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Proofreading *in vivo*: editing of homocysteine by methionyl-tRNA synthetase in the yeast *Saccharomyces cerevisiae*

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Homocysteine thiolactone is a product of an error-editing reaction, catalyzed by *Escherichia coli* methionyl-tRNA synthetase, which prevents incorporation of homocysteine into tRNA and protein, both *in vitro* and *in vivo*. Here, the thiolactone is also shown to occur in cultures of the yeast *Saccharomyces cerevisiae*. In yeast, the thiolactone is made from homocysteine in a reaction catalyzed by methionyl-tRNA synthetase. One molecule of homocysteine is edited as thiolactone per 500 molecules of methionine incorporated into protein. Homocysteine, added exogenously to the medium or overproduced by some yeast mutants, is detrimental to cell growth. The cost of homocysteine editing in yeast is minimized by the presence of a pathway leading from homocysteine to cysteine, which keeps intracellular homocysteine at low levels. These results not only directly demonstrate that editing of errors in amino acid selection by methionyl-tRNA synthetase operates *in vivo* in yeast but also establish the importance of proofreading mechanisms in a eukaryotic organism.

Key words: cysteine biosynthesis/energy cost of editing/error rate in translation/homocysteine thiolactone/methionine biosynthesis

Introduction

Error-editing reactions are essential factors in maintaining the accuracy of protein synthesis. Some amino acids are so similar in structure that the enzymes responsible for their selection, the aminoacyl-tRNA synthetases, have special hydrolytic activities to remove products of misactivation (Norris and Berg, 1964; Baldwin and Berg, 1966). Aminoacylation of tRNA is a two-step reaction. In the first step, an amino acid (AA) is activated to form enzyme (E)-bound aminoacyl adenylate.

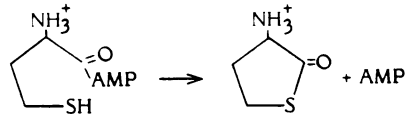
$$E + AA \rightarrow E.AA{-}AMP + PP$$

In the second step the amino acid is transferred from the adenylate to tRNA.

$$E.AA{-}AMP + tRNA^{AA} \rightarrow E + AA{-}tRNA^{AA} + AMP$$

As directly demonstrated *in vitro*, editing can occur by the hydrolysis of the misformed aminoacyl adenylate (Jakubowski, 1978b, 1980; Jakubowski and Fersht, 1981) or the aminoacyl tRNA (Eldred and Schimmel, 1972; Yarus, 1972; Fersht and Kaethner, 1976). Theoretical schemes of enhancing accuracy in macromolecular synthesis have also been presented (Hopfield, 1974, 1980; Ninio, 1975).

Recently, the *in vivo* relevance of editing mechanisms has been addressed (Jakubowski, 1990). *In vitro* studies demonstrated that *Escherichia coli* methionyl-tRNA synthetase edits misactivated homocysteine by cyclization of homocysteinyl adenylate with the formation of homocysteine thiolactone (Jakubowski and Fersht, 1981).



This editing reaction has subsequently been shown to occur *in vivo* in *E.coli* (Jakubowski, 1990). Here I report that homocysteine thiolactone is also synthesized by the yeast *Saccharomyces cerevisiae*. The data indicate that the thiolactone synthesis in yeast is due to *in vivo* editing of homocysteine by methionyl-tRNA synthetase, thus establishing the importance of error-editing mechanisms in eukaryotic cells.

Results

Detection of [^{35}S]homocysteine thiolactone in S.cerevisiae

In yeast, homocysteine is a precursor of both methionine and cysteine (Figure 1). Homocysteine is converted into methionine by the product of the *MET6* gene and into

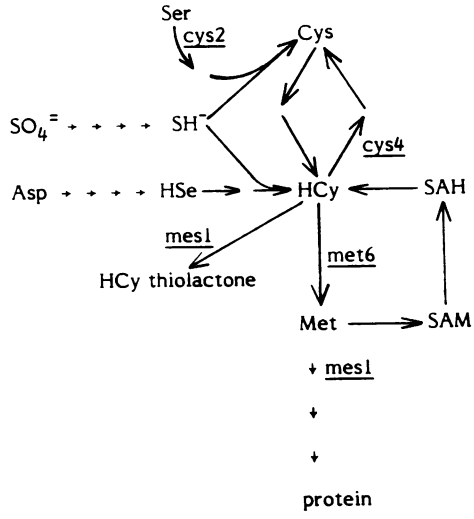


Fig. 1. Schematic representation of homocysteine metabolism in *S.cerevisiae*. Each arrow represents a step catalyzed by a separate enzyme. Mutations utilized in this study are indicated. HCy, homocysteine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; HSe, homoserine.

