Two Separate Peptides in *Escherichia coli* Methionyl-tRNA Synthetase Form the Anticodon Binding Site for Methionine tRNA†

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ABSTRACT: The amino acid residues Asn391, Arg395, and Trp461 in methionyl-tRNA synthetase (MetRS) of *Escherichia coli* are involved in the anticodon-dependent recognition of its cognate tRNAs [Ghosh, G., Pelka, H., & Schulman, L. H. (1990) *Biochemistry 29*, 2220–2225; Ghosh, G., Kim, H. Y., Demaret, J. P., Brunie, S., & Schulman, L. H. (1991) *Biochemistry 30*, 11767–11774]. While tryptophan at position 461 was shown to bind directly to the wobble base at position 34 in the $tRNA^{Met}$ anticodon, the role of residues 391–395 was not thoroughly explored. To gain further insight into the role of the 391–395 residues and nearby residues, appropriate mutations were analyzed for aminoacylation activity, as well as tRNA binding. Mutations of the phylogenetically conserved asparagine at position 391 increased the K_m for aminoacylation of $tRNA^{Met}$ 18–40-fold. Further analysis using fluorescence titration indicated that the mutation affected initial complex formation, since the K_d for $tRNA^{Met}$ binding had increased at least 15-fold over wild type. Kinetic analysis of mutationally altered derivatives of MetRS with a series of $tRNA^{Met}$ derivatives containing base substitutions in the anticodon revealed sequence-specific interaction between the amino acid residue at position 391 and the U_{36} of the anticodon of $tRNA^{Met}$. In addition to position 391, position 387 was also found to affect $tRNA^{Met}$ binding and aminoacylation, indicating a possible significant role in interaction of the enzyme with the anticodon of $tRNA^{Met}$. These results indicate that the peptide segment containing residues 391–395 is involved in the direct recognition of the 3′ end of the anticodon.

Aminoacyl-tRNA synthetases catalyze the following ATP-dependent activation of amino acids and their subsequent transfer to the 3′ terminus of appropriate cognate tRNAs:

$$AA + ATP \rightarrow AA\text{-}AMP + PP_i \quad (1)$$

$$AA\text{-}AMP + tRNA^{AA} \rightarrow AA\text{-}tRNA^{AA} + AMP \quad (2)$$

where AA represents one of the 20 amino acids and $tRNA^{AA}$ represents a tRNA specific for that amino acid. Selection of the appropriate tRNA substrate for amino acid attachment occurs by formation of RNA–protein contacts unique to each cognate tRNA–synthetase pair. One of the factors complicating the characterization of aminoacyl-tRNA synthetases is that, despite their common biological role, these enzymes exhibit a great diversity in size and oligomeric structure, making categorization difficult (Burbaum et al., 1991; Nagel et al., 1991; Moras, 1992).

While considerable progress has been made in identifying important nucleotide contacts in the tRNA (Normanly & Abelson, 1989), little is known about the structural features in the synthetases which are responsible for making these contacts. Indeed, the best characterized aminoacyl-tRNA synthetase is the *Escherichia coli* glutaminyl-tRNA synthetase (GlnRS),[1] for which an X-ray cocrystal structure with $tRNA^{Gln}$ and bound ATP has been solved to 2.5 Å (Rould et al., 1989, 1991). The structure reveals large areas of contacts between amino acid side chains of the synthetase and each of the three anticodon bases of the tRNA. In addition, sequence-specific interactions occur with the acceptor stem of the tRNA and possibly several additional sites. A high-resolution cocrystal structure of a yeast AspRS-$tRNA^{Asp}$ complex has also been described and has provided insight into the nature of the protein–RNA interactions which occur in this system (Ruff et al., 1991). In addition to these cocrystal complexes, high-resolution structures of uncomplexed aminoacyl-tRNA synthetases have also been reported for *Bacillus stearothermophilus* TyrRS (Brick et al., 1988), for *E. coli* SerRS (Cusack et al., 1990), and for a proteolytic fragment of *E. coli* MetRS (Brunie et al., 1990). The crystal structures of tRNA-synthetase complexes provide a detailed picture of the many contacts involved in the interaction of the two molecules. It should be noted that in both structures the tRNA molecule is disordered in a way which cannot be predicted from model building. However, many aspects of initial complex formation and catalysis will only become clear by carrying out site-directed mutagenesis using the crystal structure as a guide in selecting residues for study.

