Saccharomyces cerevisiae*, methionine originates from a complex pathway whose different branches also lead to threonine (steps 1, 2, 3, Fig. 1) and to cysteine (steps 5, 6, 7, 8) biosyntheses. On the basis of regulation studies (8, 9, 47, and present data), the sulfate assimilation pathway (steps 5 to 8) will be considered herein as a part of methionine biosynthesis. As far as synthesis of homocysteine from homoserine is concerned, previous studies (8) have provided evidence for the biosynthetic role of the enzyme catalyzing direct formation of homocysteine (step 9) from sulfide and *O*-acetyl-homoserine which is an obligatory intermediate (step 4).

It has been shown that methionine represses the synthesis of enzymes catalyzing steps 3, 4, 5, and 9 [homoserine dehydrogenase, homoserine-*O*-transacetylase, adenosine triphosphate (ATP) sulfurylase, and homocysteine synthetase, respectively; references 8, 30, 45]. As far as steps 4, 5,

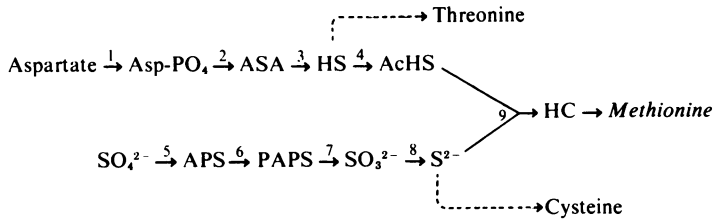


FIG. 1. *Biosynthesis of methionine in Saccharomyces cerevisiae. Step numbers correspond to enzymes as follows. 1: Aspartokinase (L-aspartate 4-phosphotransferase, EC 2.7.2.4); 2: ASA dehydrogenase (L-aspartate-β-semialdehyde:NADP oxidoreductase (phosphorylating), EC 1.2.1.11); 3: HS dehydrogenase: (L-homoserine NAD oxidoreductase, EC 1.1.1.3); 4: (L-homoserine O-transacetylase (Nagai and Flavin, 36); 5: ATP sulfurylase (ATP: sulfate adenylyl transferase, EC 2.7.7.4); 6: APS kinase (ATP:adenyl sulfate-3'-phosphotransferase, EC 2.7.1.25); 7: PAPS reductase (Pasternak et al., 42); 8: sulfite reductase (hydrogen sulfide:NADP oxidoreductase, EC 1.8.1.2); 9: homocysteine synthetase (Wiebers, and Garner, 60). Abbreviations: Asp-PO4, β-aspartyl phosphate; ASA, L-aspartate-β-semialdehyde; HS, L-homoserine; AcHS, O-acetyl-L-homoserine; APS, adenosine 5'-phosphosulfate; PAPS, 3'-phospho-adenosine-5'-phosphosulfate; HC, homocysteine.*

and 9 are concerned, it was found that a mutation leading to resistance to ethionine (*eth2*^{r}) considerably lowers the repressibility of the corresponding enzymes. In view of the recessivity of the *eth2*^{r} allele and of the absence of linkage between the gene *eth2* and the structural genes *met2* and *met8* (also unlinked to each other) corresponding, respectively, to homoserine-*O*-transacetylase (step 4) and homocysteine synthetase (step 9), it was postulated that the product of gene *eth2* participates in a pleiotropic methionine repressor system (8, 9).

On the other hand, it was previously observed that the repressibility of homoserine dehydrogenase (step 3) is not modified by the presence of the *eth2*^{r} allele (8). It was then suggested that at least two distinct methionine repressors exist in *S. cerevisiae* that could differ in the nature of either the apo- or the corepressor (or both).

Studies with various microorganisms in which the activity of a given transfer ribonucleic acid (tRNA) synthetase was modified either by mutation or by the use of an analogue pointed to the importance of these enzymes in cellular regulation and suggested that the control exerted by a given amino acid on its respective biosynthetic pathway might require synthesis of the corresponding amino-acyl tRNA. This seems to be the case for histidyl-tRNA synthetase from *Escherichia coli*, *Aerobacter aerogenes*, and *Salmonella typhimurium* (37, 48, 50, 52), valyl-tRNA synthetase from *E. coli* (3, 13, 15), isoleucyl tRNA synthetase from *E. coli* (12, 56) and from *S. cerevisiae* (34), leucyl-tRNA synthetase from *S. typhimurium* (4, 5, 17), tryptophanyl-tRNA synthetase from *E. coli* (27, 28), arginyl-tRNA synthetase from *Neurospora crassa* (40), and threonyl-tRNA synthetase from *E. coli* (39) and *S. cerevisiae* (38). On the other hand, a mutation which modifies methionyl-tRNA synthetase activity from *S. typhimurium* does not lead to derepression of the two methionine biosynthetic enzymes tested (23).

However, the synthesis of every enzyme implicated in a given pathway need not necessarily be controlled by the same regulatory mechanism, especially when the corresponding structural genes are unlinked. An example of this can be found in the pathway leading to the synthesis of isoleucine, valine, and leucine in *E. coli*. The biosynthesis of all the enzymes implicated in this complex pathway is affected by a modification in leucyl-tRNA synthetase activity, whereas an impaired valyl-tRNA synthetase affects only the enzymes in the synthesis of isoleucine and valine. Among these enzymes, only the three which are linked in an operon are affected by an impairment of the isoleucyl-tRNA synthetase activity (5). Other results unfavorable to the idea that amino acyl-tRNA participates in the regulation of the corresponding amino acid synthesis are the findings that, in *E. coli*, 5-methyltryptophan and two tyrosine analogues act as specific exogenous repressors without being activated by the corresponding synthetases (11, 44) and that *E. coli* mutants with altered tryptophanyl- or arginyl-tRNA synthetases do not show derepressed levels of the corresponding biosynthetic enzymes (11, 26). In view of these conflicting results, it was of interest to see if methionyl-tRNA participates in repression of the methionine biosynthetic enzymes of *S. cerevisiae*. For this purpose, we studied a methionyl-tRNA synthetase mutant of *S. cerevisiae* isolated by McLaughlin and Hartwell (24, 25, 33).

In this paper, we show that exogenous methionine represses, in addition to the enzymes previously studied, the synthesis of enzymes catalyzing step 1 (aspartokinase which is also subject to threonine mediated repression; reference 46)

and step 8 (sulfite reductase). It will be shown that the effect of gene *eth2* extends to the regulation of sulfite reductase synthesis. Detailed information will be provided on the quantitative aspects of methionine-mediated repression in ethionine-resistant and ethionine-sensitive strains. Moreover, we report data indicating the dual involvement of methionyl-tRNA (and more likely a minor species of it) and the product of gene *eth2* in coordinate repression of at least four enzymes which belong to the same regulatory group.

