The Appended C-Domain of Human Methionyl-tRNA Synthetase Has a tRNA-Sequestering Function†

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ABSTRACT: An ancillary RNA-binding domain is appended to the C-terminus of human methionyl-tRNA synthetase. It comprises a helix−turn−helix (HTH) motif related to the repeated units of the linker region of bifunctional glutamyl-prolyl-tRNA synthetase, and a specific C-terminal KGKKKK lysine-rich cluster (LRC). Here we show by gel retardation and tRNA aminoacylation experiments that these two regions are important for tRNA binding. However, the two pieces of this bipartite RNA-binding domain are functionally distinct. Analysis of MetRS mutant enzymes revealed that the HTH motif is more specifically endowed with a tRNA-sequestering activity and confers on MetRS a rate-limiting dissociation of aminoacylated tRNA. Elongation factor EF-1α enhanced the turnover in the aminoacylation reaction. In contrast, the LRC region is most probably involved in accelerating the association step of deacylated tRNA. These two nonredundant RNA-binding motifs strengthen tRNA binding by the synthetase. The native form of MetRS, containing the C-terminal RNA-binding domain, behaves as a processive enzyme; release of the reaction product is not spontaneous, but may be synchronized with the subsequent step of the tRNA cycle through EF-1α-assisted dissociation of Met-tRNA^{Met}. Therefore, the eukaryotic-specific C-domain of human MetRS may have a dual function. It may ensure an efficient capture of tRNA^{Met} under conditions of suboptimal deacylated tRNA concentration prevailing in vivo, and may instigate direct transfer of aminoacylated tRNA from the synthetase to elongation factor EF-1α.

Aminoacyl-tRNA synthetases are RNA binding proteins that catalyze the esterification of amino acids to the 3′-end of tRNAs (*1*). With few exceptions, the proper orientation of the acceptor stem of tRNA in the active site of these enzymes involves conjunction of RNA−protein interactions with the catalytic domain (binding of the acceptor stem of the RNA molecule) and with the anticodon-binding domain of the synthetase. However, a productive positioning of the 3′-terminal adenosine of the RNA molecule can often be achieved with minihelix substrates mimicking the acceptor-TΨC domain of tRNA (*2*). Additional RNA-binding domains are primarily appended to eukaryotic enzymes and may serve as cis- or trans-acting cofactors. In yeast, the RNA-binding protein Arc1p is associated with MetRS and GluRS and functions as a cofactor (*3*, *4*). Plant MetRS possesses a C-terminal polypeptide chain extension related to Arc1p (*5*). A similar RNA-binding domain is also recovered in the mammalian multisynthetase complex (*6*). Its crystal structure revealed an OB-fold conformation characteristic of numerous RNA binding proteins (*7*, *8*). A large, functionally redundant, 228-amino acid N-terminally appended domain is associated with yeast GlnRS (*9*−*11*). In contrast, *Bombyx mori* GlyRS diplays a short 50-amino acid C-terminal RNA-binding domain corresponding to a single helix−turn−helix (HTH) motif (*12*−*14*). Finally, yeast LysRS and human AspRS and AsnRS share with class IIb aminoacyl-tRNA synthetases another type of RNA-binding module (*15*−*17*).

Methionyl-tRNA synthetase (MetRS) displays an especially variable structural organization throughout evolution. As compared with the minimal monomeric enzyme found in the eubacteria *Aquifex aeolicus* (GenBank accession number AE000731), N- and/or C-terminal polypeptide extensions are frequently appended to the core enzyme that associates a catalytic module organized around a Rossmann fold with an α-helical anticodon-binding domain (*18*, *19*). In eukaryotes, a supplementary, nonspecific RNA-binding domain built a C-terminal polypeptide extension associated in cis with plant MetRS (*5*) or is provided in trans to the yeast enzyme through protein−protein interaction (*3*, *4*). In the latter case, yeast MetRS possesses a large N-domain interacting with the N-domain of Arc1p, the RNA-binding cofactor.

In mammals, methionyl-tRNA synthetase is one of the components of a multisynthetase complex containing the nine synthetases specific for amino acids Glu, Pro, Ile, Leu, Met, Gln, Lys, Arg, and Asp, as well as three auxiliary proteins (*20*). Following controlled trypsin digestion of the purified complex, it has been shown that the 103 kDa polypeptide corresponding to MetRS is cleaved into 96, 77, and 68 kDa

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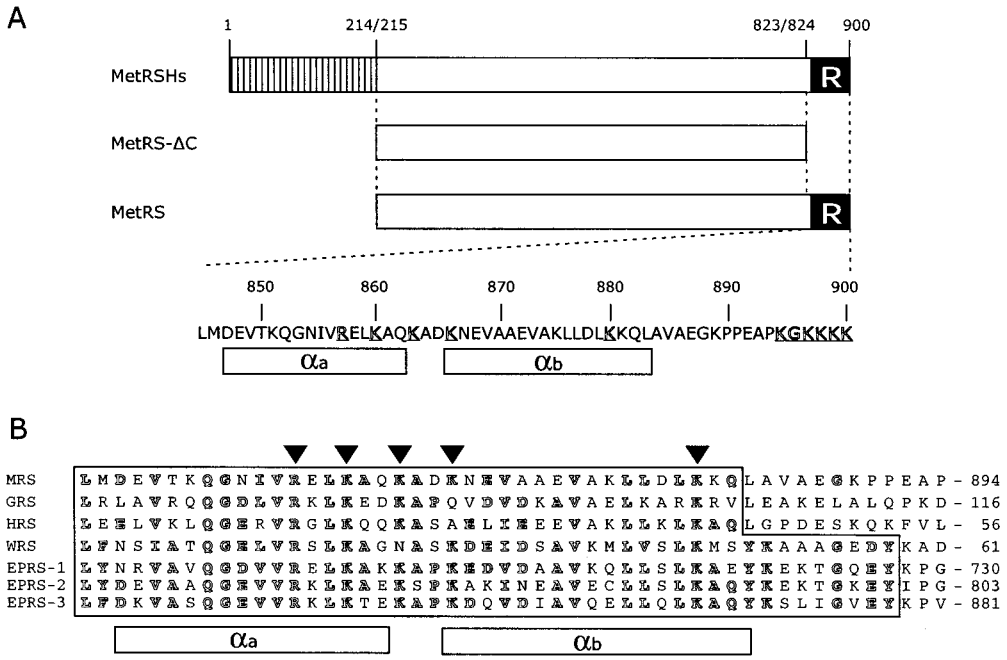

