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Arginine-395 Is Required for Efficient in Vivo and in Vitro Aminoacylation of tRNAs by *Escherichia coli* Methionyl-tRNA Synthetase†

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ABSTRACT: We have previously shown that the anticodon of methionine tRNAs contains the major recognition site required for aminoacylation of tRNAs by *Escherichia coli* methionyl-tRNA synthetase (MetRS) and have located part of the anticodon binding domain on the enzyme at a site close to Trp461 [Schulman, L. H., & Pelka, H. (1988) *Science 242*, 765–768; Ghosh, G., Pelka, H., & Schulman, L. H. (1990) *Biochemistry 29*, 2220–2225]. In order to gain information about other possible sites of contact between MetRS and its tRNA substrates, we have examined the effects of mutations at a series of positively charged residues on the surface of the C-terminal domain of the enzyme. Conversion of Arg356, Arg366, Arg380, or Arg453 to Gln had little or no effect on enzyme activity. Similarly, conversion of Lys402 or Lys439 to Asn failed to significantly alter aminoacylation activity. Conversion of Arg380 to Ala or Arg442 to Gln produced a 5-fold reduction in k_{cat}/K_m for aminoacylation of $tRNA^{fMet}$, with no effect on methionine activation, indicating a possible minor role for these residues in interaction of the enzyme with the tRNA substrate. In contrast, mutation of a phylogenetically conserved residue, Arg395, to Gln increased the K_m for aminoacylation of $tRNA^{fMet}$ about 30-fold and reduced k_{cat}/K_m by 25 000-fold. The mutant enzyme was also shown to be highly defective by its inability to complement a strain of *E. coli* having an altered chromosomal MetRS gene. Examination of the kinetic parameters for ATP–PP_i exchange catalyzed by the Gln395 enzyme showed little or no effect of the mutation on interaction of MetRS with methionine or ATP, indicating that the major role of Arg395 is in tRNA recognition. Mutation of a second conserved residue, Asn391, to Ala specifically increased the K_m for aminoacylation of $tRNA^{fMet}$ 20-fold with little or no effect on the other kinetic parameters. Examination of the crystal structure of MetRS [Brunie, S., Zelwer, C., & Risler, J.-L. (1990) *J. Mol. Biol. 216*, 411–424] shows that Trp461 is located on a separate peptide, at a distance of 10–20 Å from Asn391 and Arg395. Extensive molecular dynamics simulation studies have revealed that the peptide loop containing Trp461 is the most flexible part of MetRS and that residues in this loop can move more than 5 Å with little energy cost. This suggests that tRNA binding may induce a conformational change in MetRS that allows simultaneous interaction of the anticodon loop of the tRNA with the two peptides containing Trp461 and Asn391–Arg395. Such a substrate-induced conformational change may be an important feature of the tRNA recognition mechanism.

Aminoacyl-tRNA synthetases catalyze the ATP-dependent activation of amino acids and their subsequent transfer to the 3′ terminus of appropriate cognate tRNAs. The mechanism of differentiation between cognate and noncognate tRNAs depends on sequence-specific contacts between protein–tRNA pairs and optimal fit of the surfaces of the two macromolecules. The overall accuracy of protein synthesis is dependent on the outcome of this selection process.

The details of only one synthetase–tRNA interaction are available to date. A high-resolution X-ray crystal structure of *Escherichia coli* glutaminyl-tRNA synthetase (GlnRS)[1] complexed with $tRNA^{Gln}$ and ATP has been solved at 2.5-Å

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[1] Abbreviations: GlnRS, glutaminyl-tRNA synthetase; MetRS, methionyl-tRNA synthetase; MetRS547, monomeric MetRS truncated at amino acid residue 547; R395Q, MetRS547 with arginine-395 replaced by glutamine; other MetRS547 mutants are indicated in a similar fashion using the one-letter amino acid code; TyrRS, tyrosyl-tRNA synthetase; $tRNA^{fMet}$, initiator methionine tRNA; Met-AMP, methionyladenylate; MD, molecular dynamics.

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resolution (Rould et al., 1989, 1991). The structure reveals extensive contacts between amino acid side chains of the protein and each of the anticodon bases of the tRNA. In addition, sequence-specific interactions occur with the acceptor stem of the tRNA, and possibly several additional sites. The crystal structure nicely explains earlier genetic and biochemical experiments implicating the anticodon and acceptor stem of $tRNA^{Gln}$ in its specific recognition by GlnRS [reviewed by Rogers and Söll (1990)]. More recent in vitro studies have provided a quantitative assessment of the contribution of specific nucleotide bases in these regions to the efficiency of aminoacylation by the glutamine enzyme, confirming the important role of the anticodon and acceptor stem in $tRNA^{Gln}$ recognition (Jahn et al., 1991). In addition to these sequence-specific contacts, a series of positively charged amino acid residues line the backbone of the tRNA structure in the complex, providing additional stabilizing interactions.

Specific positively charged residues on the surface of *Bacillus stearothermophilus* TyrRS have been shown by site-directed mutagenesis techniques to play an important role in aminoacylation of $tRNA^{Tyr}$. The crystal structure of the uncomplexed tyrosine enzyme has been solved at 2.3-Å resolution (Brick et al., 1989), and results of the mutagenesis studies have been used to model the interaction of the active-site domain with the acceptor arm of the tRNA (Bedouelle & Winter, 1986; Labouze & Bedouelle, 1989). A high-resolution crystal form of a yeast AspRS–$tRNA^{Asp}$ complex has been obtained that is expected to provide details of this interaction as well (Ruff et al., 1988, 1991).