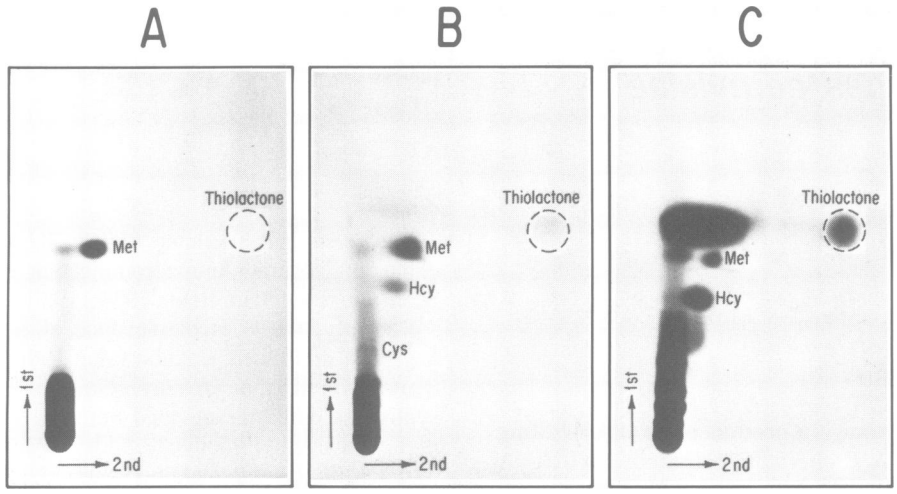


Fig. 2. Two-dimensional TLC separation of sulfur containing compounds from *S.cerevisiae* cultures. First dimension, butanol/acetic acid/water (4:1:1, by vol); second dimension, 2-propanol/ethyl acetate/ammonia/water (25:25:0.05:8, by vol). Autoradiograms of the two-dimensional separation of formic acid extracts from the following [^{35}S]sulfate-labeled yeast cells maintained in the low-sulfate medium are shown: wild-type S288C (**A**), a *cys4* prototroph JW1-2C-R1 (**B**), and a *cys2 cys4* auxotroph JW1-2C (**C**).

cysteine by a two step reaction, the first step of which is catalyzed by the product of the *CYS4* gene. Therefore it is to be expected that either a *met6* or a *cys4* mutation would lead to accumulation of homocysteine in yeast. If homocysteine is subsequently edited by methionyl-tRNA synthetase, as it is in *E.coli* (Jakubowski, 1990), these mutants should also produce much higher levels of homocysteine thiolactone than wild-type cells.

Wild-type, *cys4* prototrophic and *cys2 cys4* auxotrophic yeast strains were labeled with [^{35}S]sulfate. After 3 h labeling the cells were collected and extracted as described in Materials and methods. ^{35}S-Labeled compounds in the extracts were resolved by two-dimensional TLC on cellulose plates. Autoradiograms of these chromatograms are presented in Figure 2. As shown in Figure 2A, homocysteine thiolactone was essentially undetectable in the wild-type strain under these conditions. However, as expected, the *cys4* strains produced significant levels of homocysteine thiolactone (Figure 2B, C). Quantification of the sulfur amino acid spots from the chromatograms shown in Figure 2 is presented in Table I. Intracellular levels of homocysteine thiolactone in the *cys4* and *cys2 cys4* strains are at least 20- and 120-fold, respectively, higher than in the wild-type and correlate well with the intracellular homocysteine levels.

Table I. Intracellular levels of sulfur amino acids in the yeast *S.cerevisiae*

Yeast strain genotype	Intracellular concentration (pmol/10^7 cells)			
	Met	Cys	HCy	HCy thiolactone
Wild-type	80	<6	<4	<0.3
cys4	120	48	40	7.5
cys2 cys4	92	–	275	40

Table II. Effect of *mes1* mutation on levels of sulfur amino acids in the yeast *S.cerevisiae*

Yeast strain genotype	Intracellular concentration (pmol/10^7 cells)			
	Met	Cys	HCy	HCy thiolactone
met6	N.D.	325	360	175
met6 mes1	N.D.	170	65	<2
mes1	87	8	2	N.D.