MATERIALS AND METHODS

Strains. The haploid strains of *S. cerevisiae* used for this investigation are the following: D6: α; *met2*: *ura* (gene number unknown; from M. Grenson's collection). JE2: α; *ade2*; *ade* (gene number unknown); *hom2* (from R. K. Mortimer's collection). A364A: *a*; *ade1*; *ade2*; *ura1*; *tyr1*; *his7*; *lys2*; *trp1* and H.19.3.4: *a*; *ade1*; *leu2*; *lys11*; *his4* or *5*; *ts-296* (from L. H. Hartwell's collection). 4094-B: α; *ade2*; *ura1*; *eth1*^{s}; *eth2*^{s} (from F. Sherman's collection). CH82-7A: α; *ura1*; *eth1*^{r}; *eth2*^{s}; CC30-1D: α; *ura1*; *eth1*^{s}; *eth2*^{r}; CH82-7D: α; *ade2*; *ura1*; *eth1*^{r}; *eth12*^{r}; YS5: α; *ade2*; *ade* (gene number unknown; from our own collection).

The following diploid strains were made: CC138 = H.19.3.4 × CC30-1D and CC139 = H.19.3.4 × CH82-7D.

Cultures. For repression studies, the media and cultures were previously described by Cherest et al. (8); methionine was added to the medium at concentrations given below. For experiments with the temperature-sensitive mutant and the corresponding parental strain (H.19.3.4 and A364A), the cultures were grown overnight at the indicated temperature. In every case, the cells used as inoculum were grown at 28 C. In these experiments, plating of the cells at the end of culture showed the absence of revertants.

Extracts. The cells from a 1-liter culture were collected and washed twice in the appropriate buffer (depending upon the enzyme to be assayed; *see below*). They were then suspended in 6 ml of the same buffer; 8 g of glass beads (0.12-mm diameter) were added to the cell suspension, and the cells were broken in a Braun disrupter cooled by liquid CO_2 (20 sec at speed 1 followed by 40 sec at speed 2). The beads and cellular debris were removed by centrifugation (17,000 × *g*, 10 min). The resulting crude extract was used for enzymatic assays. When necessary, parallel extracts in different buffers were made. Sulfite reductase activity was assayed in 100 mM potassium phosphate buffer (*p*H 7.5) extracts. Homoserine transacetylase and homocysteine synthetase activities were assayed by using extracts made either in 100 mM potassium phosphate buffer (*p*H 7.5) or in 100 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 8.0. Other enzymatic activities were assayed in extracts made in 100 mM Tris-hydrochloride buffer, *p*H 8.0.

Chemostats. The chemostats were operated as described by Novick and Szilard (41) at 28 C. The strains used in chemostats were auxotrophs for methionine, so that the growth rate could be limited by the methionine concentration (the growth-limiting concentration of methionine was determined for each strain from preliminary growth curves obtained at different exogenous methionine concentrations). At a given flow rate, after some time, the optical density in the growth vessel was constant. Fractions of about 1 liter of cell suspension, collected in an ice-cooled recipient to stop growth immediately, were treated as described above; the enzymatic activities were determined for each fraction. The growth vessel was usually inoculated with 4×10^7 to 4.5×10^7 cells/ml grown in "control" conditions.

Enzyme assays. The activity of aspartokinase was assayed as described by Stadtman et al. (53), homoserine dehydrogenase activity was assayed as described by Black and Wright (2), and glutamic dehydrogenase [L-glutamate: NAD oxidoreductase (deaminating) EC 1.4.1.2] was assayed as described by Strecker (54). ATP sulfurylase activity was measured by the method of Wilson and Bandurski (61) as described by de Vito and Dreyfuss (59). The activity of homoserine-*O*-transacetylase was assayed as previously described (45). Homocysteine synthetase activity was assayed by the method of Wiebers and Garner (60) at 30 C instead of 37 C, and the homocysteine formed was estimated as described by Kredich and Tomkins (31). The sulfite reductase activity was assayed by the method of de Vito and Dreyfuss (59) and the sulfide formed was estimated by the method of Siegel (51). In our hands, sulfite reductase is cold sensitive. Consequently, where this enzyme had to be assayed, samples of the crude extracts were kept at room temperature and the activity was assayed within 1 hr after extraction. The protein estimation was carried out by the biuret method (20) with bovine serum albumin as reference.

Specific activities. For the sake of convenience, the specific enzymatic activities are expressed as nanomoles per minute per milligram of protein, i.e., in 10^{-3} international units.

Determination of the fraction of aminoacylated tRNA in vivo. (i) The degree of tRNA charged in vivo was determined by a combination of the methods described by McLaughlin et al. (34) and Folk and Berg (14).

A 1-liter culture grown in a specified medium was centrifuged and the cells were suspended in 100 ml of distilled water (23 C). A 1-ml amount of a 3% solution of sodium dodecyl sulfate was added, followed immediately by 100 ml of an 80% solution of phenol in water (w/w). The mixture was shaken vigorously for 10 min at 23 C, and the two phases were separated by centrifugation at 6,000 × *g* for 15 min. The aqueous layer was washed twice with 200 ml of cold ether containing 20 ml of 20% potassium acetate, *p*H 5.2. A 200-ml amount of ethyl alcohol (−15 C) was then added, and the RNA was allowed to precipitate for at least 30 min at −15 C.

The precipitate was collected by centrifugation at 27,000 × *g* for 10 min and dissolved in 6 ml of 100 mM potassium acetate, *p*H 4.6. At this stage, the solution was divided into two 3-ml portions. One was treated with 1 ml of a freshly prepared 10 mM sodium periodate solution (in 100 mM potassium acetate buffer, *p*H 4.6) and the other with 1 ml of the potassium acetate buffer alone. After 30 min in the dark at room temperature, 1.3 ml of potassium acetate (1 M, *p*H 4.6)

and 11 ml of cold ethyl alcohol (−15 C) were added to each portion, and the RNA which precipitated was collected immediately by centrifugation at 27,000 × *g* for 10 min. The excess of periodate was destroyed by dissolving the precipitate in 4 ml of ethylene glycol (100 mM) in potassium acetate buffer (100 mM, *p*H 4.6) and allowing to stand in the dark at room temperature for 10 min. In the two fractions, the RNA was precipitated again with 1.3 ml of potassium acetate (1 M, *p*H 4.6) and 11 ml of cold ethyl alcohol (−15 C) and collected by centrifugation at 27,000 × *g* for 10 min. Each precipitate was then dissolved in 4 ml of bicarbonate buffer 50 mM, *p*H 10.2, and kept at 37 C for 20 min to hydrolyze amino acyl-tRNA bonds. Each solution was neutralized by 1 N HCl and dialyzed overnight in 1 mM phosphate buffer, *p*H 7, containing 1 mM magnesium chloride. It was then lyophilized and the residue was dissolved in 0.250 ml of water before assaying.