A proteolytic fragment of *E. coli* MetRS that retains full biological activity has also been crystallized (Zelwer et al., 1982; Brunie et al., 1987), and the structure of the enzyme complexed with ATP has recently been refined to 2.5-Å resolution (Brunie et al., 1990). Unlike GlnRS, the methionine enzyme has been shown to recognize its tRNA substrates primarily, if not exclusively, through contacts with the anticodon nucleotides, with little or no contribution from sequences in the acceptor stem region (Schulman & Goddard, 1973; Schulman & Pelka, 1983, 1988; Schulman, 1991; Lee et al., 1991). Cross-linking experiments identified a site in MetRS (Lys465) within 14 Å of the anticodon binding domain (Valenzuela & Schulman, 1986; Leon & Schulman, 1987; Schulman et al., 1987). Subsequent site-directed mutagenesis experiments indicated a role for Trp461 in specific recognition of the wild-type anticodon sequence and exclusion of tRNAs containing non-methionine anticodons (Ghosh et al., 1990). Additional studies have recently confirmed the important role of this residue in tRNA recognition by MetRS (Meinnel et al., 1991).

Despite the apparent absence of sequence-specific contacts outside of the anticodon region, additional interactions are expected to occur between MetRS and the sugar–phosphate backbone that assist in proper alignment of the tRNA and protein to maximize the efficiency of aminoacylation at the active site of the enzyme. In the present work, we have explored the possible role of a series of positively charged residues on the surface of the C-terminal domain of MetRS in this process, using site-directed mutagenesis. These studies have revealed an additional site in the enzyme, located at some distance from Trp461, that is critical for $tRNA^{Met}$ recognition.

Materials and Methods

Materials. ^{35}S-Labeled methionine was purchased from Amersham, and ^{32}P-labeled sodium pyrophosphate was obtained from New England Nuclear. Enzymes for cloning were purchased from New England Biolabs. $tRNA^{fMet}$ having a specific activity for methionine acceptance of 1650 pmol/A_{260} was obtained from Boehringer-Mannheim. Deoxyoligonucleotides were synthesized on an Applied Biosystems DNA synthesizer by a core facility of the Albert Einstein College of Medicine.

Construction of MetRS Mutants. Site-directed mutagenesis of a truncated form of MetRS (MetRS547) was carried out as described earlier (Ghosh et al., 1990). The following oligonucleotides, complementary to the sequence of the gene, were used for the mutagenesis experiments: Arg356 → Gln, 5′GTAGTAGTATTGCAGGC3′; Arg366 → Gln, 5′ATCATCAATCTGCGAAG3′; Arg380 → Gln, 5′GGCATTCACTTGCTGAA3′; Arg395 → Gln, 5′AGCCCGCATTCTGGGAGG3′; Lys402 → Asn, 5′CGTCAAAACGATTGTTGA3′; Lys439 → Asn, 5′CGCGCACGGCGTTACCA3′; Arg442 → Gln, 5′CATGATTTCCTGCACGG3′; Arg453 → Gln, 5′ATCGACATACTGGTTAG3′. The underlined nucleotides indicate mismatches to the corresponding sites in the single-stranded DNA template. The expected mutations were confirmed by dideoxy sequencing.

Purification of Enzymes. Wild-type MetRS547 and the Gln395 and Gln442 mutant enzymes were purified from overproducing strains carrying the corresponding MetRS genes in the plasmid pGG3 as described before (Ghosh et al., 1990). The Gln356 mutant enzyme was produced in lower amounts and was purified by polymin P precipitation of an S-100 extract prepared from 10 L of culture, followed by 40–70% ammonium sulfate fractionation of the supernatant. Successive chromatography was then carried out by step elution from DEAE-Sepharose (fast flow) in buffer A (20 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride, and 10% glycerol) plus 100 mM KCl followed by gradient elution on a DE-52 column (20–250 mM KCl in buffer A). Fractions containing MetRS activity were pooled, concentrated by precipitation with 70% ammonium sulfate, and chromatographed on G-100 (superfine) in buffer A plus 10 mM KCl. The pool of MetRS547 activity was concentrated by Centricon 30 centrifugation and chromatographed on a Mono Q column using a gradient from 0 to 600 mM NaCl in 20 mM Tris-HCl, pH 8.1, 1 mM DTT, and 10% glycerol. All of the proteins except for the Gln356 mutant were greater than 95% pure as judged by SDS–polyacrylamide gel electrophoresis and contained no detectable native MetRS. The R356Q enzyme was 40% pure and free of native MetRS. ATP–PP_i exchange and aminoacylation assays on the purified proteins were carried out as detailed elsewhere (Ghosh et al., 1990).

Assay of Crude Cell Extracts. Crude cell extracts from late-log cultures of overproduced enzymes were prepared and assayed for methionine acceptor activity as described before (Ghosh et al., 1990). Activity was calculated from initial rates, which were linear with time and proportional to protein concentration. No correction was made for small variations in the levels of MetRS produced, as determined by Western blot analysis.