Native *E. coli* MetRS is a homodimeric protein with each protomer containing 676 amino acids. Limited trypsin digestion removes a carboxyl-terminal fragment which forms the dimerization interface, leaving a biologically active monomeric enzyme containing 551 amino acids (mol wt 64 000; Cassio & Waller, 1971). The crystal structure of this fragment has been solved to 2.5 Å with ATP (Brunie et al., 1990) and to 2.0 Å without ATP (unpublished results). While

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[1] Abbreviations: MetRS, methionyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase; MetRS547, monomeric MetRS truncated at amino acid residue 547; N391D, MetRS547 with asparagine 391 replaced by aspartic acid; 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.

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the structures of the uncomplexed synthetases can be used to predict the interaction between tRNA and synthetase on the basis of a rough model building, ultimately these models must be verified with cocrystal structures, cross-linking experiments, or mutational analysis. Previous work from our laboratory on the mechanism of substrate recognition by the *E. coli* MetRS indicated that the anticodon contains the major determinants for recognition of tRNAs by MetRS. An extensive set of mutants of both $tRNA^{fMet}$ and $tRNA^{Met}$ shows that all three anticodon bases are required for efficient aminoacylation by the cognate synthetase, with the largest effects seen at C_{34} in the wobble position (Schulman & Pelka, 1985, 1988, 1989). Cross-linking experiments indicated that Lys465 in MetRS lies 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 5′ nucleotide of the anticodon, C_{34}, and a role in exclusion of tRNAs containing non-methionine anticodons (Ghosh et al., 1990; Meinnel et al., 1991). Because Trp461 is thought to interact only with the wobble nucleotide but yet all three nucleotides of the $tRNA^{Met}$ anticodon are required for efficient aminoacylation, it seemed likely that additional residues would be found to interact with the other two anticodon bases. More recent studies have suggested that a separate region in the enzyme, located at some distance from Trp461 and containing Arg395, is also important for $tRNA^{Met}$ recognition (Ghosh et al., 1991b). In this paper we report the effect of site-directed mutagenesis of amino acids in the second region and that we have identified residues responsible for specific interactions with the 3′ end of the anticodon of $tRNA^{Met}$.

MATERIALS AND METHODS

Materials. ^{35}S-Labeled methionine was purchased from Amersham, and ^{32}P-labeled sodium pyrophosphate was obtained from New England Nuclear. Native $tRNA^{fMet}$ having a specific activity for methionine acceptance of 1450 pmol/A_{260} was purchased from Boehringer-Mannheim. Other tRNAs were prepared by in vitro transcription as described before (Ghosh et al., 1990). Deoxyoligonucleotides were synthesized on an Applied Biosystems DNA synthesizer by a core facility of the Albert Einstein College of Medicine. Enzymes for plasmid constructions were obtained from New England Biolabs.

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 MetRS mutants were prepared: Glu375→Ala, Gln379→Ala, Asn382→Ala, Asp384→Ala, Asn387→Ala, Lys388→Ala, Val390→Ala, Asn391→Asp, Ser394→Ala, Arg395→Lys, Asn396→Ala, and Arg501→Ala. The expected mutations were confirmed by dideoxy DNA sequencing.

Purification of Enzymes. Wild-type MetRS547 and the mutant enzymes were purified from overproducing *E. coli* strains carrying the corresponding MetRS genes in the plasmid pGG3 as described before (Ghosh et al., 1990). Cells from overnight cultures grown in enriched medium were sonicated in 20 mM potassium phosphate, pH 7.0, 0.5 mM EDTA, 0.1 mM PMSF, 10% glycerol, and 10 mM 2-mercaptoethanol and centrifuged at 100000*g* for 1h. The crude extracts were loaded onto a column of DEAE-Sepharose (Pharmacia) equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 0.1 mM PMSF, 10% glycerol, and 10 mM 2-mercaptoethanol) containing 20 mM KCl. The protein was eluted with the same buffer containing 80 mM KCl and further purified on a Mono-Q column with a gradient of 0–600 mM KCl in 20 mM Tris-HCl, pH 7.5, 1 mM DTT, and 10% glycerol. The protein eluted at 350 mM KCl and was concentrated by Centricon 30 centrifugation. All of the proteins used were greater than 95% pure as judged by SDS–polyacrylamide gel electrophoresis and contained no detectable native MetRS. ATP–PP_i exchange and aminoacylation assays with the purified proteins were carried out as detailed elsewhere (Ghosh et al., 1990).