(ii) Acceptor activity of the tRNA was determined by measuring the amount of ^{14}C-amino acid which can be esterified in an incubation mixture containing an excess of amino acyl tRNA synthetase. The assay mixture is similar to that used by Cassio et al. (7). It contains, in a volume of 0.1 ml: 10 μmoles of potassium buffer (*p*H 7.4), 1 μmole of KCl, 1 μmole of $MgCl_2$, 0.2 μmole of ATP (Na), 0.2 μmole of dithiothreitol, 0.04 μmole of ^{14}C-L-methionine (2 × 10^6 counts/min), and excess enzyme. For each tRNA sample, two assays were performed: one with 5.5 and the other with 11 A_{260} units of tRNA. After incubation at 30 C for 10 min, the reaction was stopped with 1 ml of cold 0.1% trichloroacetic acid solution followed by 2 ml of a cold 10% trichloroacetic acid solution containing 0.5% ^{12}C-DL-methionine. The resulting precipitates were filtered through Whatman glass fiber circles and washed with a cold 5% trichloroacetic acid solution containing 0.5% ^{12}C-DL-methionine. The filter was dried and the radioactivity was counted in a scintillation counter (Intertechnique SL 30). Proportionality between the amount of amino acid esterified and the amount of tRNA added indicated that the reaction had gone to completion. The enzyme used was partially purified as described below. As a control, the isoleucyl tRNA present in each tRNA sample was determined under the same conditions with the same enzyme preparation as above, but with 0.004 μmole of ^{14}C-L-isoleucine and both 1.1 and 2.2 A_{260} units of the tRNA samples. The acceptor activity of each tRNA preparation was determined on the periodate-treated and the untreated samples. Results are expressed as the per cent of tRNA charged, i.e., the ratio of the acceptor activity of periodate treated over acceptor activity of the untreated tRNA × 100.

(iii) For amino-acyl-tRNA synthetases, a preparation essentially free of tRNA was used. The strain YS5 was grown in 2 liters of minimal medium. Cells were collected at the end of the exponential phase and extracted in 20 mM phosphate buffer (*p*H 7.5) containing 1 mM 2-mercaptoethanol (buffer A). Nucleic acids were precipitated with 0.4% protamine sulfate and removed by centrifugation after 2 hr at 4 C. The resulting supernatant was brought to 90% saturation by solid ammonium sulfate, and the precipitate was dissolved in the minimal volume of buffer A. The solution was then dialyzed for 2 hr against 2 liters of buffer A and applied to a Sephadex G200 column (80 × 1.5 cm) equilibrated with buffer A containing 100 mM KCl (the ratio of the volume of the solution applied over the column volume was 1/100). Fractions of 1 ml were collected. They were assayed for methionyl-tRNA synthetase and isoleucyl-tRNA synthetase activities with commercial yeast tRNA. The tubes containing the peak of specific activity (the same for the two enzymes) were pooled and dialyzed overnight in 1 liter of buffer A containing 60% (w/v) of glycerol; it was thus concentrated four times. The resulting methionyl-tRNA synthetase was purified 15 times with respect to crude extract and used for determinations of the acceptor activity of tRNA.

Genetic techniques. Sporulation of diploids was induced by the method of McClary et al. (32). Ascospores were isolated as described by Johnston and Mortimer (29).

Chemicals. *O*-acetyl-DL-homoserine was synthesized for us by M. Cherest by the method of Sakami and Toennies (49).

L-Aspartate-β-semialdehyde was prepared by the method described by Black and Wright (1). ^{14}C-DL-3,3′,4,5-isoleucine and ^{14}C-L-methyl methionine were purchased from C.E.A., France. The ^{14}C-methionine was further purified by thin-layer chromatography on cellulose powder plates (solvent: *n*-butanol-acetic acid-water, 120:30:50). The purified ^{14}C-methionine was kept in 10 mM 2-mercaptoethanol. Sodium dodecyl sulfate was recrystallized in ethyl alcohol. Commercial yeast tRNA was purchased from Schwartz Bio-Research.

RESULTS

Study of enzymes submitted to methionine-mediated repression in methionine auxotrophs. It has been shown previously that, in *S. cerevisiae*, exogenous methionine represses the synthesis of four enzymes involved in its own biosynthesis. A fifth enzyme, sulfite reductase, implicated in the sulfate assimilation pathway, has now been found sensitive to methionine repression. Such a repression was not observed by de Vito and Dreyfuss (59). Conversely, we have not found the repressive effect of cysteine described by these authors. However, they used a medium in which methionine or cysteine were the only sulfur source, whereas, in our conditions, sulfate was always present. It is then possible that these drastically different conditions of growth might explain the differences in repression effects.

The comparison between excess methionine and methionine-limited cultures (chemostat), using methionine auxotrophs, provided us with a means to obtain more information about the variation in the specific activities of these enzymes. Two strains were used: D6 deficient in homoserine-*O*-transacetylase and JE2 deficient in aspartic semialdehyde dehydrogenase. The latter strain was used especially to study the variation of homoserine-*O*-transacetylase synthesis.

Since, in preliminary studies, the synthesis of sulfite reductase seemed to be slightly more sen-

sitive to exogenous methionine concentration in a wild-type strain (*see below*) than the other enzymes studied, a special chemostat was run with strain D6, with DL-methionine used at a concentration of 0.06 mM instead of 0.08 mM as in other experiments. In this special chemostat, homocysteine synthetase, which can be extracted under the same conditions as sulfite reductase, was then assayed concomitantly. Results are shown in Table 1. Besides glutamic dehydrogenase, which has been taken as a reference enzyme, the synthesis of all the enzymes studied is enhanced under conditions of methionine limitation. As far as aspartokinase is concerned, synthesis of this enzyme was already found to be sensitive to threonine mediated repression (46). The present finding of its derepression under methionine limitation shows that both end products, threonine and methionine, exert a control over the synthesis of the enzyme catalyzing the first step of the pathway. Enzymes specifically involved in methionine biosynthesis (sulfate group I enzymes, including the enzymes of the assimilation pathway) respond more extensively than enzymes common to threonine and methionine biosynthesis (group II enzymes). Enzymes of the second group show mostly derepression, whereas enzymes of the first group show pronounced repression in the presence of an excess of methionine as well as derepression in chemostat. If follows that ratios between maximally derepressed and maximally repressed levels are much greater for the metionine-specific enzymes than for the others.

It was thus tempting to assume that mechanisms involved in the regulation of these two groups of enzymes were not alike. Differences could be expected in the nature of either the apo- or the corepressor. The existence of a thermosensitive mutant with an impaired methionyl-tRNA synthetase now proved to be a useful tool to provide information on the nature of one of the methionine corepressors.

Study of enzymes submitted to methionine-mediated repression in a methionyl-tRNA synthetase mutant. The strain H.19.3.4, which carries the mutation *ts⁻296*, exhibits a methionine requirement which does not segregate from either the thermosensitivity or the absence of methionyl-

TABLE 1. *Effect of exogenous methionine concentration upon synthesis of enzymes involved in methionine biosynthesis*

	Enzymes	Growth conditions[a]			Ratio
		Repression[b]	Control[c]	Chemostat[d]	(max. derepression/ max. repression)
	Strain D6				
Group II	Aspartokinase	16	18	62	3.9
	Homoserine dehydrogenase	120	210	645	5.4
Group I	Homocysteine synthetase	14	97	433	31
	Homocysteine synthetase*	25	150	715	28
	ATP sulfurylase	2	100	280	140
	Sulfite reductase*	0.155	0.650	4.00	25
	Glutamic dehydrogenase	17	24	19	1
	Strain JE-2				
Group I	Homoserine-*O*-transacetylase	0.075	0.460	1.43	19
	ATP sulfurylase	2	81	220	110
	Glutamic dehydrogenase	9	10	7	1

[a] Results are specific activities expressed as nanomoles per minute per milligram of protein.

[b] For the two strains, cultures were made in the same medium as in the chemostat but with 2 mM DL-methionine.