In Vivo Complementation. E. coli strain LS50 (*metG146 recA:Tn10* F′) carries a defective chromosomal gene for MetRS encoding a protein that has a high K_m for methionine and therefore requires elevated concentrations of methionine for growth (Ghosh et al., 1990; Somerville & Ahmed, 1977). Wild-type and mutant MetRS547 genes on pGG3 were tested for their ability to complement strain LS50 by growth of the transformed cells in minimal medium in the absence of added methionine.

Molecular Dynamics Simulations. Molecular mechanics and dynamics (MD) calculations were performed on a vectorial VAX/9210, using XPLOR version 2.1 (Brünger et al., 1987).[2] Because the calculations were performed in vacuo, the effects of the solvent in damping the electrostatic forces were modeled by reducing the charge densities on surface residues according to the method described by Mouawad et al. (1990): the charge densities of the atoms exposed to the solvent (except those of the main chain) were lowered by as much as 70% when the atoms were not involved in H-bond interactions and up to 30% otherwise. In addition, when the most polar residues (Asn, Asp, Arg, Gln, Glu, and Lys) were more than 30% exposed to the solvent, the charge densities of the atoms which did not form H-bonds with atoms other than those of the main chain were set to zero. This procedure has been extensively detailed in Demaret et al. (1990). Sigmoid cutoff functions were also applied to all external energy terms, which were zeroed between 6.5 and 7.5 Å.

Energy minimization was carried out mainly to relax the constraints induced by the bad contacts which remain in the crystal structure. The MetRS was energy-minimized with a Fletcher–Powell algorithm, with decreasing harmonic constraints on all atoms during the first few hundred cycles to smoothly remove the bad contacts. Calculations stopped after about 2000 minimization cycles, upon reaching the convergence criterion of 0.01 kcal mol^{-1} $Å^{-1}$.

MD calculations were accomplished on MetRS using the following procedure: (i) a 12-ps thermalization, from 0 to 300 K in 5 K steps (the temperature is defined by assigning a Maxwellian distribution of velocities to the atoms with a variance equal to this temperature; (ii) a 20-ps equilibration with reassignment of the velocities every 0.40 ps; (iii) a 30-ps equilibration with rescaling of the velocities every 0.40 ps; (iv) a 100-ps "productive" MD simulation (i.e., MD which is not thermalization or equilibration). The integration of the Newtonian equations of motion was performed using the Verlet algorithm (Verlet, 1967) with a 1-fs integration step, using SHAKE constraints (Ryckaert et al., 1977; van Gunsteren & Berendsen, 1977) on the bonds involving a hydrogen atom. The nonbonded interactions lists were updated every 0.01 ps. Finally, "mean" (time-averaged over a dynamics simulation and energy-minimized) structures were calculated by averaging the coordinates over the "productive" dynamics and energy-minimizing the structure with 200 Fletcher–Powell cycles to remove the bad contacts.

Results

Synthesis and Activity of MetRS547 Mutants. Site-directed mutagenesis has previously been used to insert a UAA stop codon following Lys547 of the MetRS gene cloned in a high copy number plasmid (Ghosh et al., 1990). Expression from the endogenous promoter of the altered gene results in overproduction of a truncated protein (MetRS547; molecular weight 62 400) corresponding to the biologically active crystallized monomeric fragment of MetRS (Mellot et al., 1989). This protein can be readily distinguished from the native dimeric enzyme of molecular weight 2 × 76 000 (Koch & Bruton, 1974). Site-directed mutagenesis has now been used to alter a series of positively charged amino acid residues found on the surface of the carboxy-terminal domain of the enzyme. In each case, the hydrophilic character of the target residue has been preserved while the positive charge of the wild-type amino acid has been removed (Arg → Gln and Lys → Asn). Residues at several sites of particular interest were also converted to Ala to determine the effect of complete removal of the hydrophilic side chain.

Table I: Aminoacylation Activity of MetRS547 Mutants in Crude Cell Extracts[a]

position	amino acid: wild type	amino acid: mutant	relative activity [(mutant/wild type) × 100]
356	Arg	Gln	50[b]
366	Arg	Gln	100
380	Arg	Gln, Ala	50, 20
391	Asn	Ala	4
395	Arg	Gln, Ala	<1, <1
402	Lys	Asn	50
439	Lys	Asn	100
442	Arg	Gln	10
453	Arg	Gln	33
465	Lys	Asn,[c] Ala	50,[c] 33
469	Arg	Gln[c]	33[c]

[a] Crude extracts of cells overproducing wild-type and MetRS547 mutants were assayed for methionine acceptor activity as described under Materials and Methods. The enzymes were overproduced to a similar extent, and no correction was made for small variations in yield. The activity of each extract was compared with that of wild-type MetRS547 and rounded off to the nearest fold difference, e.g., none, 2-fold, 3-fold, etc. Data previously obtained for mutations at sites of positively charged amino acid residues in the C-terminal domain of MetRS are also included in the table. [b] The R356Q mutant was not overproduced. Value given is based on the activity of enzyme of 40% purity. [c] Data taken from Ghosh et al. (1990).

