Role of Carboxy-Terminal Region in Proofreading Function of Methionyl-tRNA Synthetase in *Escherichia coli*†

Wenwu Gao, Emanuel Goldman, and Hieronim Jakubowski*

Department of Microbiology & Molecular Genetics, Graduate School of Biomedical Sciences and New Jersey Medical School, University of Medicine and Dentistry of New Jersey, Newark, New Jersey 07103

Received February 4, 1994; Revised Manuscript Received July 8, 1994®

ABSTRACT: The synthetic and editing functions of three forms of *Escherichia coli* methionyl-tRNA synthetase with different C-terminal sequences have been compared *in vivo* and *in vitro*. These forms include a full-length wild-type dimer (MRS676), a truncated monomer (MRS547) believed to be equivalent to the biologically active large tryptic fragment, and a third form denoted MRS581*. DNA sequencing revealed that MRS581* is predicted to contain 18 additional amino acids from the wild-type full-length sequence at the carboxy terminus of truncated form MRS547, and this is then fused to an additional 16 amino acids encoded by vector pBR322. Both MRS676 and MRS581* were found to edit endogenous homocysteine about 20-fold more efficiently than MRS547 *in vivo*. However, the three methionyl-tRNA synthetases edited exogenously supplied homocysteine in bacterial cultures to similar extents. Purified proteins exhibited no significant differences in editing function *in vitro*. Synthetic activity of purified MRS676 *in vitro* was found to be about 2.5-fold higher per subunit compared to the shorter forms of the enzyme. The C-terminal region in *E. coli* methionyl-tRNA synthetase is thus suggested to play an important role in editing *in vivo*, most likely by allowing interaction of the enzyme with the methionine biosynthetic pathway. These data support a model of channeling of at least some metabolites in bacterial protein synthesis.

One of the key functions of aminoacyl-tRNA synthetases is to discriminate cognate and noncognate amino acids. The mechanism for noncognate amino acid editing (proofreading) has been intensively studied on some individual aminoacyl-tRNA synthetases [reviewed in Jakubowski and Goldman (1992)]. Studies with purified aminoacyl-tRNA synthetases indicate that misactivated amino acids can be edited via different pathways such as the adenylate pathway and the misacylation–deacylation pathway. The first *in vivo* evidence of proofreading was discovered with methionyl tRNA synthetase in *Escherichia coli* (Jakubowski, 1990), followed by evidence in a eukaryotic organism, *Saccharomyces cerevisiae* (Jakubowski, 1991) and in some mammalian cells (Jakubowski & Goldman, 1993). The editing mechanism of methionyl-tRNA synthetase (MetRS)[1] is directed toward homocysteine (Hcy), which is the immediate precursor of methionine in the methionine biosynthetic pathway. This involves misactivation of Hcy to form the enzyme-bound Hcy-AMP (eq 2) which is then enzymatically converted to homocysteine thiolactone (Jakubowski & Fersht, 1981) through the adenylate pathway (Jakubowski, 1978, 1980). The reactions of MetRS with methionine and homocysteine are shown in eqs 1 and 2, respectively.

$$\text{MetRS} + \text{Met} + \text{ATP} \underset{\text{PP}_i}{\rightleftarrows} \text{MetRS·Met-AMP} \overset{\text{tRNA}^{\text{Met}}}{\rightleftarrows} \text{Met-tRNA}^{\text{Met}} + \text{MetRS} + \text{AMP} \quad (1)$$

$$\text{MetRS} + \text{Hcy} + \text{ATP} \underset{\text{PP}_i}{\rightleftarrows} \text{MetRS·Hcy-AMP} \rightarrow \text{Hcy-thiolactone} + \text{MetRS} + \text{AMP} \quad (2)$$

Homocysteine thiolactone can be visualized directly under UV light, has an absorption peak at 240 nm, and also gives yellow color upon staining with ninhydrin (Jakubowski & Fersht, 1981). Furthermore, it can be well-separated from other sulfur-containing compounds in living cells by two-dimensional thin-layer chromatography. These studies not only demonstrated a proofreading mechanism operating in living cells, but also provided a useful system for further dissection of the molecular basis of translational accuracy at the level of aminoacylation.

The three-dimensional structure of a biologically active monomeric truncated form of MetRS (large tryptic fragment) has been resolved (Zelwer et al., 1982), also in its complex with ATP (Brunie et al., 1990). The structure is composed of two domains, N-terminal and C-terminal; the cavity in the N-terminal domain has been proposed to contain the catalytic center. The relevance of several amino acid residues in this catalytic center has been confirmed by site-directed mutagenesis studies (Ghosh et al., 1991a,b; Fourmy et al., 1991; Mechulam et al., 1991; Kim et al., 1993). A model of the active site of MetRS has been proposed in which an amino acid substrate is partitioned between synthetic and editing pathways (Kim et al., 1993). Hydrogen bonding and hydrophobic interactions direct the cognate methionine through the synthetic pathway and prevent it from entering the editing pathway. These interactions are missing with the noncognate homocysteine, which therefore enters the editing pathway.

The crystallographic structure of (truncated) MetRS reveals that the C-terminus of the protein overhangs the cavity which forms the active center (Brunie et al., 1990). Thus, it is possible that a C-terminal portion of the full-length protein contributes to catalytic function. It has been reported that truncation of the C-terminal region of MetRS by trypsin, producing a large tryptic fragment, does not affect synthetic activity (Cassio & Waller, 1971; Lawrence et al., 1973). A 547 amino acid version of MetRS (MRS547), believed to be similar to the

†This work was supported by grants from the NIH (GM 27711) and the NSF (MCB 9218358).

* Corresponding author.

® Abstract published in *Advance ACS Abstracts,* September 1, 1994.

[1] Abbreviations: MetRS, methionyl-tRNA synthetase; Hcy, homocysteine; 2D-TLC, two-dimensional thin-layer chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

0006-2960/94/0433-11528$04.50/0

large tryptic fragment, is also reported to have full synthetic activity, while further shortening of the protein leads to loss of synthetic function (Mellot et al., 1989). A domain equivalent to the C-terminal region of the *E. coli* enzyme is absent in yeast methionyl-tRNA synthetase, which is also less efficient than the *E. coli* enzyme in editing ability *in vivo* (Jakubowski, 1991). In this study, we present *in vivo* evidence for the relevance of a C-terminal portion of *E. coli* MetRS in editing function; further, in our hands, there is also some enhancement of synthetic function by the C-terminal portion of the protein, as well. Our results suggest that the C-terminal region of MetRS allows association of the synthetase with the methionine biosynthetic pathway in such a manner that any excess of endogenous homocysteine that cannot be transmethylated to methionine is channeled to MetRS for editing, as previously proposed (Jakubowski, 1990; Jakubowski & Goldman, 1992).