[c] D6: For the study of all the enzymes except sulfite reductase, cells were grown in a medium containing, in addition to uracil (20 mg/liter), *O*-acetyl-DL-homoserine (1 mM). Since sulfite reductase activity was found strongly repressed in these conditions, a medium containing DL-homocysteine (0.2 mM) instead of *O*-acetyl-homoserine was used for the study of sulfite reductase (*). In this experiment, we assayed homocysteine synthetase as control (*). JE-2: In addition to adenine (20 mg/liter), cells were grown in a medium containing 2 mM DL-homoserine.

[d] D6: In addition to uracil, the medium contained 0.06 mM DL-methionine for the study of sulfite reductase and 0.08 mM DL-methionine for the other enzymes studied. JE-2: In addition to adenine and DL-threonine (2 mM), the medium contained 0.04 mM DL-methionine. At least four fractions, each one of them corresponding to approximately one doubling of the cellular mass, were collected and analyzed. The values recorded in the table correspond to the fraction which gave the maximal derepression.

tRNA synthetase activity in vitro (33). Moreover, it is striking that the methionine requirement of the mutant is exhibited at all temperatures and that methionyl-tRNA synthetase remains undetectable even in extracts obtained from cells grown at the permissive temperature. McLaughlin and Hartwell concluded that the recessive mutation *ts⁻296* is responsible for the complex phenotype of the strain H.19.3.4 and that methionine is required either to stabilize or to improve saturation of the impaired methionyl-tRNA synthetase in vivo. If this is correct, it seems then that temperature sensitivity is a secondary effect which only accentuates the enzyme defect.

Since methionine was always necessary in cultures of the mutant strain, it was thought that a complete medium (YPGA) would provide the easiest conditions to study the possible regulatory consequences of such a mutation.

In view of the previous assumptions, cells were grown at permissive and semipermissive temperatures (28 and 32 C, respectively), using both parental and mutant strains. Results are presented in Table 2. The major finding resides in that, whatever the temperature of growth, the specific activities exhibited by the four methionine-specific enzymes (group I enzymes), namely, homoserine-*O*-transactylase, homocysteine synthetase, ATP sulfurylase, and sulfite reductase, are much higher in the mutant than in the parental strain. Despite some dispersion in specific activities between several experiments, the extreme values found in the parental and mutant strains do not overlap (with one exception). Moreover, within experiments, the differences between the two strains were always of the same order of magnitude. Smaller differences are observed between the two strains, in the case of the two enzymes involved in threonine-methionine biosynthesis (group II enzymes). Since differences of the same order of magnitude were observed for the reference enzyme, glutamic dehydrogenase, the response of group II enzymes seems unspecific (see also Table 4).

Growth of a wild-type strain in YPGA medium leads to repressed levels of the enzymes studied (9). The high enzymatic levels found in such a medium for the mutant strain indicate that, in this strain, repression of these enzymes is rendered ineffective. Thus, it strongly suggests that methionyl-tRNA synthetase activity plays a role in this regulatory mechanism. In addition, these results support our previous assumption that the methionyl-tRNA synthetase impairment is also expressed, in vivo, in the mutant at the permissive temperature. It is tempting to assume that in the YPGA medium, the methionine concentration is such that, in the strain H.19.3.4, methionyl-tRNA synthetase activity is sufficient to insure growth but is limiting for its regulatory function. If this is the case, it could be expected that methionine concentration would influence the regulatory pattern. To test this hypothesis, experiments were run in synthetic medium at various exogenous methionine concentrations.

Since methionine is always necessary for growth of the mutant, a minimal concentration had first to be defined which supports growth of the mutant but minimally affects enzyme levels in the parental strain. DL-Methionine (0.1 mM) was chosen as the reference concentration (9), and enzymatic activities obtained under these conditions were taken as a basis of comparison for further experiments. Consequently, results in Table 3 are expressed in per cent of these reference values for each strain. Since independent values obtained from cultures in synthetic medium show a lower dispersion than those obtained from cultures in YPGA, only average values are given in Table 3. As previously, in each separate experiment, the differences in en-

TABLE 2. *Comparative study of enzymatic activities in parental and temperature-sensitive strains*[a]

Enzymes	Parent (A364A)		Mutant (H.19.3.4)	
	28 C	32 C	28 C	32 C
Aspartokinase	15 (11-20)[b]	15 (10-22)	38 (18-65)	41 (35-51)
Homoserine dehydrogenase	191 (114-270)	320 (146-465)	256 (107-390)	426 (270-560)
Homoserine-*O*-transacetylase	0.06 (0.02-0.12)	0.06 (0.04-0.14)	0.88 (0.28-1.40)	0.76 (0.41-0.93)
Homocysteine synthetase	13 (3-36)	14 (7-31)	128 (40-256)	160 (54-350)
ATP sulfurylase	12 (4-22)	12 (3-32)	111 (85-145)	90 (20-148)
Sulfite reductase	0.016 (0.010-0.023)		1.14 (1.01-1.26)	1.66 (1.00-2.33)
Glutamic dehydrogenase	3.2 (1.8-6.4)	4.7 (2.5-8.0)	6.1 (1.3-11.0)	7.5 (3.5-14.0)

[a] The cells were grown in YPGA medium, at two temperatures. For all enzymes but sulfite reductase (only two experiments), specific activities recorded here represent the mean values of at least eight independent experiments for cultures at 28 C and at least four independent experiments for cultures at 32 C.

[b] Numbers in parentheses correspond to the extreme values obtained.

zymatic activities between the parental and mutant strains were always of the same order of magnitude.

For the group II enzymes, no major difference appears between the behavior of the two strains. However, for the group I enzymes, a twofold increase in the exogenous methionine concentration (0.2 instead of 0.1 mM) already leads to an important repression of these enzymes in the parental strain, whereas enzyme levels are almost unaffected in the mutant strain. Nevertheless, the use of a much higher methionine concentration (4 mM) leads to a significant recovery of repressibility of the group I enzymes in the latter strain at semipermissive as well as permissive temperatures. This finding corroborates our previous conclusion that the main phenotypic expression of the mutation *ts⁻296* does not reside in the temperature effect but in the methionine requirement of this strain. It follows that repressibility of the methionine-specific enzymes can be restored in conditions which supposedly favor methionyl-tRNA synthetase activity in the mutant strain.

When the ratios between enzymatic activities found in the mutant and the parental strains after various growth conditions are considered (*see* Table 4), the classification of the four methionine-specific enzymes in the same regulatory group is further established (lower part of the

TABLE 3. *Study of various enzymes after growth of parental (P) and mutant strains (M) in different conditions*

Enzymes	Minimal medium plus DL-methionine (0.1 mM)[a]		Minimal medium plus[b]			
			DL-methionine (0.2 mM)		DL-methionine (4 mM)	
	P	M	P	M	P	M
28 C[c]						
Glutamic dehydrogenase	4.14	4.84	97	100	100	100
Aspartokinase	32.3	37.2	80	90	75	78
Homoserine dehydrogenase	482	421	68	100	78	86
Homoserine-*O*-transacetylase	0.750	0.625	25	77	11	45
Homocysteine synthetase	199	153	19	83	10	24
ATP sulfurylase	104	70	25	75	12	44
Sulfite reductase	0.582	1.002	3.5	63	3	43
32 C[c]						
Glutamic dehydrogenase	3.95	2.33	103	118	89	168
Aspartokinase	55.5	61	72	85	70	63
Homoserine dehydrogenase	507	346	68	118	88	103
Homoserine-*O*-transacetylase	0.477	0.320	15	68	3	20
Homocysteine synthetase	180	150	14	92	6	33
ATP sulfurylase	136	78	9	98	8	23
Sulfite reductase		1.100		92		24

[a] Specific activities recorded here correspond to the mean value of at least four independent experiments.
[b] Activities expressed in per cent of the specific activity obtained in minimal medium plus 0.1 mM DL-methionine of the corresponding strain.
[c] Growth temperature.