Table II: Aminoacylation Activity of MetRS547 Mutants[a]

enzyme	K_m^{tRNA} (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} $μM^{-1}$)	relative k_{cat}/K_m
wild type	1.2 ± 0.2	3.2 ± 0.2	2.7	1.0
R356Q	0.6 ± 0.1	0.8 ± 0.1	1.4	0.5
R380A	10 ± 1	5.0 ± 0.2	0.5	0.2
N391A	22 ± 4	1.6 ± 0.2	0.07	0.03
R395Q	39 ● 9	(4.7 ± 0.6) × 10^{-3}	1.2 × 10^{-4}	4 × 10^{-5}
R395A	113 ± 31	(1.7 ± 0.4) × 10^{-2}	1.5 × 10^{-4}	6 × 10^{-5}
R442Q	3.5 ± 0.8	1.9 ± 0.2	0.5	0.2
W461F[b]	75 ± 15	4.3 ± 0.6	0.06	0.02

[a] Aminoacylation assays were carried out using 17 μM methionine and 2 mM ATP as described earlier (Ghosh et al., 1990). Kinetic constants were determined using native $tRNA^{fMet}$ concentrations of 0.5–6 μM for wild-type MetRS547, R356Q, and R442Q, 5–30 μM for R380A and N391A, and 5–40 μM for R395Q and R395A. [b] Data taken from Ghosh et al. (1990).

The level of expression and the methionine acceptor activity of crude extracts of cells producing each MetRS547 derivative were determined in order to screen for defective synthetase mutants. Table I summarizes the activity of the overproduced enzymes. In cases where extracts of the mutants showed significantly lower activity than the extract containing wild-type enzyme, the possibility of unexpected mutations was ruled out by completely sequencing the DNA of each of the defective genes. The mutant proteins were then purified to homogeneity for biochemical characterization. In addition, mutant R356Q, produced in lower amounts, was partially purified for determination of enzyme activity.

Characterization of the Kinetic Parameters for Aminoacylation of Native $tRNA^{fMet}$ by Purified MetRS547 Mutants. Table II compares the kinetic parameters for aminoacylation of native *E. coli* $tRNA^{fMet}$ by the wild-type and mutant enzymes. Conversion of Arg356 to Gln had little effect on the

[2] A previous set of calculations was performed with CHARMm version 21 (Brooks et al., 1983), on a STAR ST-100 array-processor, with a similar protocol, during 65 ps. Results comparable to those described here were obtained. However, to eliminate a possible artifact and to check the reproducibility of the observed high fluctuation of peptide 460–477, the calculations were carried out on the vectorial VAX/9210, during 100 ps.

Table III: Kinetic Constants for MetRS547 Mutants in the ATP–PP_i Exchange Reaction[a]

enzyme	K_m^{Met} (μM)	K_m^{ATP} (μM)	k_{cat}[b] (s^{-1})
wild type	21 ± 5	528 ± 91	74 ± 8
R356Q	16 ± 2	540 ± 30	40 ± 2
N391A	25 ± 3	529 ± 56	56 ± 3
R395Q	30 ± 8	515 ± 129	15 ± 2
R395A	23 ± 3	544 ± 105	41 ± 3
R442Q	29 ± 2	540 ± 136	32 ± 3

[a] ATP–PP_i exchange assays were carried out as described earlier (Ghosh et al., 1990). Kinetic constants for methionine were determined at 2 mM ATP and methionine concentrations of 10–100 μM. K_m values for ATP were determined at 2 mM methionine and ATP concentrations of 125–1000 μM. [b] k_{cat} is the average of the values determined in assays in which the methionine and ATP concentrations were varied.

reaction, slightly decreasing both K_m for the tRNA and k_{cat}. The R442Q mutation produced a 3-fold increase in K_m for $tRNA^{fMet}$ and a small decrease in k_{cat}, lowering k_{cat}/K_m by 5-fold. The R380A mutation also decreased k_{cat}/K_m 5-fold. In contrast, conversion of Arg395 to Gln had a major effect on both kinetic parameters. The K_m for $tRNA^{fMet}$ was increased about 30-fold, and k_{cat} was reduced 700-fold, lowering the overall specificity for the tRNA substrate, k_{cat}/K_m, by 25 000. A second mutation at this site, R395A, also led to production of a highly defective enzyme. Conversion of Asn391 to Ala reduced k_{cat}/K_m about 35-fold, a value similar to that previously observed for the anticodon binding mutant W461F (Table II).

Activity of the MetRS547 Mutants in the ATP–PP_i Exchange Reaction. Mutations that altered the aminoacylation activity of MetRS were also tested for their effect on synthesis of methionyl adenylate by measuring the rate of exchange of radioactively labeled pyrophosphate into ATP in the presence of each enzyme. Determination of K_m values for methionine and ATP in this reaction showed that there was no significant effect of any of the amino acid substitutions on initial complex formation with these substrates (Table III). Measurement of k_{cat} for each of the mutant enzymes revealed insignificant (about 2-fold) decreases in the rates of catalysis by the R356Q, N391A, R395A, and R422Q enzymes and only a 5-fold decrease in the rate of exchange catalyzed by the R395Q mutant. These data clearly indicate that the major effect of all the mutations is on interaction of the enzyme with its tRNA substrate.

In Vivo Complementation. The R356Q, N391A, R395A, R395Q, and R422Q mutant enzymes were tested for their ability to support growth of an *E. coli* strain having a defective chromosomal MetRS gene. *E. coli* strain LS50 (*metG146*) cannot grow in minimal medium unless supplemented with methionine due to the presence of a mutation in the MetRS gene that greatly increases the K_m of the enzyme for its cognate amino acid. Transformation of this strain with plasmids carrying wild-type MetRS547, or the R356Q, N391A, or R442Q mutant enzyme, restored growth to LS50 in the absence of methionine. In contrast, the R395Q and R395A mutant synthetases failed to complement the chromosomal defect. Growth of LS50 in the presence of methionine and plasmid bearing the Gln395 or Ala395 gene was normal and resulted in at least 50-fold overexpression of the mutant proteins, indicating that the altered enzymes are not toxic to cells. Together, these results show that MetRS containing mutations at position 395 is highly defective as a methionine synthetase in vivo as well as in vitro and that the mutant enzymes do not significantly mischarge noncognate tRNAs in vivo.