FIGURE 1: Structure of human MetRS. (A) The wild-type enzyme (MetRSHs) comprises a 214-amino acid N-terminal extension (hatched box), a core domain, and a 77-amino acid C-terminal region containing an RNA binding motif (R) initially described as repeated units in the multifunctional GluProRS (*24*). Two derivatives, with (MetRS) or without (MetRS-ΔC) this C-terminal domain, were expressed in *E. coli*. The amino acid sequence from the RNA binding motif is shown; conserved Arg857 and Lys860, -863, -866, and -880 residues that were changed into Ala are underlined. In addition, the four Lys residues from the very C-terminal LRC motif, removed in the MetRS-ΔK mutant, are marked. The two α-helices that build the HTH motif are indicated. (B) Alignment of the single motifs of human MetRS (MRS), GlyRS (GRS), HisRS (HRS), and TrpRS (WRS) and of the three repeated motifs of human GluProRS (EPRS). Residues conserved in at least four sequences are outlined.

polypeptides (*21*). The two 103 and 96 kDa species retained their ability to associate with the complex, in contrast with the 77 and 68 kDa polypeptides which were released as monomeric entities. The analysis of human MetRS cDNA (*22*; M. Lazard and M. Mirande, unpublished data) revealed a tripartite structural organization (see Figure 1A) and allowed us to rationalize the aforementioned biochemical data. A 214-amino acid N-terminal extension and a 77-amino acid C-terminal domain are appended to the main catalytic body of the enzyme, made of 609 amino acids that are 27% identical to the monomeric *Escherichia coli* enzyme. The native polypeptide was converted into a 93 or 77 kDa species by the removal of either the N- or C-terminal module. Deletion of both extensions led to the 68 kDa form. The free monomeric species of 77 and 68 kDa lack the large N-terminal appendage involved in complex assembly and differ by the presence of the C-terminal domain in the 77 kDa protein. The latter domain is a member of a family of general RNA-binding domains (*13*, *23*) initially found in the linker region of multifunctional glutamyl-prolyl-tRNA synthetase (*24*). The RNA-binding capacity of an isolated domain is weak [10 μM (*13*)].

We previously determined that the RNA-binding domain of plant MetRS acts as a cofactor for aminoacylation (*5*). Because plant and human MetRSs possess unrelated RNA-binding domains of different origins, we now analyzed the functional significance of the extra human domain. To unravel the role of this discrete RNA binding module, deletions and point mutations were introduced into the C-domain of human MetRS. This analysis revealed that the supplementary RNA-binding domain of MetRSs from higher eukaryotes contributes an unexpected tRNA-sequestering activity. These results have strong implications for the organization of translation in metazoan cells.

MATERIALS AND METHODS

Protein Overexpression and Purification. The cDNA fragment encoding the catalytic domain of human MetRS (MetRS-ΔC; from Ala215 to Lys823) was obtained by PCR with oligonucleotides MHs01 (5′-CCCCATATGGCTGT-CACCAATGAGCCT) and MHs02 (5′-AAAAAGCTTAG-GTCGACTTTGCCTGGCCCCCTCCAAA) and inserted into the *Nde*I−*Hin*dIII sites of the bacterial expression vector pET-28b (Novagen) to give pET/MHsΔC. The cDNA encoding the C-terminal extension was amplified with oligonucleotides MHs03 (5′-GGGGTCGACCCCGAAGC-CAGCAGTTGT) and MHs04 (5′-CCCCTCGAGTTACTTT-TTCTTCTTGCC) and inserted into the *Sal*I−*Xho*I sites of pET/MHsΔC to give pET/MHs. Deletion of the four C-terminal lysine residues of MetRS was performed by inserting into pET/MHsΔC a *Sal*I−*Xho*I fragment obtained by PCR with MHs03 and MHs05 (5′-CCCCTCGAGTTAGC-CTTTAGGGGCTTCAGGGGGT) to give pET/MHsΔK. Site-directed mutagenesis of Arg857, Lys860, Lys863, Lys866, and Lys880 into Ala was performed according to the method of Ho et al. (*25*). All constructs were verified by DNA sequencing.

The proteins encoded by the recombinant plasmids were expressed in *E. coli* BL21(DE3) grown in LB medium supplemented with kanamycin (50 μg/mL). Cultures (4.5 L) were grown at 37 °C to an A_{600} of 0.25 and transferred at 28 °C, and expression was induced at an A_{600} of 0.5 by addition of 1 mM IPTG for 4 h. Cells were washed with ice-cold buffer N1 [20 mM Tris-HCl (pH 8.0), 10 mM imidazole,

500 mM NaCl, and 10 mM 2-mercaptoethanol], resuspended in the same buffer (1.5 mL/g of cell pellet) containing 1 mM diisopropylfluorophosphate, and sonicated. After centrifugation at 45000*g* for 20 min, the lysate was incubated with 5 mL of Ni-NTA Superflow matrix (QIAGEN) at 4 °C. After it had been washed with buffer N1, MetRS was eluted stepwise with buffer N2 (buffer N1 containing 200 mM imidazole). After dialysis against buffer Q1 [20 mM Tris-HCl (pH 7.0), 10 mM NaCl, and 2 mM dithiothreitol], the solution was fractionated by anion-exchange chromatography with a Mono Q HR 5/5 column (Amersham Pharmacia Biotech) developed with a linear gradient of 10 to 300 mM NaCl. Fractions containing MetRS were dialyzed against 20 mM Tris-HCl (pH 7.0), 50 mM NaCl, 2 mM DTT, and 55% glycerol and stored at −20 °C. Protein concentrations were determined by using calculated absorption coefficients of 1.27 and 1.42 A_{280} units mg^{-1} cm^2 for MetRS and MetRS-ΔC, respectively.

Sedimentation Equilibrium. Ultracentrifugation experiments were conducted as described previously (*26*) in a Beckman Optima XL-A analytical ultracentrifuge, using an An 60 Ti rotor and a double-sector cell with a path length of 12 mm. Equilibrium was verified from the superimposition of duplicate scans recorded at 4 h intervals.

The experimental sedimentation equilibrium data were fitted to a model for a single homogeneous species following the equation

$$c(r) = c(r_{ref}) \exp\{[M_r(1 - \bar{\nu}\rho)\omega^2/2RT](r^2 - r_{ref}^2)\}$$

where $c(r)$ is the protein concentration at radial position r, $c(r_{ref})$ is the concentration of the protein at an arbitrary reference radial distance r_{ref}, M_r is the molecular mass, $\bar{\nu}$ is the partial specific volume (0.732 and 0.730 at 4 °C for MetRS and MetRS-ΔC, respectively) of the solute, ρ is the density of the solvant, ω is the angular velocity of the rotor, and R and T are the molar gas constant and the absolute temperature, respectively.