TABLE 4. *Ratios between enzymatic activities in temperature-sensitive and parental strains after growth under different conditions*

Enzymes	YPGA		MM[a] + DL-methionine			
			0.2 mM		4 mM	
	28 C	32 C	28 C	32 C	28 C	32 C
Glutamic dehydrogenase	1.6	2.7	1.0	1.1	1.0	1.9
Aspartokinase	2.2	2.4	1.1	1.2	1.1	0.9
Homoserine dehydrogenase	1.5	1.9	1.3	1.7	1.1	1.2
Homoserine-*O*-transacetylase	17.5	17.5	3.1	4.5	4.1	6.7
Homocysteine synthetase	12.3	14.1	4.4	6.5	2.4	5.5
ATP sulfurylase	14.4	12.8	3.0	11.0	3.7	2.9
Sulfite reductase	41.0		18.0		14.4	

[a] MM, minimal medium.

table). For the other enzymes (upper part of the table), the variations previously noticed are restricted to values obtained from YPGA cultures, especially in the case of aspartokinase. Furthermore, it appears that the ratios are also higher for group I enzymes after growth in YPGA as compared to growth in synthetic medium. Consequently, the small and quite unspecific effects observed after growth in YPGA medium upon synthesis of group II enzymes cannot be explained by our present data.

Study of in vivo tRNA^{met} acylation in various growth conditions. Results presented in the previous section strongly suggest that methionyl-tRNA synthetase or a product of its activity is involved in the mechanism which regulates the synthesis of methionine-specific enzymes. The most likely candidate for such a role is the corresponding amino-acyl tRNA, as already postulated in other systems (loc. cit.).

A study was thus undertaken to correlate the degree of tRNA^{met} charging in vivo with the specific activities of methionine biosynthetic enzymes. For this purpose, cultures were grown under conditions strictly identical with those used in repression studies. Results are summarized in Table 5. For both the mutant and the parental strains, the in vivo degree of charged tRNA^{ile} (considered as a control in this series of experiments) remains quite constant and almost maximal under all growth conditions used. For the parental strain, the same result is obtained in the case of the tRNA^{met}. However, for the mutant strain, growth conditions greatly affected the amount of met-tRNA^{met} formed in vivo. Conditions which lead to no, or low, enzymatic repression in the mutant (YPGA, synthetic medium plus 0.1 or 0.2 mM DL-methionine) also lead to the lowest degree of charged tRNA^{met}, whereas conditions which restore repression (synthetic medium plus 4 mM DL-methionine) are accompanied by a concomitant recovery of a high level of methionyl-tRNA. Since there is no detectable methionyl-tRNA synthetase activity in the mutant strain H.19.3.4 (33), even after growth and extraction in the presence of protective amounts of methionine (M. Tingle, *unpublished data*), it seems likely that only one such enzyme exists in *S. cerevisiae*. On the other hand, as the only difference between the parental and the mutant strains resides in the monogenic mutation *ts⁻296*, there is no reason to suppose that the two phenomena observed—differences between the two strains in the activites of methionine group I enzymes and in the amount of met-tRNA in vivo—do not result from the same genetic event. The study of two segregants from a cross between *ts⁻296* and a wild-type strain, one with a wild-type phenotype and the other with a mutant phenotype, led to results strictly identical with those obtained from the parental strains. Moreover, a spontaneous revertant of the strain H.19.3.4 (mutation *ts⁻296*) showed a parallel recovery of repression and charging of tRNA^{met}. If the bulk met-tRNA as such is acting as the corepressor, it could be expected that some variation in the level of met-tRNA would result from growth of the parental strain in repressive and nonrepressive conditions. Our results show that this is not the case. However, evidence has already been presented for the existence of more than one tRNA^{met} species in yeast (18, 43, 57, 58). The absence of detectable variation in the parental strain of the in vivo charging of tRNA^{met} could be taken as an indication that the tRNA^{met} active in regulation is only a minor species of the total tRNA^{met}. Our main conclusion from these experiments is that methionyl-tRNA, and more likely a subspecies, certainly plays a role in the regulation of synthesis of methionine group I enzymes either as the corepressor itself or as a necessary intermediate in the synthesis of the

TABLE 5. *Degree of tRNA charged in the parental and mutant strain*[a]

Strain	Medium	Charged tRNA^{met} in vivo (%)	Charged tRNA^{ile} in vivo (%)
A364A (parental)	YPGA	95 (4)	92 (3)
	G 21	89 (3)	94 (2)
	G 21 + DL-methionine (0.1 mM)	97	100 (2)
	G 21 + DL-methionine (0.2 mM)	84	87
	G 21 + DL-methionine (4 mM)	96 (2)	90 (2)
H19.3.4 (mutant)	YPGA	54 (3)	100 (3)
	G 21 + DL-methionine (0.1 mM)	38 (3)	100 (3)
	G 21 + DL-methionine (0.2 mM)	47 (2)	97 (2)
	G 21 + DL-methionine (4 mM)	86 (2)	100 (2)

[a] Cells were grown at 28 C. The percentage of tRNA charged in vivo was calculated as described in Materials and Methods. When average values are given, the number of determinations is indicated in parentheses.

final repressor.

Effects of the regulatory gene eth2. It has been shown previously that the presence of the recessive allele *eth2*^{r} leads to decreased repressibility of two enzymes which belong to the group I enzymes, namely homoserine-*O*-transacetylase and homocysteine synthetase, whereas it does not modify repressibility of at least one of the group II enzymes, namely homoserine dehydrogenase. More recently, a third enzyme of group I, ATP sulfurylase, was also found to respond similarly (9). Since, according to the data presented above, sulfite reductase also belongs to the group I enzymes, a further investigation was carried out to see if the synthesis of this enzyme also responds to the gene *eth2*. It must be recalled that in strains carrying the *eth2*^{r} allele, no derepression of synthesis of the group I enzymes occurs, but that repression of these enzymes, at a given exogenous methionine concentration, is less pronounced than in strains carrying the *eth2*^{s} allele. It thus seemed of interest to obtain more information on the quantitative aspects of methionine-mediated repression.