Molecular Dynamics Simulation and Molecular Graphics Modeling. The finding of a second domain at some distance from Trp461 that is essential for efficient aminoacylation of $tRNA^{Met}$ suggested that the anticodon of the tRNA may be buried in a cleft between these two peptides. Attempts were made to model the interaction of the tRNA with MetRS using the 3-Å crystal structure of yeast $tRNA^{fMet}$ (Schevitz et al., 1979; Basavappa & Sigler, 1991), a tRNA which is an excellent substrate for the *E. coli* enzyme (Takeishi et al., 1968; RajBhandary & Ghosh, 1969). No satisfactory docking of the tRNA and the synthetase could be achieved without introducing a large conformational change in the peptide loop containing amino acid residues 450–480. In order to examine the possibility of a significant conformational change in this part of the enzyme structure on complex formation with the tRNA, the flexibility of the MetRS structure was studied by molecular dynamics simulation. The data obtained revealed that the peptide containing residues 460–477 is the most flexible region in the entire enzyme structure, with a large rms fluctuation centered at about residue 465 (Figure 2).

Discussion

Little is known about the detailed mechanism of recognition of tRNAs by aminoacyl-tRNA synthetases. The cocrystal structure of the GlnRS·$tRNA^{Gln}$ complex shows that this protein–tRNA interaction involves extensive contacts between the two molecules, extending from the anticodon loop and stem along the inside of the L-shaped tRNA structure to the 3′ terminus of the tRNA (Rould et al., 1989, 1991).

GlnRS and MetRS are both members of the class I group of aminoacyl-tRNA synthetases (Eriani et al., 1990). The enzymes in this group share two short consensus sequences (HIGH and KMSKS) and contain a conserved structural motif (Rossmann fold) in the N-terminal domain that forms the ATP binding site (Risler et al., 1981; Rould et al., 1989). Recent detailed structural comparisons of MetRS complexed with ATP and GlnRS complexed with ATP and $tRNA^{Gln}$ have revealed two motifs in addition to the Rossmann fold that are structurally homologous in the two enzymes, although they contain no conserved primary sequences (Perona et al., 1991). These motifs encompass residues 102–124 and residues 340–378 in MetRS (Figure 1). The first conserved region is believed to play a structural role in the two enzymes and does not directly contact the tRNA in the GlnRS cocrystal. The second motif consists of an α-helix–turn–β-strand–α-helix. In GlnRS, the structure of this region is complementary to that of $tRNA^{Gln}$ along the bottom of the acceptor stem, the D stem, and the anticodon stem, and orients the 3′ end of the tRNA toward the active site. The β-strand of this motif in GlnRS closely approaches $tRNA^{Gln}$, and several direct contacts are made between this region of the protein and the sugar–phosphate backbone at the inside corner of the L-shaped tRNA (Perona et al., 1991). Conservation of this important α-helix–turn–β-strand–α-helix motif in MetRS has led to the suggestion that this enzyme and possibly other members of the class I group of synthetases may bind their tRNA substrates in a manner analogous to that seen in the GlnRS·$tRNA^{Gln}$ complex, and a model of the proposed MetRS·$tRNA^{Met}$ interaction has been described (Perona et al., 1991).

β-Strand F in MetRS (Figure 1) corresponds to the β-strand of GlnRS that contacts $tRNA^{Gln}$. The sequence of this peptide is Arg356-Tyr357-Tyr358-Tyr359-Thr360. We have recently examined the effect of several mutations in this region (Tyr358 → Ala and Tyr359 → Ala) on interaction of MetRS with $tRNA^{Met}$ (Ghosh, et al., 1991). Surprisingly, these changes had no effect on interaction of the enzyme with its tRNA

FIGURE 1: Schematic drawing of the structure of MetRS. The regions of structural homology with GlnRS (residues 104–124 and residues 340–378, including β-strand F) are shown in black. The Rossmann fold, containing the active site of the enzyme, is shaded in gray. The figure was drawn using the programs MAXIMAGE/PREMA kindly provided by Mark Rould, Yale University.

substrate, but rather had dramatic effects on steps involved in methionine activation. In the present work, we have explored the role of an additional residue in this region, Arg356. Conversion of this residue to Gln had a negligible (2–3-fold) effect on tRNA aminoacylation (Table II) and no effect on methionine activation (Table III), suggesting a nonessential role for this amino acid in MetRS.

Earlier cross-linking experiments with $tRNA^{fMet}$ containing a 14-Å-long cross-linker attached to the D loop had shown that the tRNA could be covalently coupled to two lysine residues in the C-terminal domain of MetRS, Lys402 and Lys439 (Valenzuela & Schulman, 1986; Leon & Schulman, 1987; Schulman et al., 1987). In the present work, these lysines have been converted to asparagine residues with little or no effect on aminoacylation by MetRS (Table I). This is in keeping with previous results showing that modification of these sites by the cross-linker had no significant effect on enzyme activity (Schulman et al., 1981).