N.D., not detectable.

Methionyl-tRNA synthetase mutation abolishes synthesis of homocysteine thiolactone

In order to determine if methionyl-tRNA synthetase catalyzes synthesis of homocysteine thiolactone *in vivo*, the effect of a *mes1* mutation on the thiolactone accumulation in yeast was studied. The *mes1* mutant has a defect in methionyl-tRNA synthetase which results in methionine auxotropy (Fasiolo *et al.*, 1981). A double mutant *met6 mes1* was constructed. As shown in Table II the intracellular levels of homocysteine thiolactone in the *met6 mes1* strain were >100-fold lower than in the *met6* strain. Although there was also a 6-fold drop in homocysteine levels in the *met6 mes1* strain, this cannot account for the precipitous drop in the thiolactone levels in the double mutant. Thus, it is concluded that the *mes1* mutation prevents synthesis of homocysteine thiolactone in yeast. Similar results were obtained with a *cys2 cys4 mes1* mutant (not shown).

Rate of homocysteine thiolactone synthesis is proportional to cellular levels of methionyl-tRNA synthetase

The cellular level of methionyl-tRNA synthetase in yeast was manipulated by using two plasmids bearing the methionyl-tRNA synthetase gene (*MES1*). In plasmid pM7B1, the *MES1* gene is under the control of its own promoter (Fasiolo *et al.*, 1981). In plasmid pMVT1, the *MES1* gene is under the control of *LDH1* promoter (Walter *et al.*, 1989). Expression of the *MES1* gene on the plasmids in yeast cells leads to overproduction of methionyl-tRNA synthetase. The plasmids were introduced into a *mes1 ura3* strain. The transformants and a wild-type strain were grown in the low sulfate modified SD medium. Cellular levels of methionyl-

Table III. Relationship between cellular levels of methionyl-tRNA synthetase and the rate of homocysteine thiolactone synthesis in yeast

Yeast strain	Methionyl-tRNA synthetase	Homocysteine thiolactone
S288C (wild-type)	1[a]	1[b]
pM7B1	5.2	3.7
pMVT1	23	22

[a]12.5 pmol Met-tRNA formed per 1 min at 30°C per 10^7 cells.
[b]2.5 pmol/h at 30°C per 10^7 cells.

Table IV. Effect of amino acids on synthesis of homocysteine (HCy) thiolactone by a *met6* yeast strain

Amino acid in yeast culture (1.0 mM)	[^{35}S]HCy thiolactone synthesis rate (%)
None	100
Met (1.0 mM)	0.7
(0.2 mM)	0.7
Cys	68
Leu	96
Ile	96
Val	93
Arg	99

tRNA synthetase and the rate of thiolactone synthesis in these cultures were determined as described in Materials and methods. Under the conditions of these experiments, yeast cells harboring plasmid pM7B1 or pMVT1 overproduced methionyl-tRNA synthetase 5.2- or 23-fold, respectively, over the wild-type levels, which is 6–8 times lower expression than that reported (Fasiolo *et al.*, 1981; Walter *et al.*, 1989). The differences in expression of the *MES1* gene in this study and previous reports are due most likely to different growth conditions. However, what is most important, the rate of homocysteine thiolactone synthesis in cells harboring the plasmids increased in direct proportion to the increase in methionyl-tRNA synthetase levels (Table III). Thus, there was a direct relationship between the level of methionyl-tRNA synthetase and the rate of homocysteine thiolactone synthesis in yeast cells.

Methionine inhibits synthesis of homocysteine thiolactone

If methionyl-tRNA synthetase is involved in homocysteine thiolactone synthesis in yeast as the above experiments indicate, one can expect that the thiolactone synthesis would be inhibited by the presence of methionine, but not of any other amino acid, in the growth medium. As shown in Table IV, methionine totally abolished synthesis of the thiolactone. It should be noted that the thiolactone synthesis was prevented by methionine at a concentration (0.2 mM) which does not significantly repress the methionine biosynthetic pathway.