Figure 2 shows the effect of various exogenous concentrations of methionine upon synthesis of three of the group I enzymes in strains carrying either *eth2*^{r} or *eth2*^{s} alleles in combination with *eth1*^{r} or *eth1*^{s}. Since estimation of homoserine-*O*-transacetylase is very tedious, it has not been included in these experiments. A marked difference in repressibility exists for each of these enzymes between the two types of strains. If one considers first the *eth2*^{s} strains, it appears that the methionine concentration leading to half maximal repression is almost identical for the three enzymes studied, the greatest divergence being between sulfite reductase and homocysteine synthetase which display an apparent *K* (repression) of 0.1 and 0.16 mM, respectively. Although reproducible, these differences between the three enzymes, as far as their quantitative response to exogenous methionine concentration is concerned, seem rather small. They could, of course, reflect small differences between affinities of potentially redundant operators. However, they could also reflect differences in stability of the different enzymes in vivo, especially when the repression occurring lowers the in vivo concentration of these enzymes. In any case, owing to the type of in vivo experiments, it seems unlikely that a factor of 2 could be presently considered as a significant difference.

If one now considers the *eth2*^{r} strains, the apparent *K* (repression) is changed for each of the three enzymes studied, again the most sensitive enzyme being sulfite reductase and the least sensitive being homocysteine synthetase, with an apparent *K* (repression) of 0.4 and 0.7 mM, respectively. Consequently, the most important phenomenon resulting from this comparative study resides in the quantitative nature of the mutation *eth2*^{s} → *eth2*^{r}. Moreover, it is remark-

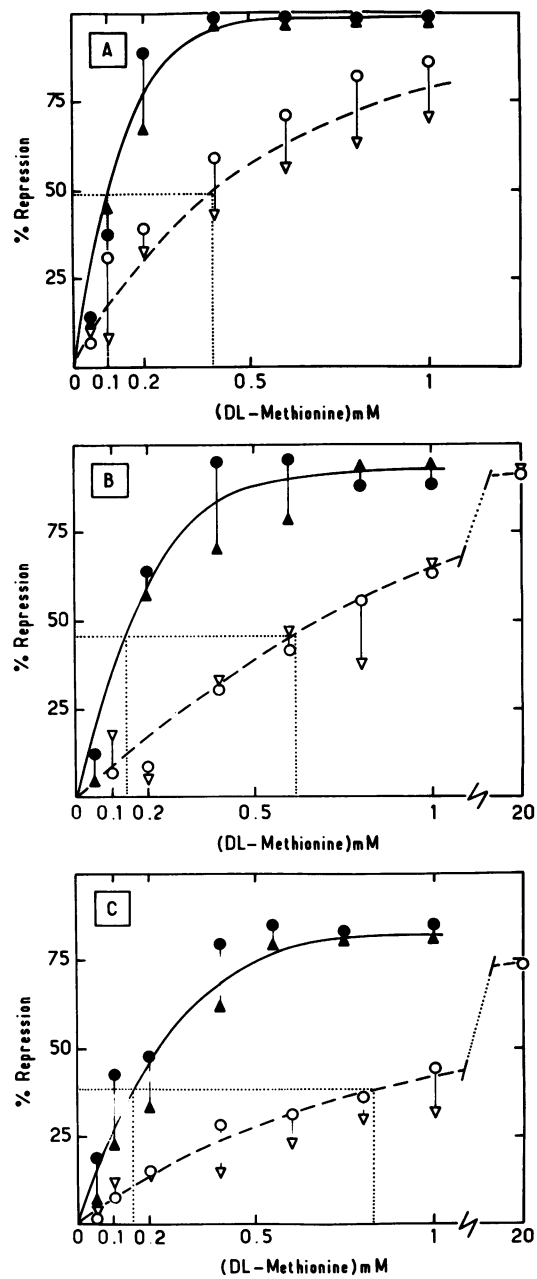


FIG. 2. *Effect of exogenous methionine concentration under repressibility of methionine specific enzymes. A, Sulfite reductase; B, ATP sulfurylase; C, homocysteine synthetase. Symbols: ●, strain CH 82-7A (eth1*^{r}*; eth2*^{s}*); ▲, strain 4094-B (eth1*^{s}*; eth2*^{s}*); ○, strain CC 30-1D (eth1*^{s}*; eth2*^{r}*); ▽, strain CH 82-7D (eth1*^{r}*; eth2*^{r}*).*

able that, by using a high concentration of methionine (20 mM), it is possible to restore repressibility of the three enzymes in the *eth2*^{r} strain to a level comparable to that attained in *eth2*^{s} strains. This is made more evident by an inverse plot of these repression data, which permits estimation of the maximal repression attainable for the three enzymes in *eth2*^{r} strains. Results in Fig. 3 indicate a repressibility near 100%, 95%, and 75% for sulfite reductase, ATP sulfurylase, and homocysteine synthetase, respectively. Moreover, this representation confirms that there is at most a factor of 2 between the apparent *K* (repression) obtained for the first and the third enzymes. It follows that the effect of the mutation *eth2*^{s} → *eth2*^{r} is, so far, best described as a decrease in the affinity of the product of the gene *eth2* either for methionine itself or for the corepressor derived from it.

As far as the *eth1* gene is concerned, there is no significant difference between strains carrying the wild-type allele *eth1*^{s} and strains carrying the mutant allele *eth1*^{r}. These data confirm our previous conclusions (8) that, although the presence of *eth1*^{r} in strains carrying *eth2*^{r} raises the level of resistance of cells towards ethionine and permits methionine biosynthesis in the presence of exogenous ethionine, it does not interfere with repressibility of the enzymes studied. Thus, the role of the gene *eth1* in the regulation of methionine biosynthesis remains unexplained.

Absence of linkage between genes eth2 and ts⁻296. The above results indicate that the enzymes affected by the mutation *ts⁻296* are also affected by the mutation *eth2*^{r}. Although the apparent affinities of methionyl-tRNA synthetase for methionine and for ethionine were found similar in *eth2*^{r} and *eth2*^{s} strains (as well as in *eth1*^{r} and *eth1*^{s} strains) with regard to the degree of charging and activation (M. Tingle, *unpublished data*), it has been verified that the two mutations are not allelic. For this purpose, two crosses were made between the strain H.19.3.4 (*ts⁻296*) and strains carrying the *eth2*^{r} allele (CC30-1D and CH82-7D). The two diploids, CC138 and CC139, respectively, were found to be temperature insensitive, prototrophic for methionine, and sensitive to ethionine. In view of the recessive character of both *ts⁻296* and *eth2*^{r}, this complementation pattern by itself shows that the two mutations are not allelic. Furthermore, the fact that, among the 47 tetrads analyzed (188 spores), 94 segregants were temperature insensitive and simultaneously protoprophic for methionine and that, in this category, only 47 were found resistant to ethionine, shows that the two genes *eth2* and *ts⁻296* segregate independently.

Coordinate synthesis of methionine group I enzymes. Taken as a whole, the above results show that synthesis of the four group I enzymes is controlled by the same regulatory system. This is shown further in Fig. 4. The rates of synthesis of all the group I enzymes varies coordinately. On the other hand, the rate of synthesis of the group II enzymes does not seem to be coordinate with that of an enzyme which belongs to group I (Fig. 5). It will be recalled that the structural genes corresponding to homoserine-*O*-transacetylase and homocysteine synthetase (*met2* and *met8*, respectively) are localized on two different chromosomes (35). Thus, the existence of coordinate synthesis of methionine group I enzymes, despite the absence of linkage between the corresponding structural genes, raises a problem which is discussed below.