Mutagenesis of positively charged residues on the surface of *B. stearothermophilus* TyrRS identified a group of nine Lys and Arg residues essential for aminoacylation of $tRNA^{Tyr}$ (Bedouelle & Winter, 1986). Six of these residues were located in the C-terminal domain of the protein, while the remainder lay close to the active site in the N-terminal domain. We have begun a similar search for positively charged residues on the surface of MetRS that might be essential for interaction of the enzyme with $tRNA^{Met}$. Most of the changes introduced to date have had little or no effect on aminoacylation [Table I and Ghosh et al. (1990)].

Lys465 was considered to be a good candidate for a site of interaction with $tRNA^{Met}$ due to its proximity to the anticodon of the enzyme-bound tRNA. Earlier studies had shown, however, that conversion of this residue to Asn had little or no effect on the aminoacylation reaction (Ghosh et al., 1990). A second mutation to Ala465 was made and confirmed that the lysine side chain plays no significant role in tRNA recognition (Table I). Arg380 is conserved in the methionine synthetases of *T. thermophilus* (Nureki et al., 1990) and yeast (Fasiolo et al., 1985), and a Lys is found at the same position in yeast mitochondrial MetRS (Tzagoloff et al., 1989). Conversion of this residue in *E. coli* MetRS to Gln had little effect on aminoacylation (Table I). A more drastic change to Ala380 increased the K_m for aminoacylation of $tRNA^{fMet}$ 8-fold and decreased k_{cat}/K_m 5-fold (Table II), suggesting the possible involvement of this residue in interaction with the sugar–phosphate backbone of the tRNA.

Conversion of Arg442 to Gln produced a 3-fold increase in K_m for $tRNA^{fMet}$ and overall 5-fold decrease in k_{cat}/K_m (Table

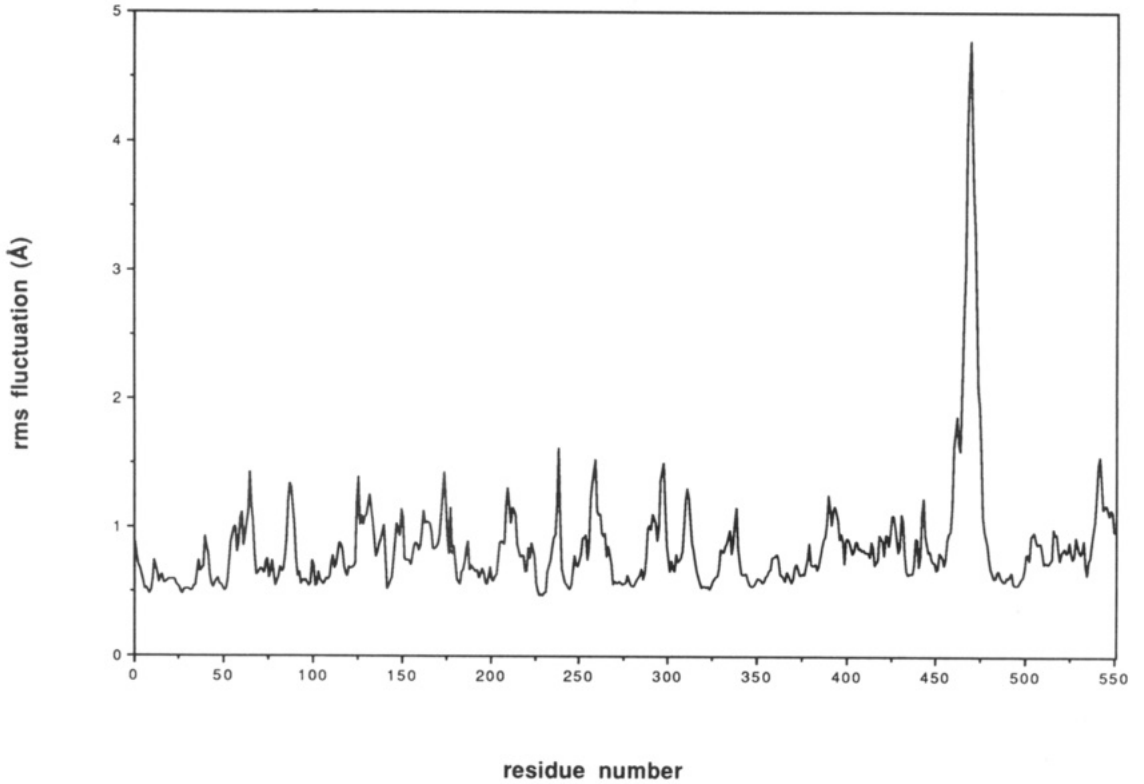

FIGURE 2: Plot of the rms fluctuation in angstroms of each amino acid residue of MetRS as derived from the molecular dynamics simulation.

		391	395
MetRS (Ec)	387	N K V V N L A S R N A G F	
MetRS (Tth)	351	D D L G N L V Q R T R A M	
MetRS (Sc)	579	A N L G N F V A R L I K F	

FIGURE 3: Conservation of the residues corresponding to Asn391 and Arg395 in *E. coli* (Ec) MetRS (Barker et al., 1982) in methionine synthetases from *T. thermophilus* (Tth) (Nureki et al., 1991) and the cytoplasm of *S. cerevisiae* (Sc) (Fasiolo et al., 1985). The synthetases have been numbered such that amino acid 1 corresponds to the second codon of the structural gene. The primary structure of mitochondrial MetRS from *S. cerevisiae* is also known (Tzagloff et al., 1989) but has been omitted here because of uncertainty in the alignment of the structure with those shown above due to a large insertion in this region of the mitochondrial enzyme.