Enzyme Assays. 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. The activity of each extract was compared with that of wild-type MetRS547. Kinetic parameters for methionine acceptance by native $tRNA^{fMet}$ were measured with purified enzymes and 0.5–40 μM tRNA. Kinetic parameters for the tRNA transcripts were determined as described for $tRNA^{fMet}$ except that reaction mixtures contained 0.5–60 μM pure tRNA and 10 mM $MgCl_2$ in addition to the $MgCl_2$ added with the tRNA transcript. Kinetic constants were calculated from the nonlinear regression analysis program Enzfitter (Biosoft). When K_m values for aminoacylation of tRNAs were greater than 100 μM, individual kinetic parameters could not be accurately determined, and k_{cat}/K_m values were obtained from the slope of the linear plot of initial velocity versus tRNA concentration.

In Vitro Transcription. Synthetic tRNA genes having a T7 RNA polymerase promoter adjacent to the 5′ end and a *Bst*NI restriction enzyme site at the 3′ end (Sampson & Uhlenbeck, 1988) were constructed from overlapping deoxyoligonucleotides and inserted into the *Eco*RI/*Sst*I site of the phagemid vector pUC119 as described before (Schulman & Pelka, 1988). Plasmids linearized with *Bst*NI were transcribed with T7 RNA polymerase, and the transcripts were purified by electrophoresis on denaturing polyacrylamide gels followed by HPLC chromatography (Schulman & Pelka, 1990).

Fluorescence Titration. The association of MetRS and its cognate tRNA was monitored by the change in the intrinsic fluorescence intensity of the enzyme. Aliquots (5 μL) of tRNA solutions were added to an enzyme solution of 1.5 mL (1.0 μM) in the quartz cell of a Perkin-Elmer LS-5B luminescence spectrometer. The enzyme solution contained 20 mM imidazole, 10 mM 2-mercaptoethanol, 1 mM DTT, and 7 mM $MgCl_2$. Excitation and emission wavelengths were 295 and 340 nm with bandwidths of 3 and 10 nm, respectively. All titration points were corrected for dilution and inner-filter effect. The corrections for the inner-filter effect were made according to the formula (Lin et al., 1988)

$$f = \frac{(P_0 + \Delta A)(1 - 10^{-P_0})}{P_0(1 - 10^{-(P_0+\Delta A)})}$$

where f is the correction factor, P_0 is the sample absorbance before titration at the excitation wavelength, and ΔA is the absorption change by the addition of tRNA.

RESULTS

Synthesis and Activity of MetRS547 Mutants. Site-directed mutagenesis was previously 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 results in the overproduction of a