Control experiments indicated that cysteine had a small, but reproducible effect on the thiolactone synthesis. This is most likely due to dilution of the [^{35}S]homocysteine pool by unlabeled homocysteine synthesized from cysteine. Several other amino acids (leucine, isoleucine, valine and arginine) did not have any significant effect on the thiolactone synthesis. These data exclude any significant participation of other enzymes in homocysteine thiolactone synthesis in yeast. Instead, the results indicate that methionyl-tRNA synthetase is the primary enzyme involved in the thiolactone synthesis in yeast cells.

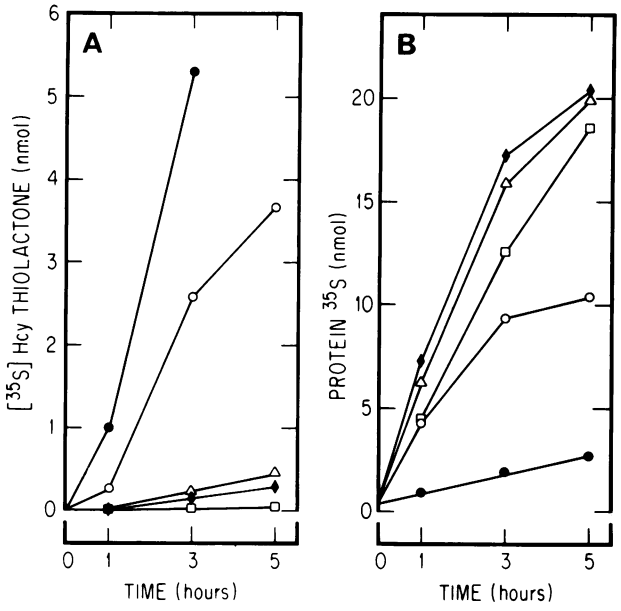


Fig. 3. Homocysteine thiolactone (**A**) and protein synthesis (**B**) in several yeast cultures. The levels of [^{35}S]homocysteine thiolactone (nmol/10^7 cells) and [^{35}S]protein (nmol/10^7 cells) in the following yeast cultures were determined at indicated time intervals: *met6* (●), *cys2 cys4* (○), a diploid prototroph ABJ-1 (△), a haploid prototroph *cys4* (◆), and a wild-type S288C (□).

The cost of homocysteine editing in vivo

One way to estimate the energy cost of editing *in vivo* is to relate the amount of edited homocysteine (in the form of thiolactone) to the amount of methionine incorporated into protein. Towards this end, incorporation of radiolabeled sulfur into homocysteine thiolactone and protein methionine was measured in yeast cultures labeled with [^{35}S]sulfate. Kinetics of the incorporation are shown in Figure 3. The levels of homocysteine thiolactone in yeast cultures after 3 h (Figure 3A) were on average 30-fold greater than the intracellular thiolactone levels (Tables I and II) which indicates that the thiolactone was secreted from cells. In fact, most of the thiolactone in yeast cultures was present in cell free medium (not shown). Parallel measurements of incorporation of ^{35}S into protein in several yeast strains are shown in Figure 3B.

The calculated ratios of the thiolactone to protein methionine are shown in Table V. In a wild-type strain S288C, one molecule of homocysteine was edited per 500 molecules of methionine incorporated into protein. While the rate of protein synthesis was not increased by overexpression of methionyl-tRNA synthetase in strains harboring plasmids pM7B1 and pMVT1, the rate of homocysteine editing did increase in proportion to the increase in the synthetase levels. The rates of homocysteine editing in two prototrophic strains, JW1-2C-R1 and ABJ-1, were ~5-fold higher than in the wild-type. The highest homocysteine editing rates were observed in a cysteine auxotroph JW-2C, which was able to grow for at least two generations in the absence of exogenous cysteine. In this particular strain, 13% of the energy used for activation of

Table V. Ratios of the rates of homocysteine editing to incorporation of methionine into protein in yeast

Yeast strain	Thiolactone: protein methionine[a]
S288C	0.0020
pM7B	0.0057
pMVT1	0.040
JW1-2C-R1	0.0095
JW1-2C	0.130
ABJ-1	0.011

[a]Calculated by assuming that 1 nmol [^{35}S]sulfur incorporated into protein corresponds to 0.63 nmol of [^{35}S]methionine.

methionine for protein synthesis was used for editing of homocysteine as opposed to 0.2% in the wild-type.