II), suggesting that this residue may also play a minor role in the aminoacylation reaction. In contrast, conversion of Arg395 to Gln dramatically affected both kinetic parameters for aminoacylation, increasing K_m for $tRNA^{fMet}$ 30-fold and decreasing k_{cat}/K_m 25 000-fold (Table II). The mutation had little or no effect on interaction of MetRS with methionine or ATP (Table III), indicating no global change in the structure of the enzyme had occurred. The large loss of specificity of MetRS for its tRNA substrate also rendered the enzyme incapable of supporting growth of *E. coli* cells by in vivo complementation of a strain containing a defective chromosomal MetRS gene. In order to determine whether the effect of the Arg395 → Gln mutation was specifically due to loss of the arginine side chain or to introduction of negative factors caused by the presence of the glutamine side chain, a second mutation at this site, Ala 395, was examined. The Ala395 mutant showed an even higher K_m for $tRNA^{fMet}$ and about 4-fold higher k_{cat} than the enzyme containing Gln395, indicating a specific role for the Arg side chain in the activity of the wild-type synthetase.

The large effect of mutations at position 395 on the aminoacylation activity of MetRS suggests that Arg395 plays a direct or indirect role in interaction of the enzyme with the $tRNA^{Met}$ anticodon, the major recognition site for this enzyme. In support of a specific function for Arg395 in tRNA selection, an arginine residue is conserved at the same site in yeast cytoplasmic MetRS (Fasiolo et al., 1985) and in *Thermus thermophilus* MetRS (Nureki et al., 1991) (Figure 3). An additional residue, Asn391, is also conserved in all three of the methionine synthetases. Conversion of this residue to Ala increased the K_m for aminoacylation of $tRNA^{fMet}$ about 20-fold with only a 2-fold reduction in k_{cat} (Table II).

Previous cross-linking experiments had identified a residue (Lys465) within 14 Å of the anticodon binding site of MetRS (Valenzuela & Schulman, 1986; Leon & Schulman, 1987; Schulman et al., 1987). Extensive analysis of the potential

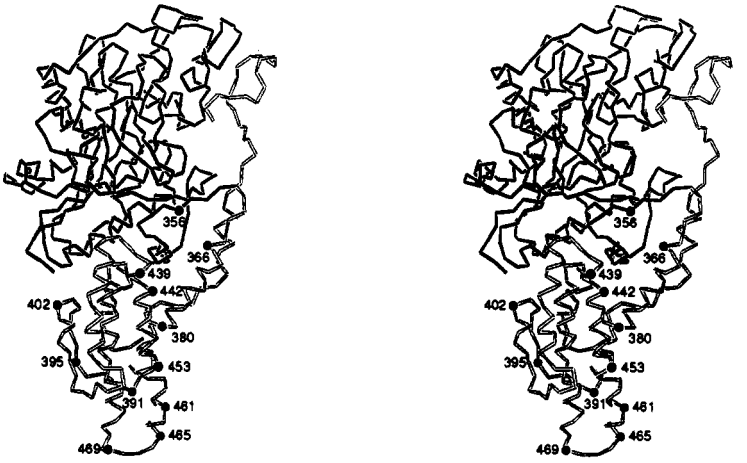

FIGURE 4: Stereoview of MetRS. The N-terminal domain of the enzyme is shown by filled lines, and the C-terminal domain is shown by open lines. The location of positively charged residues on the surface of the C-terminal domain that have been studied in the present work and by Ghosh et al. (1990) is indicated on the figure. Also shown are the locations of Asn391 and Trp461. The programs MAXIMAGE/PREMA (Mark Rould, Yale University, 1990) were used to create the figure.

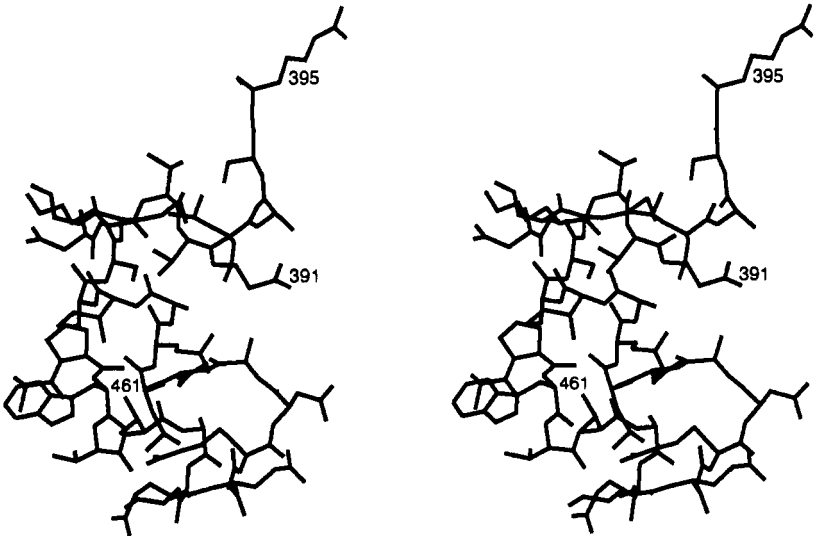

FIGURE 5: Stereoview of the proposed anticodon binding domain of MetRS.