MATERIALS AND METHODS

(1) Bacterial Strains and Plasmids. Plasmid pGG3 (Ghosh et al., 1990) contains the MetRS gene subcloned into phagemid vector pTZ18R. This produces a truncated form of MetRS, denoted MRS547, believed to be equivalent to the biologically active large tryptic fragment (Baker et al., 1982). MRS581*, described in this report, is a revertant of site-directed mutant R233Q (derived from MRS547; Ghosh et al., 1991a); both pGG3 and R233Q plasmids were obtained from the late L. Schulman (Albert Einstein College of Medicine, New York). DNA sequencing determined that the gene encoding MRS581* was identical to the gene for MetRS cloned by Baker et al. (1982; see also Results). The full-length wild-type gene carried by pBSmetG, encoding MRS676 (Dardel et al., 1984), was obtained from S. Blanquet (Ecole Polytechnique, France). The *metG*$^+$*-1* strain (Jakubowski, 1990) is a spontaneous revertant of a *metG*$^-$ strain, CS50 (F$^-$, *thr-1*, *leuB6*, Δ(*gpt-proA*)*62*, *hisC3*, *metG146*, *ara-14*, *lacY1*, *supE44*, *galK2*, λ$^-$, *rac*$^-$, *rfbD1*, *rpsL25*, *kdgK51*, *xyl-5*, *mtl-1*, *thi-1*), obtained from the *E. coli* Genetic Stock Center (Yale University). This *metG*$^-$ strain, a K_m mutant of MetRS, is auxotrophic for methionine. Because there is no synthesis of homocysteine thiolactone in *metG*$^+$*-1* (Jakubowski, 1990), this strain is a useful host for studying editing by plasmid-encoded MetRS proteins. *E. coli* K-12 *metE* was from N. Brot (Roche Institute of Molecular Biology, Nutley, NJ).

(2) DNA Sequencing and Molecular Cloning Procedures. These were as described in the supplier's instructions or in Sambrook et al. (1989). Enzymes and primers were purchased from Sigma or New England Biolabs.

(3) Protein Purification. MRS581*, MRS547, and full-length MRS676 proteins were purified by a somewhat modified procedure for MetRS purification as described by Bruton et al. (1975), Mellot et al. (1989), and Ghosh et al. (1990). Briefly, overnight cultures (500 mL in LB containing 50 μg/mL of ampicillin) were harvested by centrifugation, resuspended in 10 mM potassium phosphate (pH 7.6), 1 mM β-mercaptoethanol, and 10% glycerol, and subjected to ultrasonic disintegration. Nucleic acids were removed by precipitation of the crude cell extract with 3% streptomycin sulfate. Proteins precipitated in a 35–70% ammonium sulfate fraction were redissolved in the same buffer and loaded on a Sephacryl S-200 (Pharmacia) column (2.5 cm × 80 cm). The pooled fractions containing MetRS were further purified by a Q-Sepharose (Pharmacia) column (1 cm × 6 cm). Purification was monitored by tRNA aminoacylation assay and A_{280} measurements. The enzymes were >95% pure as determined by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [as described in Ausubel et al. (1987)]. Enzyme concentrations were determined by active site titration (Jakubowski & Fersht, 1981) and were in agreement with protein concentrations calculated from absorption at 280 nm (Mellot et al., 1989) or Bradford assay (Ausubel et al., 1987).

(4) Enzyme Activity Assays. All *in vitro* reactions were carried out at 37 °C in a standard buffer (50 mM HEPES, pH 8.0, 10 mM $MgCl_2$, 0.2 mM EDTA, and 1 mM dithiothreitol) unless otherwise indicated.

(A) Synthetic Function. $tRNA^{Met}$ aminoacylation was assayed in a standard mixture containing 10 mg/mL bulk *E. coli* tRNA (Schwarz/Mann), 1 mM ATP, 20 μM L-[*methyl*-^{3}H]methionine (50 μCi/mL, 2.5 Ci/mmol), and standard buffer. Enzyme concentrations were 0.5–2.5 nM. Charged tRNA was determined by trichloroacetic acid (TCA)-precipitable ^{3}H counts.

(B) Editing Function. This was monitored *in vivo* and *in vitro*:

(i) In Vivo Editing Function Assays. (a) Cyclization of Endogenous Homocysteine to the Thiolactone in [^{35}S]Sulfate-Labeled Cultures. Homocysteine thiolactone was separated from other sulfur-containing compounds present in [^{35}S]-sulfate-labeled cell extracts by two-dimensional thin-layer chromatography and quantitated in the scintillation counter (Jakubowski, 1990; see also Figure 1 legend).

(b) Cyclization of Exogenous Homocysteine to the Thiolactone. Bacterial cultures (cell density of 10^8 cells/mL) were incubated with 10 mM D,L-homocysteine (Sigma) in M9 medium also containing auxotrophic requirements and 50 μg/mL ampicillin. Quantitation of homocysteine thiolactone produced in the bacterial cultures was by A_{240} measurements. For homocysteine thiolactone determinations, 0.6 mL of culture was taken and clarified by centrifugation, and the spectrum from 220 to 300 nm of the cell-free medium was recorded against fresh medium as a reference using a Hewlett Packard diode array spectrophotometer, Model 8451A. The spectra obtained were identical to the spectrum of authentic Hcy-thiolactone, with a maximum at 240 nm. The standard calibration curve was prepared by A_{240} measurements of known quantities of the thiolactone in M9 medium. For determination of expression levels of plasmid-encoded MetRS proteins, cells from 0.17 mL of each culture were collected, boiled in SDS gel loading buffer, and subjected to SDS–PAGE on 10% gels (Ausubel et al., 1987). In each case, the MetRS protein was clearly seen as a prominent band that migrated exactly at a position corresponding to the purified MetRS form of that molecular weight.

(ii) In Vitro Editing Function Assays. (a) ATP Pyrophosphatase Activity of MetRS. This measures excess hydrolysis of ATP to AMP as noncognate amino acid is activated in the synthetic reaction and destroyed in the editing reaction (Jakubowski & Fersht, 1981). Reaction mixtures contained the following in standard buffer: 0.2 mM [^{3}H]-ATP (0.1 mCi/mL, 5 Ci/mmol), 0.4–100 mM homocysteine, 0.5 unit/mL yeast inorganic pyrophosphatase (Sigma), and 250 nM MetRS. AMP was separated from ATP and ADP by chromatography on PEI [poly(ethylene imine)]–cellulose (Sigma). Yeast inorganic pyrophosphatase was included to hydrolyze pyrophosphate, which might otherwise inhibit the reaction.