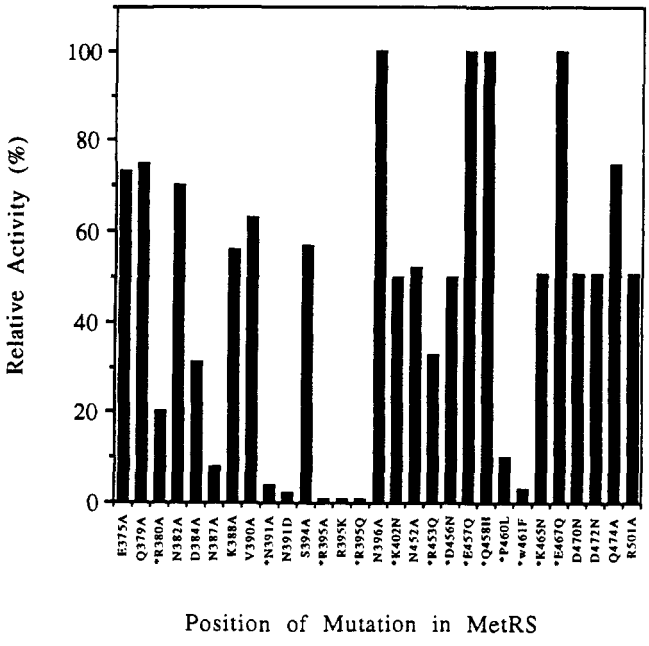

FIGURE 1: Relative aminoacylation activity at each position in MetRS. Crude extracts of cells overproducing wild-type enzyme and MetRS547 mutants were assayed for methionine acceptor activity as described under Materials and Methods. The activity of each extract was compared with that of wild-type MetRS. Asterisks indicate data previously obtained for mutations in MetRS (Ghosh et al., 1990, 1991).

truncated protein (MetRS547; mol wt 62 400) corresponding to a biologically active monomeric fragment of MetRS (Mellot et al., 1989). This protein can be readily separated from the native dimeric enzyme of molecular weight 2 × 76 000 (Dardel et al., 1984) upon purification to allow detailed kinetic analysis of mutationally altered forms. Using this truncated form of MetRS, phylogenetically conserved residues as well as charged amino acids on the surface of the C-terminal domain were altered to help identify regions of the protein involved in tRNA binding. In addition, mutations were introduced to change amino acids which modeling studies indicated were capable of forming hydrogen bonds with the tRNA anticodon. Mutations of these amino acids to alanine were chosen to thereby remove the hydrogen bonds with minimal steric disruption.

To screen for defective synthetase mutants, MetRS expression levels and methionine acceptor activity were determined from crude extracts for each MetRS547 derivative constructed. Figure 1 shows the relative aminoacylation activities of MetRS547 derivatives carrying the indicated mutation within their carboxyl-terminal domain. The entire MetRS gene was sequenced for all MetRS mutations resulting in significantly lower activity than wild type to rule out the possibility of additional unexpected mutations. The mutationally altered proteins showing less than 20% of wild-type activity were then purified to homogeneity for more complete biochemical characterization.

Characterization of Kinetic Parameters for Aminoacylation of Native tRNA^{fMet} by Purified Mutationally Altered Forms of MetRS547. Of the mutations introduced into the C-terminal domain only the 387, 391, 395, and 461 substitutions were found to have a significant effect on tRNA aminoacylation. Table I compares the kinetic parameters for aminoacylation of native *E. coli* tRNA^{fMet} by wild-type MetRS with that by the mutationally altered enzymes showing significant defects in the aminoacylation reaction. It was previously shown that conversion of Arg395 to Gln or Ala had a major negative effect on both kinetic parameters. Interestingly, the more conservative Arg to Lys change was also unable to maintain tRNA aminoacylation activity, indicating an important role for the structure of the side chain, not simply its charge, in the aminoacylation reaction. An N387A mutation produced a more modest 9-fold increase in K_m for tRNA^{fMet} and a small increase in k_{cat}, resulting in a net 8-fold decrease in k_{cat}/K_m. In contrast, conversion of Asn391 to Asp reduced k_{cat}/K_m about 80-fold, a greater effect than that observed for the N391A alteration (Table I). Substitution of Asn391 with Ala or Asp resulted in a decrease of the interaction energy between the enzyme and the tRNA^{fMet} of 2.3 or 2.7 kcal/mol, respectively (Table II). The fact that the K_m for tRNA^{Met} is larger for N391D than for the wild type suggests a possible repulsion between the tRNA and the negatively charged side chain of Asp391.

Table I: Kinetic Parameters for Aminoacylation of Native tRNA^{fMet} by Wild-Type and Mutant MetRS547[a]

enzyme	K_m^{tRNA} (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	rel k_{cat}/K_m
wild type	1.2 ± 0.2	3.2 ± 0.2	2.7	1.0
N387A	11 ± 2	3.8 ± 0.4	0.34	0.13
N391A[b]	22 ± 4	1.6 ± 0.2	0.07	0.03
N391D	48 ± 9	1.6 ± 0.2	0.033	0.012
R395A[b]	113 ± 31	(1.7 ± 0.4) × 10^{-2}	1.5 × 10^{-4}	6 × 10^{-5}
R395K	33 ± 9	(4.8 ± 0.7) × 10^{-3}	1.5 × 10^{-4}	6 × 10^{-5}
R395Q[b]	39 ± 9	(4.7 ± 0.6) × 10^{-3}	1.2 × 10^{-4}	4 × 10^{-5}
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 usinng tRNA^{fMet} concentrations of 0.5–6 μM for wild-type MetRS547, 5–30 μM for N387A, and 5–40 μM for N391D and R395K. [b] Data taken from Ghosh et al. (1991).