Gel Retardation Assay. ^{32}P-labeled tRNAs were obtained by in vitro transcription with T7 RNA polymerase and were purified on denaturing polyacrylamide gels. Protein−tRNA interactions were analyzed using a band shift assay as previously described (*5*). Free and bound tRNA species were quantified with a PhosphorImager.

The amino acid acceptor (Acc-tRNA^{Met}) and anticodon (Ant-tRNA^{Met}) RNA minihelices corresponding to rabbit liver elongator tRNA^{Met} were produced by in vitro transcription of *Bst*NI- and *Bcl*I-digested pUC118 derivatives constructed by insertion into their *Hin*dIII−*Bam*HI sites of oligonucleotides RS101 (5′-AGCTTAATACGACTCACT)/RS111 (5′-CTATAGTGAGTCGTATTA) and MR101 (5′-ATAGCCTCGTGTGAGTTCGATCCTCACACGGGGCACCAG)/MR111 (5′-GATCCTGGTGCCCCGTGTGAGGATCGAACTCACACGAGG), and RS101/RS111 and MR102 (5′-ATAGTCAGTCTCATAATCTGATCAG)/MR112 (5′-GATCCTGATCAGATTATGAGACTGA), respectively.

Aminoacylation Assay. Initial rates of tRNA aminoacylation were measured at 25 °C in 0.1 mL of 20 mM imidazole-HCl (pH 7.5), 150 mM KCl, 0.5 mM DTE, 5 mM $MgCl_2$ (except where stated otherwise), 3 mM ATP, 52 μM ^{14}C-labeled methionine (NEN; 58 Ci/mol), and saturating amounts of tRNA, as previously described (*27*). Total brewer's yeast tRNA (Roche, methionine acceptance of 9 pmol/A_{260}) or homogeneous rabbit elongator tRNA^{Met} purified from an *E. coli* overproducing strain as described in ref *28* (methionine acceptance of 1215 pmol/A_{260}) was used as the tRNA substrate. Total bovine tRNA was depleted of tRNA^{Met} by chromatography on a benzoyl-DEAE column. The incubation mixture contained catalytic amounts (1−15 nM) of enzymes appropriately diluted in 10 mM Tris-HCl (pH 7.5) and 10 mM 2-mercaptoethanol, containing 4 mg/mL bovine serum albumin. One unit of activity is the amount of enzyme producing 1 nmol of methionine-tRNA^{Met}/min at 25 °C. For the determination of K_M values for tRNA, tRNA^{Met} concentrations of 0.05−25 μM were used. Michaelian parameters were obtained by nonlinear regression of the theoretical Michaelis−Menten equation to the experimental curve using the KaleidaGraph 3.0.8 software (Abelbeck Software).

For measurement of the maximal rates of Met-tRNA^{Met} formation in the presence of EF-1α at the indicated concentrations, aminoacylation was conducted as described previously (*29*). Briefly, the incubation mixture was 20 mM imidazole-HCl (pH 7.5), 6 mM Tris-HCl (pH 7.5), 100 mM KCl, 11.5 mM NH_4Cl, 10% glycerol, 0.5 mM DTE, 5 mM $MgCl_2$, 3 mM ATP, 52 μM [^{14}C]methionine, 12 μM rabbit elongator tRNA^{Met}, and 0.6 mg/mL BSA. Where indicated, GTP and GDP were used at concentrations of 120 μM.

RESULTS

The Repeated Unit Provides Human MetRS with RNA Binding Properties. Human MetRS is a modular enzyme made of three distinct blocks (Figure 1A). The central domain, homologous to other known MetRSs, can aminoacylate tRNA in the absence of its eukaryotic-specific N- and C-terminal polypeptide extensions. To probe the function of the RNA-binding C-domain of MetRS, we expressed two derivatives of this enzyme in *E. coli* with an N-terminal His tag. Because the N-domain, involved in complex assembly, is dispensable for catalysis (ref *21*, and see below), we cloned into the pET28b expression vector the cDNA of human MetRS starting from residue 215. A derivative (MetRS-ΔC) with a deletion of residues 824−900, encompassing the RNA-binding unit, was also constructed. Purification over Ni-NTA and MonoQ columns led to homogeneous proteins that displayed the expected molecular masses (80 kDa for MetRS and 72 kDa for MetRS-ΔC) as assessed by SDS−PAGE. MetRS and MetRS-ΔC catalyzed the aminoacylation of tRNA^{Met} with similar efficiencies (specific activities of 262 and 300 units/mg of protein, respectively, when measured with saturating amounts of total yeast tRNA). The two enzyme species were subjected to equilibrium sedimentation analysis in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, and 1 mM DTT (Figure 2). Experimental data could be fitted to monodisperse solutes with M_rs of 82 345 and 77 528 for MetRS and MetRS-ΔC, respectively. Therefore, as opposed to the C-domain of *E. coli* MetRS (*30*), the C-domain of human MetRS is not a dimerization domain. The finding that human MetRS is a monomer in solution is consistent with the finding that the multisynthetase complex contains a single copy of the MetRS polypeptide (*21*).

We showed earlier that the repeated units from the linker region of GluProRS (EPRS repeats) display general RNA binding properties (*13*). However, its affinity for tRNA was