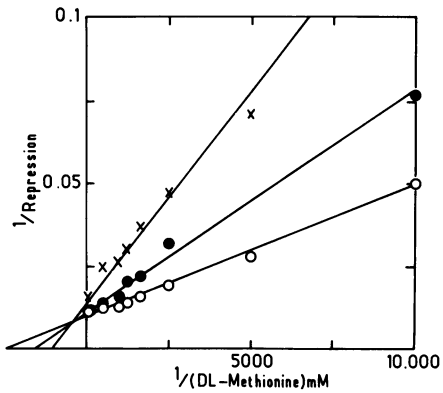


FIG. 3. *Estimation of maximal repression and apparent K (repression) in strains carrying the eth2*^{r} *allele. Inverse plot of mean values obtained from data in Fig. 2 for strains CC 30-1D and CH 82-7D. Symbols: O, sulfite reductase; ●, ATP sulfurylase; ×, homocysteine synthetase.*

DISCUSSION

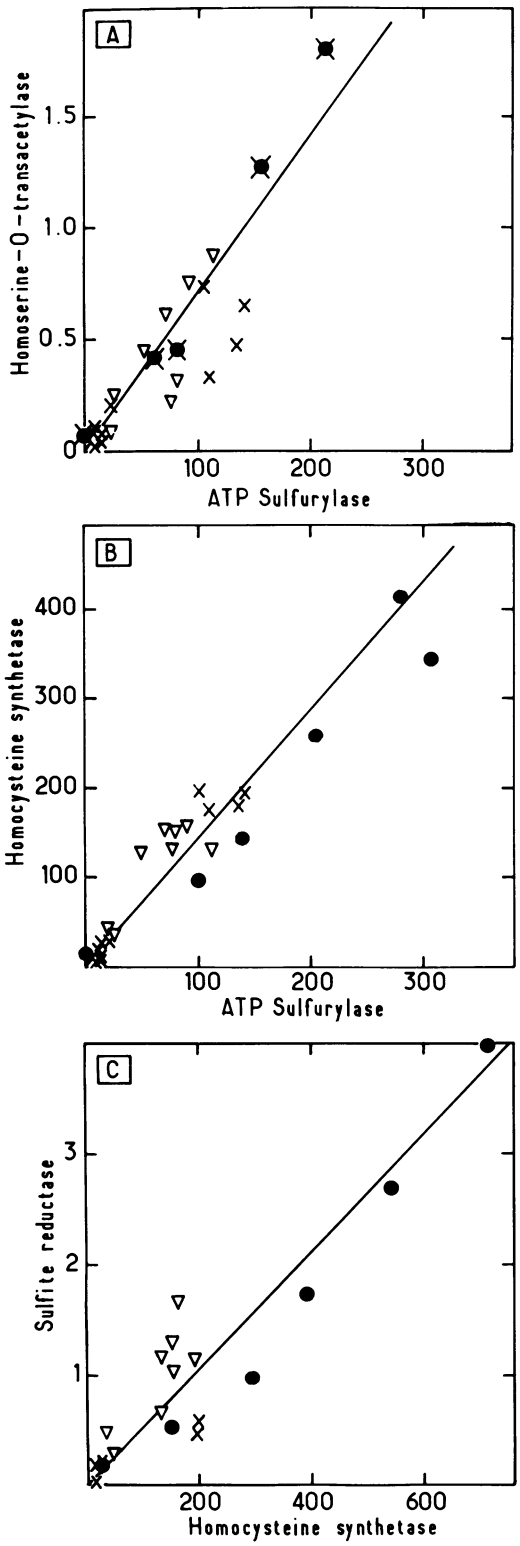
Results presented here are schematically summarized in Fig. 6. They have shown that at least four enzymes, which are involved in different parts of the complex pathway leading to methionine biosynthesis in yeast, belong to the same regulatory group, although at least two of them are coded by unlinked structural genes. This conclusion has been reached through different but complementary sets of evidence.

(i) Their synthesis is not repressed in a mutant with a defective methionyl tRNA synthetase under conditions which lead to full repression in wild-type strains.

(ii) The repressibility of at least three of them is decreased to the same extent in strains carrying a regulatory mutation *eth2*^{r}.

(iii) Their synthesis appears to be coordinate.

Each of these findings deserves some comment. Experiments with the strain carrying the



mutation *ts-296* have pointed to the pleiotropic regulatory effect of this mutation and suggest that met-tRNA^{met} is involved in this regulatory process. In the mutant strain, the in vivo charging of bulk tRNA^{met} has been found

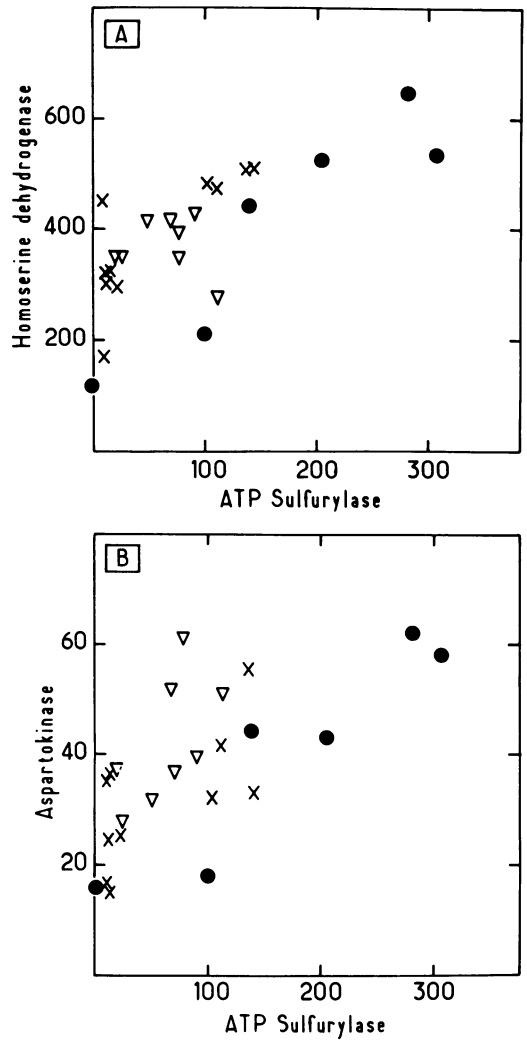


FIG. 5. *Absence of coordinacy between enzymes involved in methionine and threonine biosynthesis and one of the methionine specific enzymes. For symbols, see legend of Fig. 4.*

FIG. 4. *Coordinate synthesis of enzymes specifically involved in methionine biosynthesis. Specific activities were obtained from:* ●, *strain D6 grown in methionine limitation;* ◉, *strain JE2 grown in methionine limitation;* ▽, *strain H19.3.4 grown in the presence of various exogenous methionine concentrations;* ×, *strain A364A grown in the presence of various exogenous methionine concentrations.*

greatly reduced and, concomitantly, the synthesis of four group I enzymes was not repressed. Moreover, increased methionine concentration led to the recovery of both repressibility and $tRNA^{met}$ charging. To our knowledge, only one example of such a correlation has previously been noticed, although less extensively substantiated, in a eukaryotic organism, *N. crassa*, between arg-$tRNA^{arg}$ and synthesis of ornithine transcarbamylase (40). In the thermosensitive mutant studied here, the defect in methionyl-tRNA synthetase activity is expected to be reflected in the in vivo charging of all (major or minor) $tRNA^{met}$ species. On the contrary, in the parental strain one might assume that conditions which would affect charging of only one minor iso accepting species could escape detection. If only one such minor subspecies of $tRNA^{met}$ displays a regulatory function, it might not be surprising that variations in repression levels in the parental strain cannot cause a marked difference in the charging of the bulk of the $tRNA^{met}$.