Homocysteine inhibits cell growth

As demonstrated above, homocysteine is misactivated and edited by methionyl-tRNA synthetase in yeast. Therefore, it is to be expected that these processes, if too extensive, would lead to slower growth rates. This is, in fact, what has been observed. Two isogenic strains which overproduced homocysteine to different extents, JW1-2C (Cys^-) and JW1-2C-R1 (Cys^+) were grown in a mixed culture for 15 generations in SD minimal medium containing cysteine. The number of each kind of cells during growth was determined by plating appropriate dilutions of the mixed cultures on YEPD plates and scoring the colonies for Cys^- and Cys^+ phenotypes. Growth of the Cys^+ strain relative to the growth of Cys^- strain is presented in Figure 4. As shown in Figure 4, the strain JW1-2C which contained more intracellular homocysteine and produced more thiolactone (i.e. edited more, Table I) grew 16% more slowly than the strain JW1-2C-R1 which edited less. In the presence of exogenous homocysteine or methionine the growth disadvantage of the JW1-2C over JW1-2C-R1 in a mixed culture increased to 49% or 36%, respectively. The result that exogenous methionine led to a similar growth disadvantage for JW1-2C in a mixed culture as did the presence of homocysteine, can be explained by the existence of a pathway which produces homocysteine from methionine (via SAM and SAH, Figure 1) in yeast. It should be noted that the presence of exogenous homocysteine has a profound inhibitory effect on the growth of yeast cells. This inhibition can be reversed by exogenous methionine. The doubling times for strain JW1-2C-R1 grown in the presence of homocysteine, methionine, methionine and homocysteine and in the absence of either were 7.6 h, 2.9 h, 4.2 h and 2.9 h, respectively. Thus, a yeast strain containing high endogenous levels of homocysteine grew slower than the strain producing lower levels of homocysteine. Also, exogenous homocysteine was detrimental to growth of yeast cells.

Discussion

This study demonstrates the *in vivo* existence of a proofreading mechanism that prevents incorporation of homocysteine into tRNA and protein in the yeast *S. cerevisiae*, thereby extending the importance of proofreading to a eukaryotic organism. Both genetic and biochemical evidence presented above indicate that homocysteine is