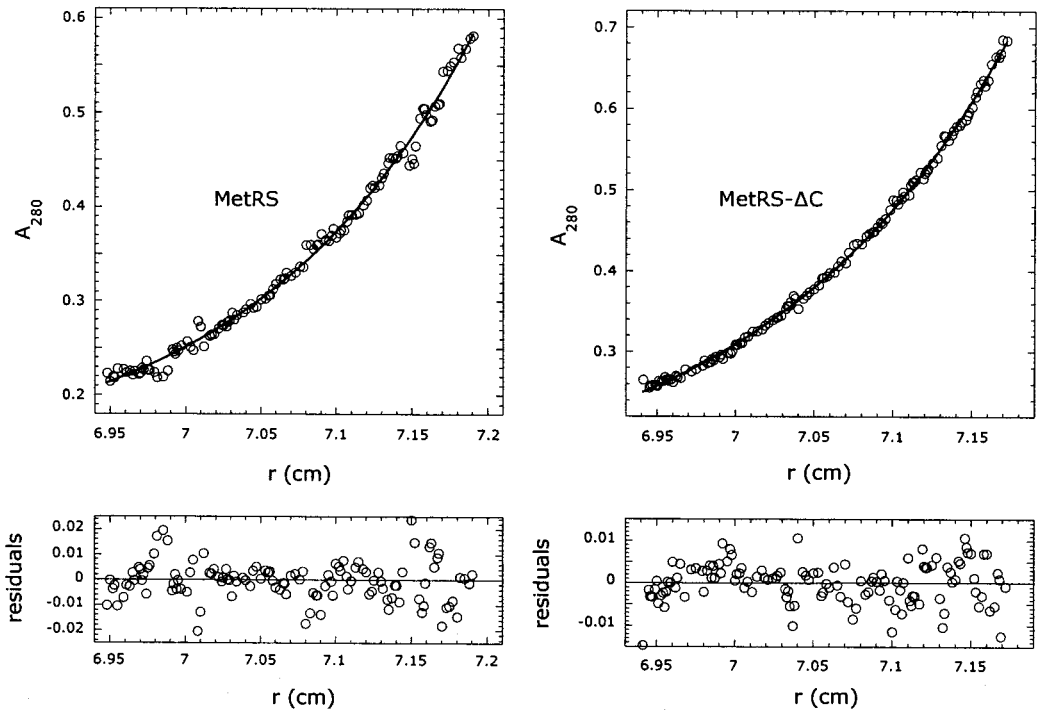

FIGURE 2: Human MetRS is a monomer in solution. MetRS and MetRS-ΔC (initial concentrations of 2.6 and 3.2 μM, respectively) were analyzed by equilibrium sedimentation at 10 000 rpm in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, and 1 mM DTT at 4 °C. Experimental values (○) were fitted (—) to monodisperse 82 345 and 77 528 Da solutes. The residuals are indicated.

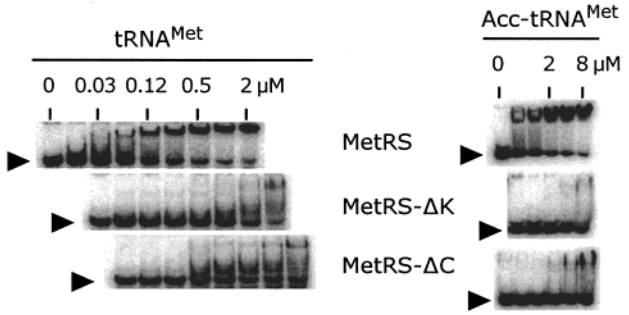

FIGURE 3: C-Domain conferring on human MetRS the ability to bind the acceptor domain of tRNA. Gel mobility shift assays of MetRS and deletion mutants to ^{32}P-labeled yeast initiator tRNA^{Met} (left) or the acceptor-TΨC domain of rabbit elongator tRNA^{Met} (right) are shown. The arrowheads point to free RNA species. Numbers refer to concentrations of MetRS and of its derivatives.

Table 1: Apparent Dissociation Constants of Wild-Type and Mutant Human MetRS for tRNA^{Met} and Acc-tRNA^{Met} Determined by a Gel Retardation Assay

	K_d for tRNA^{Met} (μM)	K_d for Acc-tRNA^{Met} (μM)
MetRS	0.1	0.5
MetRS-ΔK	1.5	~10.0
MetRS-ΔC	4.0	~10.0
MetRS-R857A	0.4	1.5
MetRS-K860A	1.5	8.0
MetRS-K863A	0.15	0.5
MetRS-K866A	0.15	0.5
MetRS-K880A	2.5	8.0

[a] Standard errors for K_d are in the range of 20−30% of the value.

weak ($K_d \sim 10\ \mu$M). Because the C-domain of human MetRS is extensively similar to these motifs (Figure 1B), we analyzed by a band shift assay the ability of MetRS and MetRS-ΔC to form stable complexes with tRNAs transcribed in vitro (Figure 3 and Table 1). Substrate (initiator yeast tRNA^{Met}) and nonsubstrate tRNAs (yeast tRNA^{Asp}, not shown) produced stable RNA−protein complexes with MetRS (apparent dissociation constants K_d of 100 and 150 nM, respectively). The C-terminally truncated protein, MetRS-ΔC, displayed a much weaker binding capacity for tRNA^{Met} (Figure 3; $K_d \sim 4\ \mu$M). These results exemplified the nonspecific general RNA binding capacity conferred on MetRS by its C-terminally appended unit. The stable association of MetRS with tRNA results from the synergy of two weak interactions between tRNA and (i) the body of MetRS (MetRS-ΔC) and (ii) its C-domain.

The EPRS repeats appended to various aaRSs have been classified into two groups according to the extent of conserved residues (*13*, *23*). The solution structure of the second repeated motif of GluProRS revealed a conserved helix−turn−helix fold followed by an Ω-loop (*13*). In the case of MetRS, only the helix−turn−helix motif is conserved (Figure 1B). The presence of a lysine-rich cluster (LRC) at its very C-terminal extremity (Figure 1) suggested that two regions of this extension could be involved in tRNA binding. We produced in *E. coli* a MetRS mutant with a deletion of the four C-terminal lysine residues, MetRS-ΔK. This mutant also displayed a severely reduced ability to bind tRNA^{Met} (Figure 3; $K_d \sim 1.5\ \mu$M). For MetRS-ΔC and MetRS-ΔK, a discrete band of the protein−tRNA complex was not easily detectable. The smear visible on the gel is most likely due to partial dissociation of the weak complex during electrophoretic separation of free and bound species of tRNAs.

To probe the region of tRNA involved in the interaction with MetRS, RNA minihelices mimicking the acceptor and anticodon stems of rabbit elongator tRNA^{Met} were produced by in vitro transcription with T7 RNA polymerase. The acceptor minihelix (Acc-tRNA^{Met}) (Figure 3), but not the anticodon minihelix (Ant-tRNA^{Met}) (not shown), formed a complex with MetRS. The apparent dissociation constant for