These considerations might explain the failure of other authors to show a correlation between a specific tRNA charging and repression of related enzymes (11, 26). However, other hypotheses can be made to explain the regulatory consequences of a mutation impairing methionyl-tRNA synthetase activity. First, one cannot exclude the possibility that this enzyme molecule might play a role in regulation. Second, methionyl-tRNA might not be the corepressor as such but an intermediary compound in the biosynthesis of the final corepressor. Alternately, the methionyl-tRNA synthetase might catalyze an additional unknown reaction specifically involved in the regulatory process. Moreover, repression might result from the concerted action of methionyl-tRNA synthetase and at least another enzymatic process. Nevertheless, the direct involvement of an iso accepting species of methionyl-tRNA in regulation remains, so far, the simplest hypothesis and the easiest one to be submitted to further experimentation.

As shown in this paper, the pleiotropic regulatory mutation *eth2*^{r} acts not only upon synthesis of homoserine-*O*-transacetylase and homocysteine synthetase but equally well upon synthesis of ATP sulfurylase and sulfite reductase. It appears, then, that the same four group I enzymes which respond to limitation in $tRNA^{met}$ charging also respond to the regulatory gene *eth2*. In view of the recessivity of the *eth2*^{r} allele and of the absence of linkage between *eth2*, *ts*$^-$*296*, and the structural genes coding for two of the group I enzymes (*met2* and *met8*), it seems plausible that the product of the gene *eth2* could be a pleiotropic aporepressor. The final repressor would

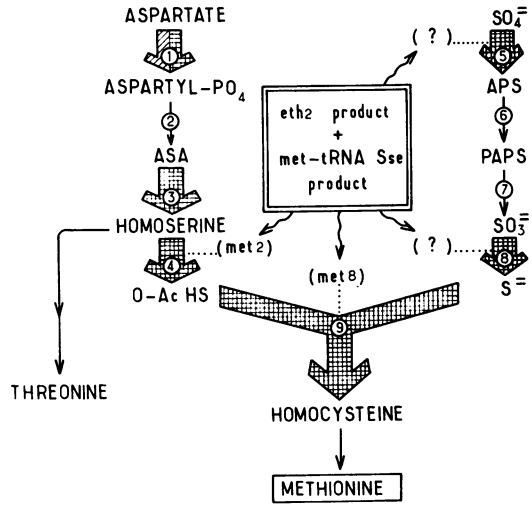


FIG. 6. *Regulation of synthesis of group I and group II enzymes. Repression by exogenous methionine (group I enzymes), horizontal and vertical cross-hatching with dots; repression by exogenous methionine (group II enzymes), horizontal and vertical cross-hatching; repression by exogenous threonine, diagonal cross-hatching. For abbreviations and enzyme nomenclature, see legend of Fig. 1.*

then result from the association of this aporepressor with the postulated "regulatory met-$tRNA^{met}$."

In an alternative hypothesis, the *eth2* product might correspond to the "regulatory $tRNA^{met}$" itself. The existence of suppressible mutations in the *eth2* locus would favor the idea that the *eth2* product is a protein. A search for such mutants is currently under way in this laboratory.

In any case, the *eth2*^{r} allele presently studied determines a very precise change in the response of the four group I enzymes to exogenous methionine. First, the sharpness of the response to methionine concentration found in the wild type was quite unexpected, especially when one considers the existence of unspecific (22, 55) as well as specific (19) methionine uptake systems and the existence of large amino acid pools in yeast (10, 55) which are able to compensate internally for a wide range of external methionine concentrations. Such a sensitivity in response of methionine group I enzymes to exogenous methionine concentration might indicate that the pleiotropic methionine apo-corepressor complex postulated here, is assembled in a compartment (perhaps the nucleus) physically separated from the metabolic pools. Such an hypothesis would certainly fit the previous assumption of the existence of a "regulatory $tRNA^{met}$" which then might be expected to be found mainly in this compartment.

The shape of the curves (see Fig. 2 and 3) show that, whatever the nature of the corepressor, its fixation on the *eth2* product is best described by a saturation process. The mutation *eth2-1*^{r} seems then to have reduced the affinity of the *eth2* product for the appropriate corepressor.

The pleiotropic character of *eth2* suggests that redundant genetic structures exist for each of the structural genes it governs. It was already pointed out that the small differences observed in the quantitative response of three enzymes, either in *eth2*^{s} or *eth2*^{r} strains, did not seem significant. This is further shown by the coordinate synthesis of the group I enzymes. Such a finding might seem surprising in view of the usual belief that coordinate synthesis is a characteristic of operon organization. However, some observations in the eukaryote, *N. crassa*, have already disproved this restricted belief for enzymes involved in acetate metabolism (L. Fincham, *personnal communication*) and in aromatic amino acid biosynthesis (6), for example. It seems then that coordinate synthesis of certain enzymes reflects mostly the existence of a regulatory system common to different structural genes, disregarding their localization in the genome.

The biosynthesis of group II enzymes also needs comment. The absence of response to the regulatory gene *eth2* and to the limitation in charging of tRNA^{met} suggests that another system(s) regulates their synthesis. In agreement with previous conclusions, it appears from Fig. 4 that the synthesis of group II enzymes is not coordinated with the synthesis of group I enzymes. However, both regulatory systems use methionine, or a product made from methionine, as a corepressor. It is thus expected that in a certain range of exogenous methionine concentrations the synthesis of enzymes belonging to both of these groups would be affected. The important difference resides in that cessation of synthesis of group I enzymes occurs before that of synthesis of group II enzymes. The complex regulation of aspartokinase synthesis is, in itself, very interesting. According to data previously obtained, there seems to be only one such enzyme coded by a single structural gene in *S. cerevisiae* (47). It follows that somehow the two end products, methionine and threonine, are expected to be involved in a multivalent type of repression (16). Some results obtained first in *E. coli* (39) and then in *S. cerevisiae* (38) by using borrelidin, an antibiotic which specifically inactivates threonyl-tRNA synthetase, indicate that threonyl-tRNA might be involved in both organisms in threonine-mediated repression. On the other hand, results obtained so far led to aspartokinase being included in the regulatory group II, rather than group I. It would seem, then, that with such a bivalent repression, although one of the end products might exert its control through the corresponding acylated tRNA, it might not be the case for the other end product.

The present work has shown that, inside a complex biosynthetic pathway such as the one studied, the existence of more than one regulatory system can be expected, differing in the aporepressors as well as in the corepressors involved. If one admits the role of methionyl-tRNA in the regulation of group I enzymes we have proposed, our results also indicate that its action might not extend over synthesis of every enzyme of the pathway which is subject to methionine-mediated repression and that, at least, one other methionine corepressor should also be operative. Beside methionine itself, *S*-adenosylmethionine might be a good candidate (8). This hypothesis seems to have recently received some experimental support, in work with *E. coli* (21).

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