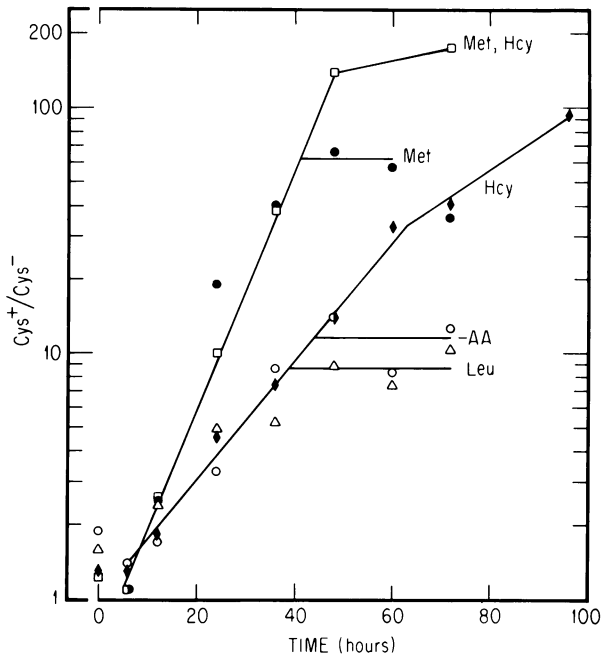


Fig. 4. Relative growth rates of JW1-2C and JW1-2C-R1. Yeast strains JW1-2C (Cys^-) and JW1-2C-R1 (Cys^+) were grown in mixed cultures in SD medium +1 mM cysteine at 25°C. The strains were inoculated at a total initial cell density of 1000 per ml in a 1:1 ratio. At indicated time intervals, the number of Cys^- and Cys^+ cells was determined by plating appropriate dilutions of the cultures on YEPD plates and scoring for the Cys^- phenotype. The logarithm of the ratio of Cys^+/Cys^- cells is plotted as a function of time of growth. The mixed cultures were grown in the absence (○) and in the presence of 0.4 mM D,L-homocysteine (◆), 0.4 mM methionine (●), 0.4 mM D,L-homocysteine and 0.4 mM methionine (□) or, as a control, 0.4 mM leucine (△). The ratios of the generation times, *A*, for JW1-2C and JW1-2C-R1 were calculated according to the equation $\log y = x \log A$ where *y* is a ratio of Cys^+ to Cys^- cells, and *x* is the number of generation times of the faster growing strain (time of growth divided by doubling time). The doubling times of the JW1-2C-R1 (Cys^+) strain were 2.9 h in the absence and presence of either 0.4 mM methionine or leucine, 7.6 h in the presence of 0.4 mM D,L-homocysteine, and 4.2 h in the presence of 0.4 mM D,L-homocysteine and 0.4 mM methionine. The following values of the ratios of the generation times, *A*, were calculated: 1.16 for mixed cultures growing in the absence (○) or presence (△) of 0.4 mM leucine; 1.49 in the presence of 0.4 mM D,L-homocysteine (◆); 1.36 in the presence of 0.4 mM methionine (●); 1.55 in the presence of 0.4 mM D,L-homocysteine and 0.4 mM methionine (□).

transformed into homocysteine thiolactone by methionyl-tRNA synthetase in *S.cerevisiae*, an editing reaction originally discovered *in vitro* (Jakubowski and Fersht, 1981) and subsequently shown to exist also *in vivo* in *E.coli* (Jakubowski, 1990). The thiolactone has not been reported in yeast before. As reported here, it is a component of sulfur amino acid pools in *S.cerevisiae* and its concentration can exceed that of methionine in some yeast strains (Tables I, II and V). However, again similar to its fate in *E.coli*, most of the thiolactone is secreted from yeast cells and can easily be detected in cell-free medium.

The relationship between the methionine and cysteine biosynthetic pathways and the proofreading reaction in yeast is depicted in Figure 1. In the last step of the methionine biosynthetic pathway, homocysteine (HCy) is methylated to methionine by the product of the *MET6* gene. In the cysteine biosynthetic pathway, homocysteine is transformed into cysteine by a two-step reaction. As shown here, homo-

cysteine is also edited as thiolactone by the product of *MES1* gene, whose major function is to provide Met-tRNA^{Met} for protein synthesis. There is a quantitative difference in homocysteine editing in wild-type yeast and *E.coli*: the former makes five times less homocysteine thiolactone than the latter. The ratio of the thiolactone formed to methionine incorporated into protein is 1:109 in *E.coli* (Jakubowski, 1990) and 1:500 in yeast (Table V). Thus, it seems that yeast expends less energy for homocysteine editing than does *E.coli*. This quantitative difference in homocysteine editing can be traced to different organization of the methionine biosynthetic pathways in the two organisms. Apparently, the existence of an additional pathway from homocysteine to cysteine in yeast (Figure 1) allows the cell to lower its intracellular homocysteine level >10 times below that present in *E.coli*. A yeast *cys4* mutant (a prototroph) is the equivalent of a wild-type *E.coli* in terms of organization of its methionine biosynthetic pathway. The mutant has relatively high intracellular homocysteine levels, as does *E.coli*, and it edits one homocysteine per 100 methionines incorporated into protein, almost exactly as does *E.coli* (Jakubowski, 1990). As demonstrated in the last section of Results (Figure 4) high levels of homocysteine retard growth. This detrimental effect of homocysteine is minimized in yeast, apparently by evolving a capability to transform homocysteine into cysteine.

There is another important difference in homocysteine editing between yeast and *E.coli*. *E.coli* is much more efficient than yeast in homocysteine editing. This is illustrated by the fact that an *E.coli metE* mutant does not accumulate homocysteine; it accumulates the thiolactone instead (Jakubowski, 1990). Apparently, in *E.coli* all homocysteine that cannot be processed to methionine is edited as thiolactone. However, this is not so in yeast. A yeast *met6* mutant, which is an equivalent of *metE* in *E.coli*, accumulates both homocysteine and the thiolactone to levels 90- and 500-fold higher, respectively, than wild-type yeast. Another yeast mutant, *cys2 cys4*, also accumulates homocysteine and the thiolactone to levels 70- and 130-fold higher than the wild-type (Tables I and II). The less efficient homocysteine editing in yeast than in *E.coli* is most likely due to different organization and, presumably, compartmentation of methionine biosynthetic pathways in the two organisms.