(b) Homocysteine Thiolactone Production Using Purified Enzymes. Reaction mixtures contained 25 mM ATP, 35 mM $MgCl_2$, 25 mM homocysteine, 50 mM HEPES, pH 8.0 buffer,

0.5 unit/mL yeast inorganic pyrophosphatase, and 10 μM purified MetRS. In experiments in which, instead of purified MetRS, crude extracts from MetRS overproducing strains were used, 1 mM of each of leucine, valine, and isoleucine were also added to saturate the respective aminoacyl-tRNA synthetases which could also edit homocysteine *in vitro* (Jakubowski & Fersht, 1981; Englisch et al., 1986, 1990). At different time points, an aliquot of reaction mixture was subjected to thin-layer chromatography (TLC) separation. Homocysteine thiolactone, visualized under UV light, was extracted from TLC plates in 1 mL of water overnight and quantitated by A_{240} measurements. The standard calibration curve of homocysteine thiolactone was prepared by A_{240} measurements of known quantities of the thiolactone subjected to the same separation procedure. The background of A_{240} absorbing material from TLC plates was negligible.

(c) Enzymatic Deacylation of Charged Met-tRNA^{fMet}. This assay is based on the discovery that methionine charged to tRNA^{fMet} is also cyclized to *S*-methylhomocysteine thiolactone at a low rate, in a reaction similar to synthesis of homocysteine thiolactone (Jakubowski, 1993a,b). Activity was determined by measuring the decrease of [^{3}H]Met-tRNA^{fMet} or the appearance of [^{3}H]-*S*-methylhomocysteine thiolactone. [^{3}H]-Met-tRNA^{fMet} was prepared by charging tRNA^{fMet} (1400 pmol/A_{260}; Sigma) with [^{3}H]methionine and purified as described previously (Jakubowski, 1993b). Deacylation reactions were carried out in mixtures containing 1 μM [^{3}H]Met-tRNA^{fMet} and 5 μM MetRS in standard buffer. The loss of charged Met-tRNA was determined by TCA precipitation. We also confirmed that *S*-methylhomocysteine thiolactone was produced, as expected.

(5) Cell Growth and ^{35}S-Labeling Conditions. These were as described in Jakubowski (1990). The auxotrophic requirements for *metG*$^+$*-1* were satisfied by 40 μg/mL each of threonine, leucine, proline, and histidine.

(6) Quantitation of Expression Level of MetRS from Gels. This was as described in Sipley et al. (1991). Briefly, after SDS–PAGE separation, protein bands corresponding to the different forms of MetRS were cut out from gels and rehydrated with 100 mL of H_2O for 30 min, followed by addition of 10 mL of Fluorosol, a one-step scintillator/protein digestant (National Diagnostics). Samples were kept shaking for 2–3 days at 37 °C to elute all the radioactivity from the gel slices prior to scintillation counting.

RESULTS

(1) Isolation of an Altered Form of MetRS with High Editing Activity. In studies of site-directed mutants of MetRS defective in charging, we encountered revertants which also showed exceptionally high levels of Hcy-thiolactone. For example, R233Q (Ghosh et al., 1991a) produces a MetRS protein which is inactive in both synthetic and editing function (Kim et al., 1993). On a minimal plate (M9 plus auxotrophic requirements and ampicillin, but without methionine), an interesting revertant was obtained from strain *metG*$^-$ bearing R233Q. A plasmid isolated from the revertant conferred the Met$^+$ phenotype upon transformation of fresh *metG*$^-$ cells. The *metG*$^-$ strain containing this revertant plasmid, later named MRS581*, not only grew on minimal plates but also showed high *in vivo* editing function.

Radioactively labeled cultures bearing these MetRS overproducing constructs were subjected to SDS–polyacrylamide gel electrophoresis. MRS581* was found to be slightly heavier than MRS547; the relative mobility was about 66 kDa for MRS581* versus 64 kDa for MRS547 and R233Q. The subunit of full-length wild-type (MRS676) migrated, as expected, at 76 kDa (Figure 1D).

This interesting revertant plasmid was subjected to DNA sequencing to determine the reason for the higher molecular weight, as well as the change(s) associated with higher editing function. The complete sequence revealed two mutated sites: the first mutation occurred at the nucleotide triplet coding for Q (Gln) 233, CAG, which was mutated back to CGC coding for R (Arg) 233; the second mutation was at the stop codon TAA. This stop codon was originally engineered to replace the amino acid at position 548 in the truncated parent (MRS547) and is preserved in the R233Q plasmid (Ghosh et al., 1990). In MRS581*, this TAA has mutated to GAA, which codes for Glu and which happens to be the original amino acid at that position in native MetRS. Thus, MRS581* encodes a longer protein, which goes through the rest of the *metG* sequence in the pGG3 construct (52 nucleotides for $17^1/_3$ codons before fusion to the vector sequence), on into part of the pBR322 sequence linked immediately downstream of the *metG* gene. Presumably, translation stops at the first in frame stop codon (another 50 nucleotides downstream before TAG to give $16^2/_3$ additional codons). The fused codon encodes the same amino acid as found in native MetRS at that location. Thus, MRS581* gives rise to a chimeric protein with 16 amino acids from the vector sequence as a carboxy-terminal tail in addition to 547 + 18 = 565 amino acids of the MetRS sequence (starting from the N-terminus). Compared to MRS547, another 34 amino acids are added. Since the DNA sequence of the gene for MRS581* is exactly the same as that of the MetRS gene in pBR322/EcoMTS-p8 originally reported by Baker et al. (1982) when the *metG* sequence was first cloned, we are not showing the sequence here. Because of two separated sequence changes (Q back to original R, and TAA to GAA), the gene for MRS581* may have arisen from homologous recombination between plasmid R233Q and the chromosomal copy of the mutant gene for MetRS in the *metG*$^-$ strain.