Table II: Apparent Variations of the Interaction Energy between MetRS and tRNA^{fMet}

	k_{cat}/K_m	ΔG_{app}[a] (kcal/mol)
wild type	2.7	
N391A	0.07	2.3
N391D	0.033	2.7
W461F	0.06	2.4

[a] $\Delta G_{app} = -RT \ln[(k_{cat}/K_m)_{mutant}/(k_{cat}/K_m)_{wt}]$ (Fersht, 1985).

Activity of the MetRS547 Mutations in the ATP–PP$_i$ Exchange Reaction. Using the mutationally altered MetRS derivatives described above, the effects on synthesis of methionyl adenylate were investigated by measuring the rate of exchange of ^{32}P-labeled pyrophosphate into ATP catalyzed by MetRS. Determination of K_m values for methionine and ATP in the ATP–PP$_i$ exchange reaction showed that there was no significant effect of any of the amino acid substitutions on the initial complex formation with these substrates (Table III). Measurement of k_{cat} for each of the mutant enzymes revealed insignificant (approximately 2-fold) decreases in the rates of catalysis by the N391D, R395K, and W461F enzymes. These data clearly indicate that the major effect of all the mutations is on the interaction of the enzyme with its tRNA substrate and/or the formation of the aminoacyl-tRNA bond. As shown below, the effect is primarily on the binding of the tRNA^{Met}.

Titration of MetRS547 Wild-Type and Mutant Enzymes with tRNA^{fMet}. tRNA binding can be studied by monitoring the quenching of the intrinsic protein fluorescence upon ligand binding (Blanquet et al., 1973). Using this approach, dissociation constants were deduced for the MetRS547-tRNA^{fMet} complex using the wild-type MetRS and mutants at positions 387, 391, 395, and 461. Titrations were carried out in parallel, using the wild-type MetRS547 and mutationally

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
R380A	21 ● 3	538 ± 56	51 ± 6
N387A	22 ± 2	525 ± 84	63 ± 5
N391A[c]	25 ± 3	529 ± 56	56 ± 3
N391D	24 ± 6	566 ± 21	33 ± 3
R395A[c]	23 ± 3	544 ● 105	41 ± 3
R395K	27 ± 4	539 ± 84	29 ± 5
R395Q[c]	30 ± 8	515 ± 129	15 ± 2
W461F	27 ± 3	550 ± 71	28 ± 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 by 2 mM methionine and ATP concentrations of 100–1000 μM. [b] k_{cat} is the average of the values determined in assays in which the methionine and ATP concentrations were varied. [c] Data taken from Ghosh et al. (1991).

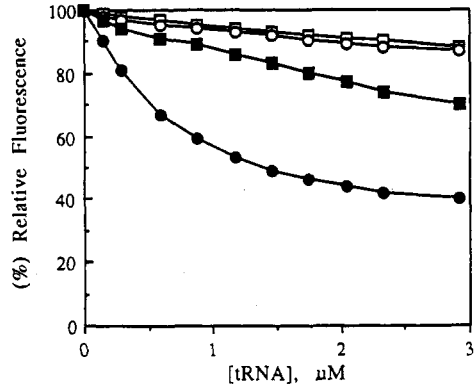

FIGURE 2: Binding of $tRNA^{fMet}$ to MetRS547 wild-type and mutant enzymes: wild type (●); N387A (■); R395A and R395K (○); and W461F, N391A, and N391D (□). The titrations were carried out as described in Materials and Methods.

altered enzymes simultaneously. Figure 2 shows the profile of protein fluorescence quenching upon binding of $tRNA^{fMet}$. The dissociation constant for the wild-type MetRS547 enzyme with $tRNA^{fMet}$ was 0.9 ± 0.3 μM with 65% maximum enzyme fluorescence quenching. This K_d was similar to the K_m for tRNA (1.2 ± 0.2 μM) in the aminoacylation reaction. In the case of the mutationally altered MetRS derivatives, a small decrease of fluorescence could be observed upon addition of $tRNA^{fMet}$. However, even at 5 μM tRNA, saturation could not be observed with the mutant enzymes. The tRNA dissociation constants of the enzymes altered at positions 387, 391, 395, and 461 were calculated to be more than 12 μM. This result confirms that the mutations at positions 387, 391, 395, and 461 affect initial complex formation and, hence, tRNA binding.