In vitro studies with pure aminoacyl-tRNA synthetase indicate that editing of errors of misactivation of amino acids can take place by the four routes shown in Figure 5: (i) via k_1, the dissociation of the enzyme-bound aminoacyl adenylate to give free aminoacyl adenylate which hydrolyzes in solution (Jakubowski, 1978b, 1980; Jakubowski and Fersht, 1981); (ii) via k_2, the tRNA-independent hydrolysis of the enzyme-bound aminoacyl adenylate (Jakubowski, 1978b, 1980; Jakubowski and Fersht, 1981; Englisch *et al.*, 1986); (iii) via k_3, the tRNA-dependent hydrolysis of the enzyme-bound aminoacyl adenylate without transient formation of mischarged tRNA (Fersht, 1977; Jakubowski, 1980); (iv) via k_4, the hydrolysis of the enzyme-bound mischarged tRNA (Eldred and Schimmel, 1972; Yarus, 1972; Fersht and Kaethner, 1976; Fersht and Dingwall, 1979; Jakubowski and Fersht, 1981). In some cases the edited amino acid (AA*) is a distinct chemical species (Jakubowski and Fersht, 1981; Englisch *et al.*, 1986). The contribution of a particular pathway to overall editing depends on the ratios of all the rate constants in Figure 5.

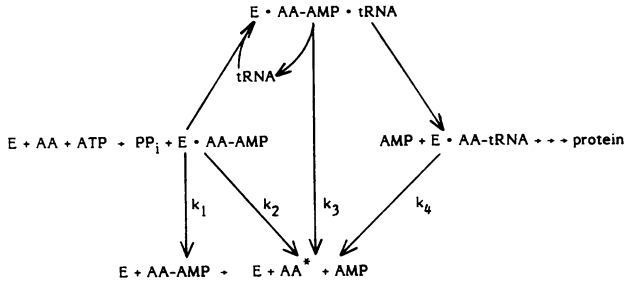


Fig. 5. Possible routes for the editing of errors of misactivation of amino acids.

Table VI. *S.cerevisiae* strains used

Strain	Genotype	Source (reference)
S288C	*MATα SUC2 mal mel gal2 CUP1*	YGSC
JW1-2C	*MATa cys2-1 cys4-1 CUP1*	YGSC (Ono *et al.*, 1988)
JW1-2C-R1	*MATa cys4-1*	This work (Ono *et al.*, 1988)
XJB3-1B	*MATα met6 gal2*	YGSC
F.F.1 sp1	*MATα mes1 ura3*	Fasiolo *et al.* (1981)
ABJ-1	*MATa cys2-1 cys4-1* + / *MATα* + + met6	This work
ABJ-8	*MATa cys2-1 cys4-1 mes1*	This work
ABJ-3D	*MATa met6 mes1*	This work

Although all the pathways are used to some extent, the available evidence indicates that routes k_2 and k_4 are of major importance. For example, methionyl-tRNA synthetase edits misactivated homocysteine via the pre-transfer route involving cleavage of homocysteinyl adenylate (k_2) with formation of homocysteinyl thiolactone. Valyl-tRNA synthetase edits misactivated threonine via the post-transfer route involving hydrolysis of mischarged Thr-tRNA^{Val} (k_4). Of these two major *in vitro* editing pathways, the k_2 pathway involving cleavage of homocysteinyl adenylate by methionyl-tRNA synthetase was shown to be important for editing of misactivated homocysteine *in vivo* by the bacterium *E.coli* (Jakubowski, 1990). Since there is an extensive sequence homology between the *E.coli* and yeast methionyl-tRNA synthetases (Walter *et al.*, 1983), the *in vivo* editing of homocysteine in yeast described in this report is most likely also through the k_2 pathway.

Materials and methods

Strains and plasmids

The *S.cerevisiae* strains used are listed in Table VI. *E.coli* HB101 was used as host for plasmid maintenance and recovery. Plasmids pM7B1 (Fasiolo *et al.*, 1981) and pMVT1 (Walter *et al.*, 1989) were obtained from F.Fasiolo (CNRS, Strasbourg, France). pM7B1 is a derivative of a high copy plasmid pFL$_1$ which contains the yeast methionyl-tRNA synthetase gene (*MES1*). pMVT1 is a derivative of the yeast/*E.coli* shuttle vector pVT103U which contains the *MES1* gene under the control of a yeast *ADH1* promoter.