(2) Comparison of in Vivo Editing Function of MetRS Forms with Different C-Terminal Sequences. The editing function of MetRS can be studied *in vivo* by determination of homocysteine thiolactone levels in cultures of cells. The extent of homocysteine thiolactone produced reflects the editing activity of the enzyme and depends on intracellular concentrations of homocysteine and methionine, as well as on the enzyme levels. To determine levels of homocysteine thiolactone, cultures producing the three different forms of the enzyme were grown in the presence of [^{35}S]sulfate. Sulfur-containing compounds were extracted and resolved by two-dimensional thin-layer chromatography (2D-TLC), and radiolabeled homocysteine thiolactone was quantitated, as described (Jakubowski, 1990). Panel A in Figure 1 shows the amounts of the thiolactone produced *in vivo* by MRS581*, MRS547, and MRS676 as a function of time. Unexpectedly, bacterial cells harboring different MetRS plasmids produced different amounts of Hcy-thiolactone. For example, after 4 h of labeling with [^{35}S]sulfate, MRS581* and MRS676 produced 50- and 15-fold more thiolactone, respectively, than MRS547 (Figure 1A). To exclude that the differences in the thiolactone levels were caused by failure of some cultures to efficiently metabolize [^{35}S]sulfate and incorporate ^{35}S into protein, and/or inefficient expression of plasmid-encoded MetRS, we determined total ^{35}S incorporation into protein (Figure 1B) and also performed SDS–PAGE to measure MetRS expression levels (Figure 1D). As shown in Figure 1B, cells harboring different MetRS plasmids all efficiently

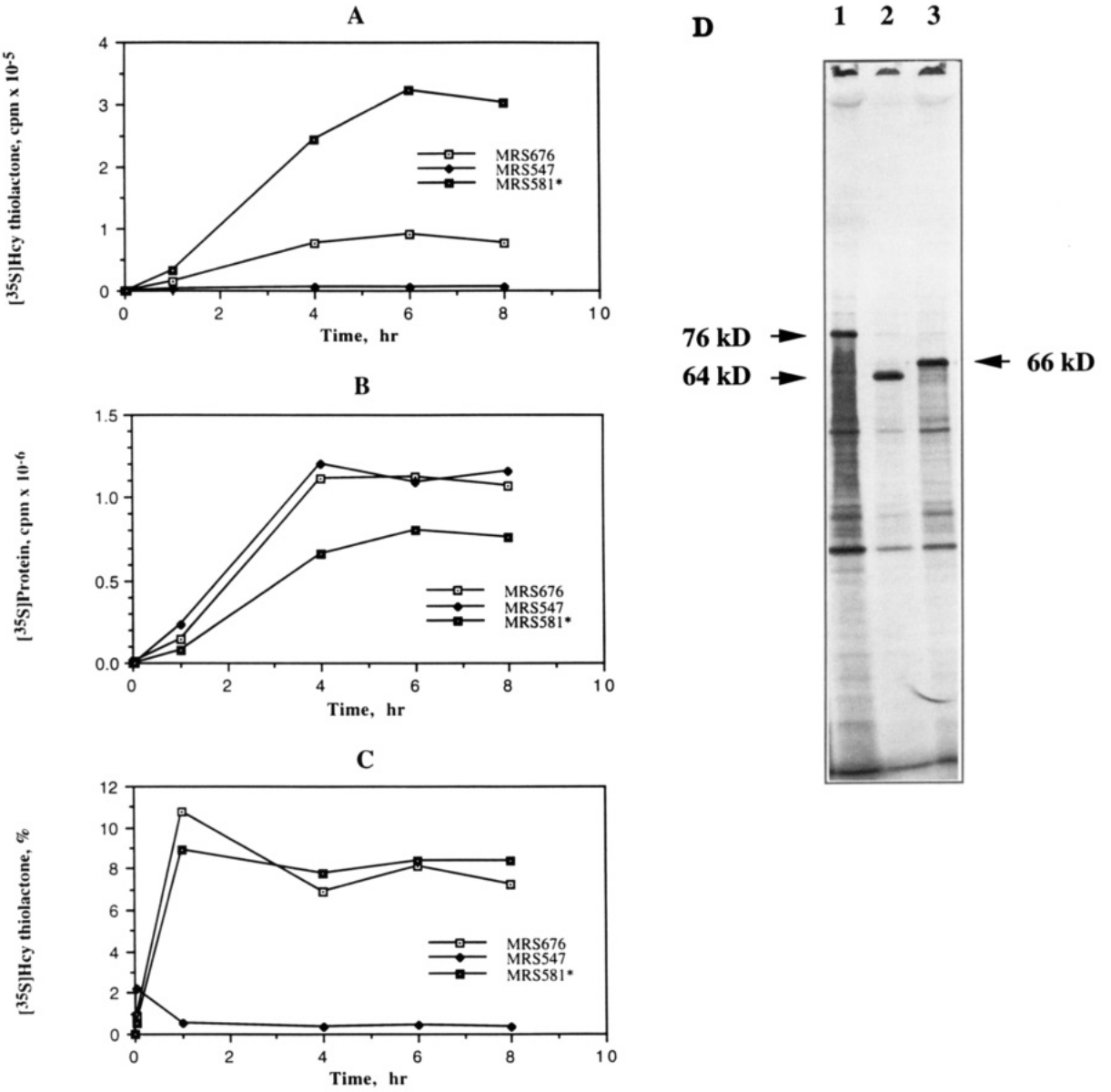

FIGURE 1: Editing of endogenous homocysteine by MetRS proteins in *E. coli*. Editing-deficient *E. coli metG*$^+$*-1* cells (10^8 cells/mL) harboring different MetRS plasmids (as indicated in the figure) were labeled at 37 °C with 10 μM [^{35}S]Na_2SO_4 (0.1 mCi/mL) in sulfur-free M9 plus auxotrophic requirements and ampicillin. At indicated time points, aliquots of 5 μL of each labeled culture were extracted with 1 M formic acid followed by 2D-TLC to determine Hcy-thiolactone. Another set of 5 μL aliquots was precipitated with 10% TCA to determine total protein. The thiolactone was measured as ^{35}S counts comigrating with authentic Hcy-thiolactone on two-dimensional TLC of [^{35}S]sulfate-labeled *E. coli* cultures. Total protein was measured as trichloroacetic acid-precipitable ^{35}S counts. Panels A and B show time courses of Hcy-thiolactone formation and protein synthesis, respectively. A third set of 5 μL aliquots was subjected to SDS–PAGE on 10% polyacrylamide gels [as described in Ausubel et al. (1987)] followed by autoradiography using Kodak XAR-5 X-ray film. As an example, an autoradiogram of the gel obtained after 6 h of labeling is shown in panel D. Cultures of *metG*$^+$*-1* cells harbored pBSmetG with full-length MRS676 (lane 1), pGG3 with MRS547 (lane 2), and pGG3 with MRS581* (lane 3). Also shown are the relative molecular masses in kilodaltons (kDa). MetRS bands were cut out from the gel, dissolved in fluorosol (Sipley et al., 1991), and counted in a scintillation counter. The counts were taken as the expression level of MetRS proteins in the corresponding cultures. Homocysteine thiolactone levels were normalized to relative MetRS expression levels, which were 0.2, 0.3, and 1 for MRS676, MRS547, and MRS581*, respectively. Similar expression levels were obtained by aminoacylation activity assays of crude extracts from the respective cultures (see Table 3). Panel C shows normalized homocysteine thiolactone levels, expressed as % of total ^{35}S incorporated into protein at indicated time points for indicated MetRS forms. The editing activity of MRS547 (panel A) was 1600 cpm/h, and the background of the host strain was 400 cpm/h.