Effect of Anticodon Base Changes on Recognition of tRNAs by MetRS Mutants. In order to test whether the amino acid side chain of Asn391 or Arg395 is involved in anticodon recognition, a series of $tRNA^{Met}$ derivatives with altered anticodon sequences were prepared by in vitro transcription and assayed with the wild-type and the N391A, N391D, and R395Q mutant enzymes (Table IV). The tRNA transcript containing the normal methionine anticodon CAU was found to exhibit kinetic parameters with MetRS547 which were the same as those observed with native $tRNA^{fMet}$ (Ghosh et al., 1990). Alterations in the anticodon sequence of $tRNA^{Met}$ affected both the K_m and the k_{cat} for aminoacylation by wild-type and mutant enzymes. However, the Asn391→Asp mutation yielded a relatively low K_m when $tRNA^{Met}$(CAC) was used as the substrate (Table IV). The data show that the effect of the two mutations together, the N391D mutant and the $tRNA^{Met}$(CAC) derivative, increased the affinity of the initial complex formation between the enzyme and the tRNA. This effect was not observed with N391A and R395Q mutants, which showed immeasurably high K_m values with all of the non-methionine anticodon derivatives tested.

DISCUSSION

The mechanism of recognition of tRNAs by aminoacyl-tRNA synthetases has been extensively studied. 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; Burbaum et al., 1991). The enzymes in this group share two short consensus sequences (HIGH and KMSKS) and contain a conserved structural motif (the so-called "nucleotide 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 that are structurally homologous in the two enzymes, although they contain no conserved primary sequences. Conservation of one of these motifs 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 rough model of the proposed MetRS-$tRNA^{Met}$ interaction has been described (Perona et al., 1991). However, because the tRNA anticodon binding domains in MetRS and GlnRS have very different 3-D structures, identification of MetRS residues involved in anticodon binding is not straightforward.

Mutagenesis of positively charged residues in the carboxyl-terminal domain of *B. stearothermophilus* TyrRS identified a group of nine lysine and arginine residues essential for aminoacylation of $tRNA^{Tyr}$ (Bedouelle & Winter, 1986). However, the C-terminal domain of MetRS is structurally different from that of GlnRS or TyrRS, and most of the positively charged residues on the surface of MetRS were found to have little or no effect on aminoacylation (Ghosh et al., 1990). Cross-linking experiments with $tRNA^{fMet}$ containing a 14-Å-long cross-linker revealed a site in MetRS (Lys465) close to the anticodon binding domain (Valenzuela & Schulman, 1986; Leon & Schulman, 1987; Schulman et al., 1987). Combining site-directed mutagenesis with molecular modeling using the crystal structure of MetRS and the cross-linking data allowed identification of a residue, Trp461, involved in anticodon recognition Figure 3. 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 interacts directly with anticodon base C_{34} (Ghosh et al., 1990).

While C_{34} in the $tRNA^{Met}$ anticodon plays the largest role in tRNA's identity, substitutions of A_{35} or U_{36} can also reduce the kinetics of aminoacylation of $tRNA^{Met}$ by MetRS by as much as 10^4 (Schulman & Goddard, 1973; Schulman & Pelka, 1983, 1988). It is possible that conformational changes occur in the anticodon when the bases are substituted. However, the major role of the anticodon in tRNA recognition suggested that numerous protein side chains might participate in the selection of this specific sequence. Indeed, the solution of the crystal structure of the GlnRS-$tRNA^{Gln}$ complex has revealed that many different protein side chains contact functional