role of hydrophilic residues[3] in this domain in anticodon recognition by site-directed mutagenesis revealed that only one such residue, Trp461, was essential for interaction with the tRNA (Ghosh et al., 1990). Mutations at this site increased the K_m for aminoacylation of $tRNA^{fMet}$ about 60-fold, with little or no effect on k_{cat} (Ghosh et al., 1990), and greatly reduced the affinity of the tRNA for the protein (Meinnel et al., 1991). Changes in all three anticodon bases (5′CAU3′) affect recognition of the tRNA by wild-type MetRS, with the wobble base C_{34} playing the most important role in $tRNA^{Met}$ identity (Schulman & Goddard, 1973; Schulman & Pelka, 1983, 1988). Examination of the kinetic parameters for aminoacylation of a series of anticodon derivatives of $tRNA^{Met}$ by MetRS containing a Trp461 → Phe mutation indicated that Trp461 was essential for recognition of the methionine CAU anticodon and exclusion of tRNAs containing non-methionine anticodons. The data suggested that this residue may directly interact with anticodon base C_{34}.

Conversion of the methionine anticodon of $tRNA_m^{Met}$ to the amber anticodon 5′CUA3′ greatly reduces the ability of the tRNA to be aminoacylated by MetRS, rendering it inactive as an amber suppressor tRNA in vivo when present as a single-copy gene (Meinnel et al., 1991). Selection of mutants of MetRS that allow aminoacylation of the mutant tRNA was found to occur at a frequency of only 1 in 10^9, indicating that such mutations are very rare. DNA sequencing showed that the activating mutation resulted in an Asp to Tyr change at position 456, a site close to Trp461. Conversion of Trp461 to Ala greatly increased the frequency with which MetRS mutants capable of aminoacylating the amber suppressor tRNA could be selected; however, no mutants were found outside of the Trp461 domain. These results led to the proposal that this domain contains all of the information required for anticodon recognition of methionine tRNAs (Meinnel et al., 1991).

The finding that mutations directly introduced into wild-type MetRS at sites containing hydrophilic side chains[3] near Trp461 fail to significantly affect the activity of the enzyme was unexpected. The major role of the anticodon in tRNA recognition suggested that numerous protein side chains might participate in selection of this specific sequence. Indeed, solution of the crystal structure of the GlnRS·$tRNA^{Gln}$ complex, where anticodon recognition also plays a major role, has revealed that many different protein side chains contact functional groups on each of the anticodon bases (Rould et al., 1991). These contacts arise from two separate domains of GlnRS that come together to form the anticodon binding site. The results presented in the present work suggest that more than one protein domain is also involved in tRNA anticodon recognition by MetRS.

Arg395 is located at the extremity of one region of the C-terminal domain of MetRS, at a distance of more than 20 Å from Trp461, and on the opposite side of the molecule (Figure 4). The distance between the Cα carbons of Trp461 and Asn391 is 10 Å; thus, it is possible for these residues to simultaneously interact with the anticodon of $tRNA^{Met}$ (Figure 5). Attempts to construct a detailed model of the interaction of the anticodon loop with the static enzyme structure using molecular graphics have not been satisfying, however, since a significant change in the conformation of the loop containing amino acid residues 450–480 would be required to dock the tRNA on the synthetase with the anticodon grasped on each side by Trp461 and Asn391. Molecular dynamics simulation (Figure 2) on the MetRS structure reveals a striking peak around residue 465, indicating a large flexibility of the loop containing residues 460–477. A more detailed analysis involving the rms fluctuations over part of the "productive" dynamics also shows a slow drift of the loop with time, indicating that changes in the conformation of this region of the protein structure can readily occur. The conformation of the anticodon loop of $tRNA^{Met}$ is also expected to be flexible and may undergo significant changes on complex formation with the protein, as has already been directly observed in the GlnRS·$tRNA^{Gln}$ and AspRS·$tRNA^{Asp}$ complexes (Rould et al., 1991; Ruff et al., 1991).

Taken together, the results presented here suggest that tRNA recognition by MetRS involves an induced-fit mechanism, with a significant conformational change occurring in the anticodon binding domain of the enzyme on interaction with the cognate tRNA, allowing simultaneous contacts to be made between the anticodon loop and the two peptides containing Asn391–Arg395 and Trp461. Unlike mutations at Asn391 and Trp461, mutations at Arg395 strongly affect the rate of aminoacylation by MetRS as well as initial tRNA binding, indicating that this residue plays a role in stabilization of the transition state for the transfer reaction. Thus, changes in the structure of the protein in this domain are coupled to changes at the active site of the enzyme, enhancing the efficiency of the transfer step. Such changes could involve a long-range effect on the conformation of reactive groups at the active site and/or a direct effect on alignment of the 3′ terminus of the tRNA at the active site. Substrate-induced conformational changes may be an important feature of other tRNA–synthetase interactions as well. To date, structural data are not available to allow direct comparison of any synthetase in both its free and its complexed state. Such data will be required to fully evaluate the extent of the substrate-induced changes in these enzymes and the role such changes may play in achieving the specificity and efficiency of aminoacylation reactions.

[3] Including Asp456, Glu457, Gln458, Lys465, Glu467, Arg469, Asp470, and Asp472.

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