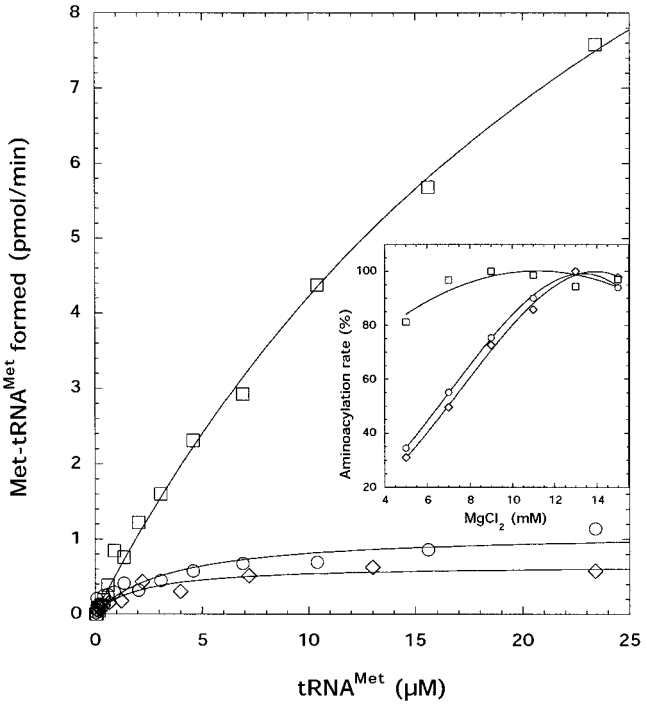

FIGURE 4: HTH motif of MetRS that severely restricts its turnover in the aminoacylation reaction. The tRNA saturation kinetics in the $tRNA^{Met}$ aminoacylation reaction were determined with nearly homogeneous rabbit elongator $tRNA^{Met}$ (methionine acceptance of 1215 pmol/A_{260} unit) in the presence of 5 nM MetRS (○), MetRS-ΔK (◇), or MetRS-ΔC (□). Experimental values (symbols) were fitted to the Michaelis−Menten equation (—). The inset shows the initial velocity ($V_{i,max}$) of MetRSs (symbols as above) measured as a function of $MgCl_2$ added to the aminoacylation mixture.

Acc-$tRNA^{Met}$ ($K_d \sim 0.5$ μM), identical with that observed for the acceptor domain of yeast $tRNA^{Asp}$ (not shown), a noncognate tRNA, was increased to ~10 μM with MetRS-ΔK and MetRS-ΔC (Figure 3 and Table 1). Therefore, the C-terminally appended domain of human MetRS is mainly involved in binding the acceptor region of tRNA.

Roles of the HTH and LRC Regions of the Bipartite RNA-Binding Domain on tRNA Aminoacylation. Homogeneous rabbit elongator $tRNA^{Met}$ (methionine acceptance of 1215 pmol/A_{260}) was used to determine the steady-state kinetic parameters in the aminoacylation reaction catalyzed by MetRS, MetRS-ΔK, and MetRS-ΔC (Figure 4 and Table 2). Although the specificity constants (k_{cat}/K_M) of the three MetRS species were similar, they resulted from large compensatory changes in the K_M and k_{cat} values. The truncated enzyme MetRS-ΔC exhibited a 9-fold increase in the K_M for $tRNA^{Met}$ but, unexpectedly, also displayed a 16-fold increase in the k_{cat} of Met-$tRNA^{Met}$ formation, as compared with those of the wild-type enzyme. As a control, we observed that the MetRS component of the multisynthetase complex displayed kinetic parameters essentially similar to those obtained with the native but ectopic MetRS used in this study (Table 2). Therefore, the presence of the C-domain significantly restricts the turnover number of the enzyme. In contrast, the removal of the four C-terminal lysine residues had no discernible effect on the k_{cat} and K_M values of MetRS-ΔK, as compared with those of the wild type. Because both mutants, MetRS-ΔC and MetRS-ΔK, had partially lost their ability to form stable complexes with tRNAs (Figure 3) but displayed radically different aminoacylation kinetics, the HTH and LRC motifs from the C-domain were quite likely to be nonredundant functional motifs.

Table 2: Apparent Kinetic Parameters[a] for the $tRNA^{Met}$ Aminoacylation Reaction[b] of Rabbit Elongator $tRNA^{Met}$ with Wild-Type and Mutant MetRS

	K_M (μM)	k_{cat} (s^{-1})
MetRS-Cx[c]	3.9 ± 1.3	0.46 ± 0.05
MetRS	3.5 ± 1.0	0.15 ± 0.04
MetRS-ΔK	2.2 ± 0.7	0.09 ± 0.02
MetRS-ΔC	32 ± 4	2.4 ± 0.5
MetRS-R857A	5.7 ± 1.1	0.47 ± 0.05
MetRS-K860A	17.2 ± 5.0	0.85 ± 0.15
MetRS-K863A	3.3 ± 0.8	0.22 ± 0.03
MetRS-K866A	3.9 ± 1.4	0.23 ± 0.04
MetRS-K880A	16.3 ± 5.7	1.03 ± 0.20

[a] Standard errors were determined from at least two independent data sets. [b] $tRNA^{Met}$ acceptance of 1215 pmol/A_{260}. [c] Multienzyme complex containing MetRS. The k_{cat} value is calculated taking into account that 1 mol of complex ($M_r \sim 1.5$ MDa) contains 1 mol of MetRS (101 kDa).

The C-domain of MetRS, including the HTH and LRC regions, is rich in basic residues (Figure 1). We surmised that positively charged compounds that may form complexes with tRNA molecules could modulate MetRS activity. The effect of Mg^{2+} and spermidine on tRNA aminoacylation by MetRS and its C-domain deletion mutants was investigated. We found that increasing the Mg^{2+} concentration from 5 to 13 mM was accompanied by an ~4-fold increase in the activity of MetRS and MetRS-ΔK, resulting from a 4-fold increase in k_{cat} without a noticeable change in the K_M value for tRNA, but had only a slight effect on MetRS-ΔC (inset of Figure 4). Likewise, addition of 6 mM spermidine in the aminoacylation mixture (in the presence of 5 mM $MgCl_2$) stimulated MetRS and MetRS-ΔK activity, but not MetRS-ΔC activity (not shown).

We previously observed that the presence of a large excess of noncognate tRNA in the aminoacylation assay may conceal kinetic effects contributed by the appended RNA-binding domains of eukaryotic enzymes (*5*). Similarly, the three enzymes MetRS, MetRS-ΔK, and MetRS-ΔC exhibited similar apparent K_M values for tRNA (0.5−0.9 μM) and identical k_{cat} values (1.2 ± 0.4 s^{-1}) when total yeast tRNA (methionine acceptance of 9 pmol/A_{260}) or partially purified beef tRNA (methionine acceptance of 80 pmol/A_{260}) was used.