Genetic and recombinant DNA methods

Genetic techniques, plasmid recovery and transformation were done by standard procedures (Sherman *et al.*, 1986).

Growth and ^{35}S-labeling conditions

Yeast cells were grown aerobically at 25°C in low-sulfate medium plus auxotrophic requirements. The low-sulfate medium was prepared as SD medium (Sherman *et al.*, 1986) except that ammonium sulfate was replaced by an equivalent amount of ammonium chloride and 0.15 mM sodium sulfate

was included. The cells were harvested by centrifugation in an Eppendorf microcentrifuge at room temperature for 30 s, washed with sulfate-free medium, resuspended at a cell density of 3×10^7 cells/ml in low-sulfate medium containing 0.15 mM [^{35}S]sulfate (675 Ci/mol) at 0.1 mCi/ml (1 Ci = 37 GBq) (New England Nuclear), and maintained at 25°C with vigorous aeration.

Preparation of ^{35}S-labeled extracts

For homocysteine thiolactone determination, aliquots (20 μl) of the ^{35}S-labeled yeast cultures were removed and extracted with 5 μl of 5 M formic acid for 30 min at 0°C. The extracts (5 μl) were analyzed by two-dimensional TLC. For sulfur amino acids determination, yeast cells were labeled with [^{35}S]sulfate for 3 h. The labeled cells from 0.5 ml aliquots of the cultures were collected on nitrocellulose filters (0.45 μm; Schleicher & Schuell) and extracted with 0.12 ml of 1 M formic acid on ice for 30 min. The filters were washed with two 0.12 ml aliquots of 1 M formic acid. The extracts and washes were combined and frozen at −80°C. After thawing the extracts were clarified by centrifugation and lyophilized. The residues were taken up in 8 μl of water and analyzed by two-dimensional TLC.

Two-dimensional TLC analysis of ^{35}S-labeled compounds

The procedure is similar to that described previously (Jakubowski, 1990) except that 20-fold less ammonia was used in the second dimension solvent. This modification improves stability of homocysteine thiolactone and leads to its essentially quantitative recovery. Extracts (4 μl) were applied as a spot on cellulose plates (20 × 10 cm; Sigma). Butanol/acetic acid/water (4:1:1, v/v) was used as the first-dimension solvent and 2-propanol/ethyl acetate/ammonia/water (25:25:0.05:8, v/v) was used as the second dimension solvent. Standard sulfur-containing compounds were cochromatographed with the ^{35}S-labeled samples. The standards were located under UV light and/or after staining with ninhydrin. ^{35}S-Labeled compounds were visualized by autoradiography using Kodak XAR-5 film. The ^{35}S-labeled spots were quantified by scintillation counting. Counting efficiency was 60%.

Measurements of protein synthesis

Trichloroacetic acid-insoluble radioactivity was used as a measure of protein synthesis in [^{35}S]sulfate-labeled yeast cultures.

Measurements of methionyl-tRNA synthetase activity

Yeast strains were grown at 30°C in the low-sulfate medium to a cell density of 6×10^7/ml. The cells from 1.5 ml cultures were harvested by centrifugation, resuspended in 50 μl of 10 mM potassium phosphate buffer, pH 6.8/10 mM 2-mercaptoethanol/10% (v/v) glycerol, and disrupted by vortexing with glass beads (450 μm) until at least 90% of cells were broken. The extract was diluted with another 50 μl of the buffer and clarified by microcentrifugation at 4°C. Levels of methionyl-tRNA synthetase in the extracts were determined by tRNA aminoacylation with [^{35}S]methionine. The tRNA aminoacylation mixture contained (in a final volume of 50 μl) 50 mM HEPES−KOH buffer (pH 8.0), 10 mM $MgCl_2$, 1 mM ATP, 6.7 mg/ml unfractionated yeast tRNA (Sigma), 2.5 mM [^{35}S]methionine (1 pmol = 300 c.p.m.), and 4% (v/v) of crude extract. After 1, 2, 4 and 8 min at 30°C, [^{35}S]Met-tRNA was precipitated and washed with ice-cold trichloroacetic acid (Jakubowski, 1978a) and quantified by scintillation counting.

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