incorporated ^{35}S into protein. Since there were some differences in the expression level of MetRS from different plasmids (Figure 1D), we normalized the thiolactone levels to constant MetRS expression, as described in the legend to Figure 1. To relate the synthesis of Hcy-thiolactone to the overall thioamino acid metabolism of bacterial cultures, ratios of normalized [^{35}S]Hcy-thiolactone to total incorporation of ^{35}S into protein were calculated. These ratios, constant with time for a given MetRS plasmid (Figure 1C), indicate that MRS581* and full-length MRS676 were roughly equivalent in editing function, and both were about 20-fold more active in editing than the truncated MRS547. Although the scales in Figure 1A,C compress the editing activity of MRS547 virtually to base line, the editing activity of MRS547 is significant and well above the background of the editing-deficient host strain (see legend to Figure 1).

Although unlikely, it was possible that the lower levels of homocysteine thiolactone in the culture producing MRS547 were a consequence of grossly different concentrations of homocysteine and/or methionine. Therefore, we measured

Table 1: Intracellular Concentrations of Sulfur-Containing Amino Acids[a]

strains *metG*+-1 with	amino acid concentrations (pmol/10^8 cells)		
	methionine	homocysteine	Hcy-thiolactone
MRS676	22.5	38.1	4.0
MRS547	22.2	24.3	0.5

[a] Cells (*metG*+-*1*) with different forms of MetRS were grown overnight in M9 medium plus leucine, proline, histidine, and threonine (40 μg/mL each), to satisfy auxotrophic requirements, and ampicillin (50 μg/mL). Cells were collected and washed with sulfur-free M9 three times and then diluted in the labeling medium which contains sulfur-free M9, auxotrophic requirements, ampicillin, and 0.1 mCi/mL [^{35}S]Na_2SO_4 (carrier free), 0.1 mM Na_2SO_4. The labeled cultures were grown to about 4×10^8 cells/mL and collected by filtering through a 0.45-μm nitrocellulose filter (Millipore). Cells were extracted with 1 M formic acid, and clarified supernatants were lyophilized. The samples were resuspended in water and subjected to 2D-TLC. Spots corresponding to methionine, homocysteine, or Hcy-thiolactone were cut out and counted in the scintillation counter.

the intracellular sulfur amino acids concentrations in cells containing MRS676 and MRS547 (Table 1). No difference was found in the concentration of methionine in the two strains. While homocysteine levels were about a third lower in the strain carrying MRS547, this difference is far too small to account for the 20-fold greater editing ability of full-length MRS676 seen in Figure 1C. In line with the greater editing ability, Hcy-thiolactone (most of which is secreted into the medium) levels are also much higher in the strain carrying MRS676 compared to MRS547. Thus, the respective editing abilities observed appear to be intrinsic properties of the two MetRS forms *in vivo*.

Homocysteine thiolactone measured in experiments with [^{35}S]sulfate originated from *endogenous* homocysteine produced in the methionine biosynthetic pathway. To test if different forms of MetRS would also similarly edit *exogenous* homocysteine supplied to the medium, bacterial cells harboring different MetRS plasmids were incubated in M9 medium containing 10 mM homocysteine, and the levels of homocysteine thiolactone were determined by A_{240} measurements. Homocysteine thiolactone absorbs UV light with a maximum at 240 nm. Absorption spectra of the media from cultures of MetRS plasmid-harboring cells incubated with homocysteine were indistinguishable from the UV spectrum of an original sample of Hcy-thiolactone (data not shown). Time courses of Hcy-thiolactone production *in vivo* determined from A_{240} measurements for three forms of MetRS are shown in Figure 2A. Under these conditions, the differences in editing of exogenous Hcy between the three MetRS forms were much less pronounced than those in the experiment of Figure 1, which shows editing of endogenous Hcy. The 2- to 3-fold higher homocysteine thiolactone levels in MRS581* and MRS676 cultures compared to MRS547 can be accounted for by similar differences in the expression levels of corresponding MetRS proteins (Figure 2B).

(3) Comparison of in Vitro Editing Function of MetRS Forms with Different C-Terminal Sequences. The three forms of methionyl-tRNA synthetase (MRS676, MRS547, and MRS581*) were purified by routine procedures as described in the Materials and Methods. The migration of the purified proteins on SDS–PAGE was consistent with molecular weights predicted from the lengths of the genes (data not shown).

Full-length methionyl-tRNA synthetase (MRS676) is a dimer of two identical subunits of 676 amino acids. The C-terminal region of this native enzyme has been shown to be responsible for dimerization (Koch & Bruton, 1974). The truncated form MRS547 is monomeric, without the C-terminal