To ascertain that noncognate tRNAs may affect the rate of $tRNA^{Met}$ aminoacylation by MetRS, the activity of MetRS was assayed using homogeneous rabbit $tRNA^{Met}$ as the RNA substrate (1215 pmol/A_{260}), with the addition of increasing amounts of total beef tRNA which had been depleted of $tRNA^{Met}$ (1:1, 1:3, and 1:6 mixtures of $tRNA^{Met}$ and depleted tRNA). At a low Mg^{2+} concentration (5 mM), addition of a 6-fold molar excess of noncognate tRNA led to a 4-fold increase in the activity of MetRS and MetRS-ΔK, but not in that of MetRS-ΔC. In the presence of 15 mM Mg^{2+} in the aminoacylation reaction mixture (resulting in a 4-fold increase in k_{cat}), no further stimulation of MetRS activity by noncognate tRNA was observed. Collectively, these results suggest that the rate-limiting step of Met-$tRNA^{Met}$ formation by human MetRS can be alleviated in vitro either by lowering the extent of the intrinsic tRNA−protein interaction (use of Mg^{2+} or spermidine, removal of the C-terminal RNA-binding domain) or by enhancing dissociation of Met-$tRNA^{Met}$ by

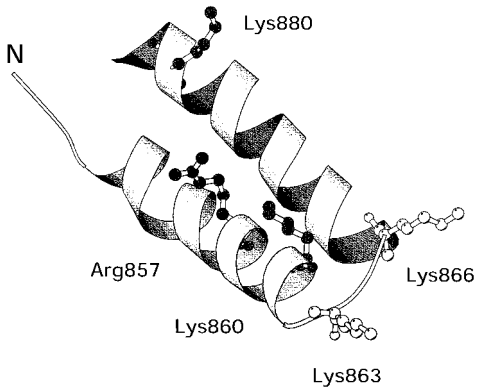

FIGURE 5: Model of tRNA binding to the HTH motif of the repeated unit of MetRS. The helix−turn−helix motif of MetRS of residues 845−883 is shown as a ribbon diagram, and the five conserved basic residues are depicted as ball-and-stick diagrams. The putative interaction region with the acceptor domain of tRNA is likely to involve residues Arg857, Lys860, and Lys880 (indicated in gray). This figure was generated using MOLSCRIPT (*43*).

competition (use of nonspecific tRNAs in excess that compete for interaction with the C-domain). The finding that noncognate tRNAs also formed stable complexes with native MetRS is consistent with their ability to displace Met-tRNA^{Met} from the synthetase.

Role of the Conserved Basic Residues from the HTH Motif. To probe the functional role of the HTH motif in a wild-type background, that is to say, independently of the removal of the LRC motif, we performed site-directed mutagenesis of its conserved, presumably functionally important residues. One arginine residue (Arg857) and four lysine residues (Lys860, -863, -866, and -880) (Figure 1B) are conserved in most of the HTH motifs reported to date (*23*). Because these residues have no structural role (*13*) and are clustered on one side of this domain (Figure 5), we tested for the impact of point mutations of these conserved basic residues on tRNA binding and on the steady-state kinetic parameters for the aminoacylation reaction. We substituted one by one these basic residues with Ala to give the R857A, K860A, K863A, K866A, and K880A mutants of human MetRS. Otherwise, all the mutant proteins contained the native LRC at their very C-termini. The five resultant mutants were expressed in *E. coli* and purified to homogeneity.

Using the gel-mobility shift assay described above, mutants K860A and K880A displayed a large decrease in their affinity for tRNA^{Met} and Acc-tRNA^{Met} (15−25-fold; Table 1). These single mutations induced a loss in affinity comparable to that observed for the deletion mutant MetRS-ΔC, which comprises a deletion of both the HTH and LRC motifs. MetRS-R857A also displayed a significant increase in K_d (3−4-fold). In contrast, mutations K863A and K866A did not alter the apparent affinity of MetRS for tRNA. It is worth mentioning that analysis of the sequences for 34 repeated units revealed that Arg857, Lys860, and Lys880 are invariant residues, as opposed to the more limiting conservation of Lys863 and Lys866 which are located in the loop of the HTH motif (27 and 26 occurrences, respectively) (*23*).

Measurement of the kinetic parameters of the aminoacylation reaction corroborated the relative tRNA binding properties of the mutants. The K_M and k_{cat} values determined for MetRS-K863A and -K866A were similar to those of the wild type (Table 2), and both values were significantly higher for mutants K860A and K880A. Basically, these two mutants recapitulated the properties of the deletion mutant MetRS-ΔC. Therefore, the HTH motif has a dominant effect over the LRC motif. The putative RNA binding site is likely to involve residues Arg857, Lys860, and Lys880 located on one side of the HTH structural motif, but not Lys863 and Lys866 located in the turn of this motif (Figure 5).

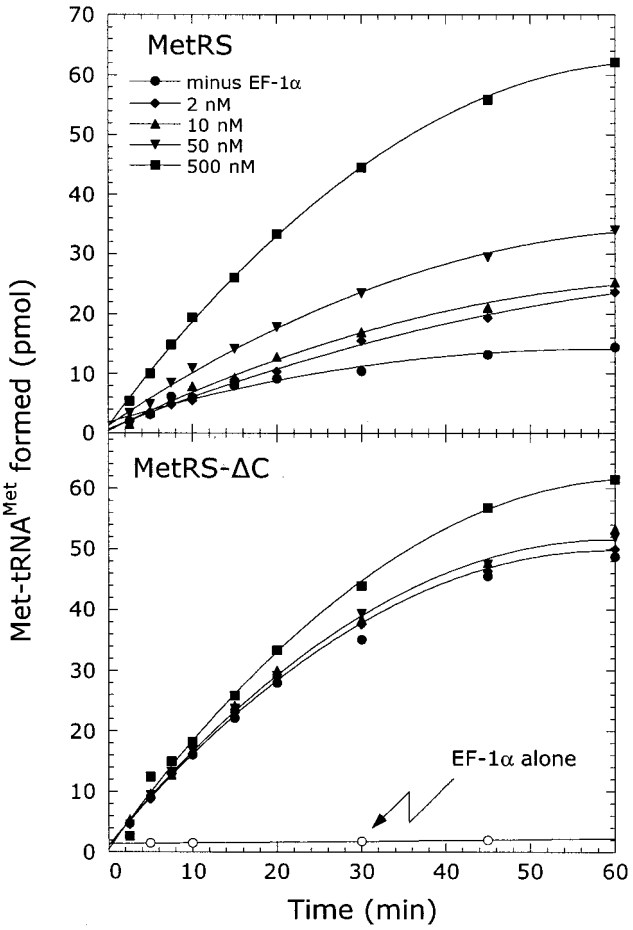

FIGURE 6: Elongation factor EF-1α stimulates MetRS activity. The time course of Met-tRNA^{Met} formation by wild-type MetRS or MetRS-ΔC was monitored in the absence or presence of increasing amounts (2−500 nM) of homogeneous EF-1α added to the aminoacylation mixture. The reaction mixtures contained 10 nM MetRS, 1 nM MetRS-ΔC, and 12 μM rabbit tRNA^{Met}. In the absence of MetRS, no detectable Met-tRNA^{Met} was formed in the presence of 500 nM EF-1α.