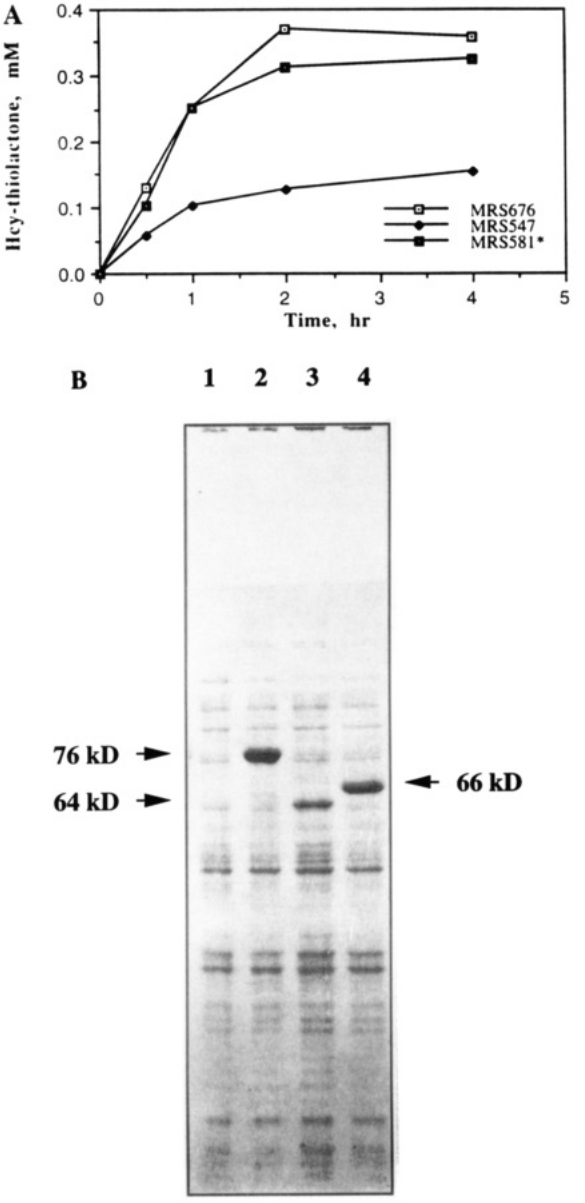

FIGURE 2: Editing of exogenous homocysteine by MetRS proteins in *E. coli*. Homocysteine thiolactone produced in bacterial cultures (10^8 cells/mL) of cells harboring different MetRS plasmids was quantitated by A_{240} measurements as described in Materials and Methods. M9 medium was supplemented with 10 mM D,L-homocysteine in addition to ampicillin and auxotrophic requirements of *metG*+-*1* cells. No detectable Hcy-thiolactone was formed in the absence of exogenous homocysteine or in the absence of a MetRS plasmid. Panel A shows time courses of Hcy-thiolactone formation in cultures of cells harboring indicated MetRS plasmids. To determine expression levels of plasmid-encoded MetRS proteins, cells from 0.17 mL of each culture (equivalent to 1.4×10^7 cells) were also subjected to SDS–PAGE. A photograph of a Coomassie Brilliant Blue-stained gel is shown in panel B. Protein patterns were from cells harboring plasmids encoding MRS676 (lane 2), MRS547 (lane 3), and MRS581* (lane 4). As a control, cells harboring plasmid pTZ18R were also analyzed (lane 1).

129 amino acids of native MetRS. Because the excision of only 5 residues from the natural C-terminus of MetRS is enough to decrease the association constant between shortened protomers by 3 orders of magnitude (Meinnel et al., 1990), MRS581* is also expected to be a monomer. We have confirmed this by nondenaturing gel electrophoresis and gel filtration. MRS581* migrated as a single band on nondenaturing polyacrylamide gels at about the same position as MRS547; MRS676, as expected, migrated considerably

Table 2: Editing Activity of Three Forms of Purified MetRS Proteins

	ATP pyrophosphatase activity[a]					
	homocysteine			methionine inhibition[b]	Hcy-thiolactone production[c]	deacylation of Met-tRNA[c]
MetRS	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	I_{50} (μM)	v/E (s^{-1})	k_{cat} (s^{-1})
MRS676	1.4	1.7	0.82	5.0	0.61	
MRS547	1.7	2.6	0.65	4.0	0.51	0.07[d]
MRS581*	0.8	1.7	0.47	5.0	0.41	0.07

[a] The ATP pyrophosphatase activity of MetRS was measured as described in Materials and Methods. Kinetic constants were calculated using Eadie–Hofstee plots. [b] I_{50} is the methionine concentration at which 50% inhibition of homocysteine-dependent ATP pyrophosphatase activity was observed. The concentrations of methionine varied from 0 to 40 μM at constant 50 mM homocysteine. [c] These assays were performed as described in Materials and Methods. [d] Identical to the previously published value (Jakubowski, 1993b).

slower, consistent with it being a dimer (data not shown). On gel filtration MRS581* eluted roughly at the same position as MRS547, with a single major activity peak and A_{280} absorption peak.

Tests of editing function *in vitro* were performed on these purified proteins. Kinetic indices of ATP pyrophosphatase activities of MRS676, MRS547, and MRS581* in the presence of homocysteine are shown in Table 2. The differences in catalytic efficiencies (k_{cat}/K_m) were within a 1.2-fold range per subunit. Editing function was further studied by direct measurements of homocysteine thiolactone production. As shown in Table 2, the three forms of MetRS exhibited similar activity. At 25 mM homocysteine, each enzyme produced from 0.4 to 0.6 mol of Hcy-thiolactone/mol of enzyme·s). Enzymatic deacylation of Met-tRNA in the absence of AMP (Jakubowski, 1993a) was also studied. This reaction, which does not involve a synthetic step, is another specific way to measure editing function. The data indicate no significant difference between MRS581* and truncated form MRS547 (Table 2); the half-life of the reaction was 10 s with each enzyme. In other experiments in which MetRS was not saturating, full-length MRS676 also showed no difference from the other forms in this assay (data not shown).

We have also measured the effect of methionine on homocysteine-dependent ATP pyrophosphatase activity of the three forms of MetRS. As expected, methionine inhibited ATP pyrophosphatase activity, and the I_{50} values (Table 2) for methionine were similar for all three enzymes.

Crude extracts from *metG*$^+$*-1* cells were also tested for a possible effect on editing function of these purified enzymes, in both *in vitro* ATP pyrophosphatase activity and homocysteine thiolactone production. As above, no significant differences between the three forms of the enzyme were observed in these *in vitro* assays (data not shown).

Thus, under the conditions we examined, similar editing activities of different forms of the enzyme *in vitro* cannot account for observed differences in editing of endogenous (formed in the methionine biosynthetic pathway) homocysteine *in vivo* (Figure 1). However, the *in vitro* editing results parallel the lack of influence of the C-terminal region of MetRS on editing activity of the enzyme observed during *in vivo* editing of exogenous homocysteine (Figure 2).

(4) Comparison of in Vitro Synthetic Function of MetRS Forms with Different C-Terminal Sequences. The synthetic function of the purified enzyme from MRS581* was studied by standard tRNA aminoacylation assays and compared to MRS547 and full-length MRS676. Table 3 shows that specific synthetic activity per subunit of full-length MRS676 is about 2.5-fold higher than both MRS581* and MRS547. The latter two had no significant difference in synthetic activity. These results are in contrast with published measurements of similar synthetic activity of the large tryptic fragment of MetRS (Cassio & Waller, 1971; Lawrence et al., 1973), MRS547 (Mellot et al., 1989), and full-length MRS676.

Table 3: Synthetic Activity of Three Forms of MetRS *in Vitro*[a]

MetRS forms	*in vitro* tRNA aminoacylation v/E (s^{-1})	rel sp act. of crude extracts[b]
MRS676	3.0	98
MRS547	0.7	11
MRS581*	0.6	23
metG$^+$*-1* with pTZ18R		1

[a] The experimental conditions are as described in the Materials and Methods. [b] Fresh crude extracts were made from 10 mL cultures of 4×10^8 cells/mL. Cells were collected by centrifugation, washed with standard enzyme buffer (10 mM potassium phosphate, pH 7.6, 1 mM β-mercaptoethanol, 10% glycerol), and resuspended in 0.4 mL of the same buffer, followed by sonication at 70 W, 15–30 s/stroke, 3–4 strokes. The same volume of crude extracts from the different cultures was used. The aminoacylation activity was determined by TCA-precipitable ^3H counts per minute. Counts per minute ranged from 120 to 11 650 for the same amount of crude extract.

Cultures of cells bearing the respective plasmids were also extracted and synthetic capabilities of the three different enzyme forms compared in an aminoacylation assay. The full-length enzyme was the most active, at least 4-fold higher than MRS581*, which in turn was about 2-fold higher than truncated form MRS547 (third column of Table 3). These results with crude extracts are in reasonable agreement with synthetic capability as measured with purified enzymes (second column of Table 3).

DISCUSSION

E. coli MetRS is a dimer of two 676 amino acid subunits (Koch & Bruton, 1974; Dardel et al., 1984). Deletion of the C-terminal 129 amino acids produces a monomer of 547 amino acids which is biologically active (Mellot et al., 1989; Ghosh et al., 1990). Sequence alignments of *E. coli* and yeast MetRS indicate that the C-terminal region responsible for dimerization of *E. coli* MetRS is absent in yeast MetRS (Walter er al., 1983); yeast MetRS is in fact a monomer (Fasiolo et al., 1985). It is not known why MetRS exists as a dimer in *E. coli*, while it can function perfectly well as a monomer both in yeast and, in modified form, in *E. coli*. One possible reason for different forms of MetRS utilized by these two microorganisms is suggested by different needs for editing between the *E. coli* and yeast MetRS enzymes. Since, in *E. coli*, homocysteine concentrations are about 10-fold higher than in yeast, *E. coli* MetRS has evolved to efficiently edit homocysteine (Jakubowski, 1990). On the other hand, because of different organization of the methionine biosynthetic pathway in yeast, homocysteine concentrations in yeast cells are low; when forced to edit, for example, by overproduction of homocysteine, yeast MetRS is less efficient *in vivo* than the *E. coli* enzyme (Jakubowski, 1991). Thus, a function of the C-terminal region of *E. coli* MetRS may be to enhance editing

activity, perhaps by directing MetRS to a point in the methionine biosynthetic pathway where homocysteine is produced or transmethylated. We report here that the truncation of *E. coli* MetRS does indeed lead to a significant deficiency of editing function *in vivo* when editing of endogenous (that is, made in the methionine biosynthetic pathway) homocysteine is measured. However, the truncation of *E. coli* MetRS does not affect *in vivo* editing of exogenous (that is, supplied in the medium) homocysteine, nor does it affect editing function of purified proteins *in vitro*. Further, in our hands, the truncation also led to somewhat diminished synthetic function, as well.

In addition to MRS676 and MRS547, another interesting form of methionyl-tRNA synthetase, MRS581*, was isolated and studied. This form contains the first 565 amino acids of MetRS fused to an additional 16 amino acids encoded by vector pBR322 at the C-terminus and is equivalent to the original recombinant clone of MetRS reported by Baker et al. (1982). Thus, the three forms of MetRS studied here differ in their C-terminal regions: MRS676 has 129 additional residues compared to MRS547, and MRS581* has an additional 34 residues compared to MRS547. *In vivo* detection of editing product homocysteine thiolactone showed that the proofreading activity of MRS581* against endogenous homocysteine was much higher (20-fold, after normalization to MetRS expression levels) than that of MRS547. Full-length MRS676 exhibited similar proofreading activity with endogenous homocysteine *in vivo* as MRS581*. Although truncated MRS547 appears to be deficient in editing of endogenous homocysteine *in vivo*, no significant difference in editing of exogenous homocysteine, nor in editing function *in vitro*, between the three forms of MetRS was observed. Editing function *in vitro* was tested in three different editing assays: ATP pyrophosphatase activity, homocysteine thiolactone formation, and enzymatic deacylation of Met-tRNA. Addition of cell extracts from *metG*$^+$*-1* cells did not result in differences between the MetRS forms in editing function in the *in vitro* reactions. Even though there were no significant differences in editing activities of the three forms of MetRS *in vitro*, synthetic activity of MRS676 was about 2.5-fold higher per subunit than that of both MRS547 and MRS581*. For unknown reasons, this differs somewhat from previously published work on synthetic function (Cassio & Waller, 1971; Lawrence et al., 1973; Mellot et al., 1989), which found no significant differences between truncated and full length.

There is a discrepancy between *in vivo* editing of endogenous (but not exogenous) homocysteine and *in vitro* editing function. We have excluded that our *in vitro* measurements of homocysteine editing by ATP pyrophosphatase activity can be differentially affected by methionine for each of the enzymes. We have also excluded some simple possible explanations for the differences between the editing of endogenous homocysteine by the enzymes *in vivo*. For example, the differences are not due to different levels of expression of MetRS proteins, regardless of whether the expression levels are determined by a chemical measurement (by counting radioactivity in ^{35}S-labeled bands on SDS–PAGE gels; Figure 1D) or by an enzymatic assay of activity (Table 3). The differences between the editing of endogenous homocysteine *in vivo* are also not due to different levels of methionine and homocysteine in the respective cell cultures. In this regard, similar discrepancies between *in vitro* and *in vivo* editing function were observed in the cases of IleRS, ValRS, and LeuRS, all of which edited homocysteine to Hcy-thiolactone *in vitro* (Jakubowski & Fersht, 1981; Englisch et al., 1986, 1990) but not *in vivo* (Jakubowski, 1990). These other three aminoacyl-tRNA synthetases are apparently excluded from interaction with homocysteine in the cell. A possible reason for this could be that homocysteine might be compartmentalized in the methionine biosynthetic pathway. Such compartmentalization is referred to as "channeling", and there is in fact some evidence in eukaryotic cells for channeling of metabolites in protein synthesis [e.g., Negrutskii and Deutscher (1991)]; however, this has not yet been demonstrated in bacterial protein synthesis.