Are Protein Factors Needed for Optimal MetRS Activity? The above results suggested that product release could be the limiting step for Met-tRNA^{Met} formation in vivo. Since elongation factor 1α carries aminoacylated tRNA to the ribosome, we surmised that it could be involved in facilitating the dissociation of Met-tRNA^{Met} from the synthetase to ensure channeling of tRNA in the cycle of protein synthesis. Therefore, we examined the effect of EF-1α [the purified factor is in the GDP form (*31*)], in the presence or absence of saturating amounts of GDP or GTP, on the rate of aminoacylation catalyzed by MetRS or by the mutant enzymes described above. Addition of EF-1α to the reaction mixture, in the presence or absence of nucleotide, increased the rate of Met-tRNA^{Met} formation by the native enzyme (Figure 6). The enhancement of MetRS activity ($V_{i,max}$) was

maximal at an EF-1α concentration of 500 nM and followed a saturation kinetics with an apparent dissociation constant K_d of 180 ± 40 nM. The extent of stimulation was independent of the addition of nucleotides [in vitro, both EF-1α−GDP and EF-1α−GTP complexes have been shown to interact with aminoacyl-tRNAs with similar affinities (*32*, *33*)]. EF-1α also increased the rate of Met-tRNA^{Met} formation by MetRS-ΔK with a similar apparent affinity (not shown). Conversely, MetRS-ΔC did not show any significant stimulation by EF-1α (Figure 6).

DISCUSSION

The C-terminal polypeptide extension provides human methionyl-tRNA synthetase with general tRNA binding properties. This domain is related to the repeated motifs initially discovered in the linker region of the bifunctional glutamyl-prolyl-tRNA synthetase. The catalytic domain of MetRS ($K_d \sim 4\ \mu$M for tRNA) and its C-domain containing a single repeat [$K_d \sim 10\ \mu$M (*13*)] act synergistically to confer on the native enzyme the ability to bind tRNA with a much higher apparent affinity ($K_d \sim 0.1\ \mu$M). Both the HTH and LRC regions of this bipartite module are essential for tRNA binding and are not functionally redundant.

The requirement for an additional RNA-binding domain is a general feature of eukaryotic MetRSs. However, neither the type of RNA-binding module nor the mode of association is conserved among species. In plants and nematodes, MetRS displays a C-terminal polypeptide extension similar to the C-domain of p43, an RNA-binding protein arranged in an OB-fold conformation, associated within the mammalian multisynthetase complex (*6*, *8*). In yeast, MetRS associates with Arc1p, a protein that provides in trans a p43-like RNA-binding domain (*4*). In higher eukaryotic species, from *Drosophila* to mammals, corresponding to the coelomate branch of metazoan organisms, MetRS displays a strikingly different structural organization. First, this enzyme is associated with a macromolecular assembly containing eight other aminoacyl-tRNA synthetases (*20*). Second, it displays an original RNA-binding domain related to the repeated units of GluProRS (*13*, *23*). Among higher eukaryotes, apart from the human enzyme, only the preliminary genomic sequence of *Drosophila* MetRS can be retrieved from GenBank (AE003798; gene CG15100). In accordance with the analysis of the enzyme purified from *Drosophila* cells (*34*), the fly MetRS is larger than the human enzyme. Its C-terminal polypeptide extension is made of three HTH motifs, and it displays a KGKKKK C-terminal sequence identical to the LRC of the human enzyme. Thus, the two distinct blocks identified within the RNA-binding domain of human MetRS are likely to be conserved from fly to human.

MetRS is one of the rare examples of a sudden change of structural organization in evolution. Why do higher eukaryotic MetRSs need an RNA-binding domain distinct from that found in MetRSs from lower eukaryotes? We had previously shown that the p43-like domain of plant MetRS contributed a 10-fold decrease in K_M for tRNA in the aminoacylation reaction catalyzed by the native enzyme, as compared with the C-terminally truncated MetRS (*5*). No evidence for k_{cat} limitation was obtained. Because the free tRNA concentration is nonsaturating within the cell, we concluded that the C-terminally appended domain of plant MetRS is required for an efficient capture of tRNA by the synthetase. In contrast, the appended domain of human MetRS not only decreases the apparent affinity for tRNA but also causes a severe turnover limitation. Because removal of the LRC region did not relieve turnover restriction (Figure 4) but impaired tRNA association at equilibrium (Figure 3), we suggest that the HTH and LRC regions have distinct roles in sustaining tRNA aminoacylation. The LRC might be involved in tRNA capture via an increase in the association rate (k_{on}) of tRNA. The five exposed lysine residues would create a high positive electrostatic potential involved in long-distance interactions with these negatively charged molecules, thus leading to a higher local concentration in tRNA. In contrast, the HTH module is likely to contribute a rate-limiting product release. Stimulation of Met-tRNA^{Met} synthesis was observed upon deletion of the entire C-terminal domain, or in the presence of the LRC region when point mutations are introduced into the HTH segment.

Polyamines have long been known as modulators of protein biosynthesis efficiency both in vivo and in vitro (*35*). Our data show that Mg^{2+} or spermidine, and to a lesser extent KCl (not shown), partially releases the rate-limiting step of Met-tRNA^{Met} dissociation observed for the native enzyme. Magnesium and polyamines are known to interact with tRNA and to contribute important interactions in maintaining the tertiary structure of tRNA (*36*−*38*). Because Mg^{2+} or spermidine did not enhance the activity of MetRS-ΔC, stimulation of MetRS or MetRS-ΔK is not related to a structural change in the tRNA used in our study. As more polyamine is added, neutralization of phosphate charges may alter the interaction of tRNA with the basic patches from the C-domain of MetRS.

The acquisition of supplementary RNA-binding domains by mammalian aminoacyl-tRNA synthetases may be related to the emergence of tRNA channeling in translation (*39*). This is supported by the finding that EF-1α may mediate the release of Met-tRNA^{Met} (this study), of Val-tRNA^{Val} (*29*), of Phe-tRNA^{Phe} (*31*), and of Asp-tRNA^{Asp} (*40*) from their cognate synthetase. This would be a rational means of ensuring EF-1α-assisted vectorial transfert of tRNA from its aminoacylation site (the synthetase) to its utilization site (the ribosome). In the case of ValRS, the channeling is mediated by stable association of this synthetase with elongation factor EF-1H, the heavy form of elongation factor 1 that comprises the three subunits EF-1β, EF-1γ, and EF-1δ and contributes the guanine nucleotide exchange activity (*41*, *42*). The finding that Met-tRNA^{Met} is not spontaneously released from the synthetase suggests that mammalian synthetases work in vivo in a processive manner. Product dissociation awaits productive interaction between the synthetase and elongation factor EF-1α. The high cellular content in EF-1α may be essential for this process to occur.