Channeling of endogenous homocysteine would imply that MetRS in the cell participates in an enzyme complex in the biosynthetic pathway of methionine. Our observations that endogenous homocysteine is edited by MRS547 much less efficiently than by MRS581* or MRS676 but that all three enzyme forms edit exogenous homocysteine with similar efficiencies are consistent with this notion. Apparently, all three enzyme forms are able to efficiently edit homocysteine when it is made available, as further confirmed by our *in vitro* measurements with purified proteins. One plausible candidate for interaction with MetRS is the *metE* gene product, which is responsible for conversion of homocysteine to methionine. From the pathway, a *metE*$^-$ strain should accumulate homocysteine, but the strain does not, accumulating homocysteine thiolactone instead (Jakubowski, 1990). This suggests direct transfer of excess homocysteine from the metE protein to MetRS. The inability of MRS547 to edit at wild-type levels *in vivo* could thus be due to an inability to interact with the metE protein. We attempted to test this by introducing the three different forms of MetRS into a *metE*$^-$ mutant strain to examine editing function. Introduction of MetRS plasmids into this strain did not result in significant increases in editing of endogenous homocysteine, most likely because of poor expression of plasmid-encoded MetRS (data not shown), as well as severalfold higher levels of chromosomally-encoded MetRS in this *metE*$^-$ strain (Jakubowski, 1990).

The data show that the tryptic fragment-like form MRS547 has some deficiencies compared to wild-type MetRS and that the C-terminal region plays other roles in enzyme function in addition to dimerization. A major functional role of the C-terminal region seems to be to allow association of MetRS with a component of the methionine biosynthetic pathway which assures that any excess of homocysteine coming from the pathway is edited. From the *in vivo* comparison of three forms of MetRS in editing function, the C-terminal region of MetRS, missing in MRS547, is shown to be important. The editing function of the 129 carboxy-terminal amino acids in full-length MRS676 can be fulfilled by the 34 C-terminal amino acids of MRS581* in *E. coli* cells. However, we do not know if only amino acids 548–565 of MetRS, or the 16 amino acids from the fused vector sequence, or both, are sufficient to supply this function. Since MRS581* doesn't appear to dimerize, dimerization seems not to be required for full editing function *in vivo*. However, dimerization may be required for full synthetic function, as our *in vitro* measurements suggest.

REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K. (1987) *Current Protocols in Molecular Biology*, John Wiley & Sons, New York.

Baker, D. G., Ebel, J., Jakes, R., & Bruton, C. J. (1982) *Eur. J. Biochem. 127*, 449–457.

Brunie, S., Zelwer, C., & Risler, J. L. (1990) *J. Mol. Biol. 216*, 411–424.

Bruton, C. J., Jakes, R., & Atkinson, A. (1975) *Eur. J. Biochem. 59*, 327–333.
Cassio, D., & Waller, J.-P. (1971) *Eur. J. Biochem. 20*, 283–300.
Dardel, F., Fayat, G., & Blanquet, S. (1984) *J. Bacteriol. 160*, 1115–1122.
Englisch, S., Englisch, U., von der Haar, F., & Cramer, F. (1986) *Nucleic Acids Res. 14*, 7529–7539.
Englisch-Peters, S., von der Haar, F., & Cramer, F. (1990) *Biochemistry 29*, 7953–7958.
Fasiolo, F., Gibson, B. W., Walter, P., Chatton, B., Biemann, K., & Boulanger, Y. (1985) *J. Biol. Chem. 260*, 15571–15576.
Fourmy, D., Mechulam, Y., Brunie, S., Blanquet, S., & Fayat, G. (1991) *FEBS Lett. 292*, 259–263.
Ghosh, G., Pelka, H., & Schulman, L. H. (1990) *Biochemistry 29*, 2220–2225.
Ghosh, G., Pelka, H., Schulman, L. H., & Brunie, S. (1991a) *Biochemistry 30*, 9569–9575.
Ghosh, G., Brunie, S., & Schulman, L. H. (1991b) *J. Biol. Chem. 266*, 17136–17141.
Jakubowski, H. (1978) *FEBS Lett. 95*, 235–238.
Jakubowski, H. (1980) *Biochemistry 19*, 5071–5078.
Jakubowski, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4504–4508.
Jakubowski, H. (1991) *EMBO J. 10*, 593–598.
Jakubowski, H. (1993a) *FASEB J. 7*, 168–172.
Jakubowski, H. (1993b) *J. Biol. Chem. 268*, 6549–6553.
Jakubowski, H., & Fersht, A. R. (1981) *Nucleic Acids Res. 9*, 3105–3117.
Jakubowski, H., & Goldman, E. (1992) *Microbiol. Rev. 56*, 412–429.
Jakubowski, H., & Goldman, E. (1993) *FEBS Lett 317*, 237–240.
Kim, H. Y., Ghosh, G., Schulman, L. H., Brunie, S., & Jakubowski, H. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11553–11557.
Koch, G. I. E., & Bruton, C. J. (1974) *FEBS Lett. 40*, 180–182.
Lawrence, F., Blanquet, S., Poiret, M., Robert-Gero, M., & Waller, J.-P. (1973) *Eur. J. Biochem. 36*, 234–243.
Mechulam, Y., Dardel, F., Le Corre, D., Blanquet, S., & Fayat, G. (1991) *J. Mol. Biol. 217*, 465–475.
Meinnel, T., Mechulam, Y., Dardel, F., Schmitter, J. M., Hountodji, C., Brunie, S., Dessen, P., Fayat, G., & Blanquet, S. (1990) *Biochimie 72*, 625–635.
Mellot, P., Mechulam, Y., Corre, D., Blanquet, S., & Fayat, G. (1989) *J. Mol. Biol. 208*, 429–443.
Negrutskii, B. S., & Deutscher, M. P. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4991–4995.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sipley, J. , Stassi, D., Dunn, J., & Goldman, E.. (1991) *Gene Expression 1*, 127–136.
Walter, P., Gangloff, J., Bonnet, J., Boulanger, Y., Ebel, J.-P., & Fasiolo, F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2437–2441.
Zelwer, C., Risler, J. I., & Brunie, S. (1982) *J. Mol. Biol. 155*, 63–81.