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REFERENCES

1. Ibba, M., and Söll, D. (2000) *Annu. Rev. Biochem. 69*, 617−650.
2. Beuning, P. J., and Musier-Forsyth, K. (1999) *Biopolymers 52*, 1−28.
3. Simos, G., Segref, A., Fasiolo, F., Hellmuth, K., Shevchenko, A., Mann, M., and Hurt, E. C. (1996) *EMBO J. 15*, 5437−5448.
4. Deinert, K., Fasiolo, F., Hurt, E. C., and Simos, G. (2001) *J. Biol. Chem. 276*, 6000−6008.
5. Kaminska, M., Deniziak, M., Kerjan, P., Barciszewski, J., and Mirande, M. (2000) *EMBO J. 19*, 6908−6917.
6. Shalak, V., Kaminska, M., Mitnacht-Kraus, R., Vandenabeele, P., Clauss, M., and Mirande, M. (2001) *J. Biol. Chem. 276*, 23769−23776.
7. Kim, Y., Shin, J., Li, R. B., Cheong, C., Kim, K., and Kim, S. (2000) *J. Biol. Chem. 275*, 27062−27068.
8. Renault, L., Kerjan, P., Pasqualato, S., Menetrey, J., Robinson, J. C., Kawaguchi, S., Vassylyev, D. G., Yokoyama, S., Mirande, M., and Cherfils, J. (2001) *EMBO J. 20*, 570−578.
9. Whelihan, E. F., and Schimmel, P. (1997) *EMBO J. 16*, 2968−2974.
10. Wang, C. C., and Schimmel, P. (1999) *J. Biol. Chem. 274*, 16508−16512.
11. Wang, C. C., Morales, A. J., and Schimmel, P. (2000) *J. Biol. Chem. 275*, 17180−17186.
12. Wu, H., Nada, S., and Dignam, J. D. (1995) *Biochemistry 34*, 16327−16336.
13. Cahuzac, B., Berthonneau, E., Birlirakis, N., Guittet, E., and Mirande, M. (2000) *EMBO J. 19*, 445−452.
14. Jeong, E. J., Hwang, G. S., Kim, K. H., Kim, M. J., Kim, S., and Kim, K. S. (2000) *Biochemistry 39*, 15775−15782.
15. Frugier, M., Moulinier, L., and Giegé, R. (2000) *EMBO J. 19*, 2371−2380.
16. Hammamieh, R., and Yang, D. C. H. (2001) *J. Biol. Chem. 276*, 428−433.
17. Beaulande, M., Kron, M., and Härtlein, M. (2001) *FEBS Lett. 494*, 170−174.
18. Mechulam, Y., Schmitt, E., Maveyraud, L., Zelwer, C., Nureki, O., Yokoyama, S., Konno, M., and Blanquet, S. (1999) *J. Mol. Biol. 294*, 1287−1297.
19. Sugiura, I., Nureki, O., Ugaji-Yoshikawa, Y., Kuwabara, S., Shimada, A., Tateno, M., Lorber, B., Giegé, R., Moras, D., Yokoyama, S., and Konno, M. (2000) *Structure 8*, 197−208.
20. Mirande, M. (1991) *Prog. Nucleic Acid Res. Mol. Biol. 40*, 95−142.
21. Mirande, M., Kellermann, O., and Waller, J. P. (1982) *J. Biol. Chem. 257*, 11049−11055.
22. Lage, H., and Dietel, M. (1996) *Gene 178*, 187−189.
23. Berthonneau, E., and Mirande, M. (2000) *FEBS Lett. 470*, 300−304.
24. Cerini, C., Kerjan, P., Astier, M., Gratecos, D., Mirande, M., and Semeriva, M. (1991) *EMBO J. 10*, 4267−4277.
25. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene 77*, 51−59.
26. Agou, F., Waller, J. P., and Mirande, M. (1996) *J. Biol. Chem. 271*, 29295−29303.
27. Mirande, M., Cirakoglu, B., and Waller, J. P. (1983) *Eur. J. Biochem. 131*, 163−170.
28. Meinnel, T., Mechulam, Y., Fayat, G., and Blanquet, S. (1992) *Nucleic Acids Res. 20*, 4741−4746.
29. Negrutskii, B. S., Shalak, V. F., Kerjan, P., El'skaya, A. V., and Mirande, M. (1999) *J. Biol. Chem. 274*, 4545−4550.
30. Cassio, D., and Waller, J. P. (1971) *Eur. J. Biochem. 20*, 283−300.
31. Negrutskii, B. S., Budkevich, T. V., Shalak, V. F., Turkovskaya, G. V., and El'skaya, A. V. (1996) *FEBS Lett. 382*, 18−20.
32. Roobol, K., and Möller, W. (1978) *Eur. J. Biochem. 90*, 471−477.
33. Crechet, J. B., and Parmeggiani, A. (1986) *Eur. J. Biochem. 161*, 647−653.
34. Kerjan, P., Cerini, C., Semeriva, M., and Mirande, M. (1994) *Biochim. Biophys. Acta 1199*, 293−297.
35. Tabor, C. W., and Tabor, H. (1984) *Annu. Rev. Biochem. 53*, 749−790.
36. Quigley, G. J., Teeter, M. M., and Rich, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 64−68.
37. Sampson, J. R., and Uhlenbeck, O. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1033−1037.
38. Frydman, L., Rossomando, P. C., Frydman, V., Fernandez, C. O., Frydman, B., and Samejima, K. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9186−9190.
39. Stapulionis, R., and Deutscher, M. P. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 7158−7161.
40. Reed, V. S., Wastney, M. E., and Yang, D. C. H. (1994) *J. Biol. Chem. 269*, 32932−32936.
41. Motorin, Y. A., Wolfson, A. D., Löhr, D., Orlovsky, A. F., and Gladilin, K. L. (1991) *Eur. J. Biochem. 201*, 325−331.
42. Bec, G., Kerjan, P., and Waller, J. P. (1994) *J. Biol. Chem. 269*, 2086−2092.
43. Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946−950.

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