Table IV: Kinetic Parameters for Aminoacylation of tRNA Transcripts by Wild-Type and Mutant MetRS

	wild type		N391A		N391D		R395Q	
anticodon	K_m (μM)	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	K_m (μM)	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	K_m (μM)	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	K_m (μM)	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)
$tRNA^{fMet}$ (CAU)	1.1 ± 0.3	2.8 ± 0.3	22 ± 4	(7.3 ± 0.9) × 10^{-2}	48 ± 9	(3.3 ± 0.2) × 10^{-2}	39 ± 9	(1.2 ± 0.2) × 10^{-4}
$tRNA_m^{Met}$ (CAG)	76 ± 11	(2.0 ± 0.2) × 10^{-3}	83 ± 26	(5.4 ± 1.2) × 10^{-4}	>100	(4.5 ± 0.1) × 10^{-6}	>100	(1.2 ± 0.1) × 10^{-6}
(CAC)	>100	(3.9 ± 0.4) × 10^{-4}	>100	(9.2 ± 0.4) × 10^{-6}	18 ± 6	(7.1 ± 1.1) × 10^{-6}	>100	(4.2 ± 0.3) × 10^{-6}
(UAU)	>100	(5.7 ± 0.5) × 10^{-4}	>100	(3.4 ± 0.1) × 10^{-4}	>100	(2.1 ± 0.1) × 10^{-5}	96 ± 19	(5.9 ± 0.3) × 10^{-5}
(GAU)	>100	(8.4 ± 0.2) × 10^{-5}	>100	(7.0 ± 0.2) × 10^{-6}	>100	(3.6 ± 0.3) × 10^{-6}	>100	(4.3 ± 0.2) × 10^{-6}

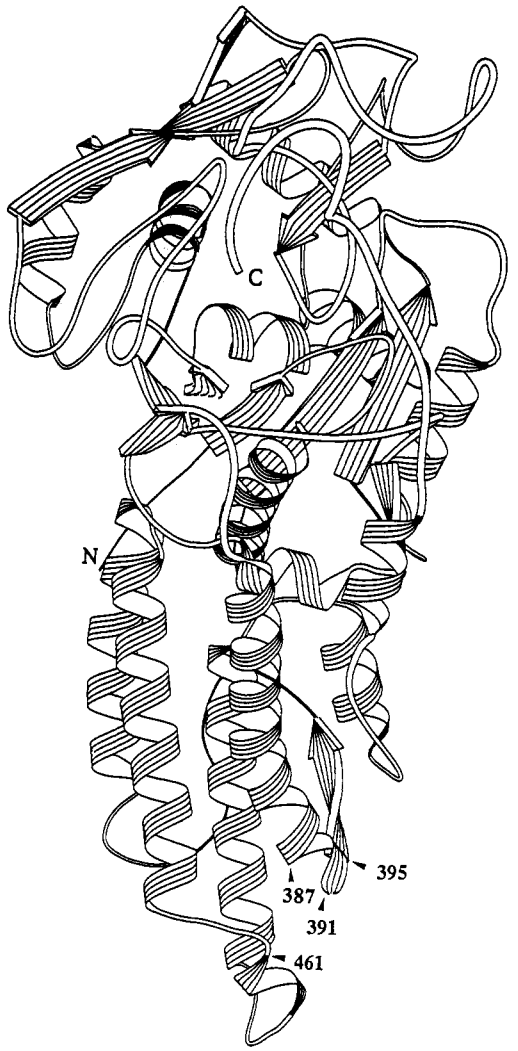

FIGURE 3: Ribbon structure of the MetRS polypeptide backbone. The locations of Asn387, Asn391, Arg395, and Trp461 are indicated on the figure.

groups on the anticodon bases (Rould et al., 1991). This suggests that, in addition to Trp461, additional residues are likely to make discriminating contacts with the $tRNA^{Met}$ anticodon, particularly with the U_{36} base. Converting Asn391 into Asp increased the K_m for $tRNA^{fMet}$ about 40-fold with only a 2-fold reduction in k_{cat} (Table I). This suggests that Asn391 might play a direct role in the recognition of the $tRNA^{Met}$ anticodon as important as the role of Trp461. However, the small effect of the mutations at position 391 on aminoacylation indicates that although a strong interaction between the anticodon and the enzyme has been lost, the overall orientation of the tRNA molecule on the MetRS molecule is conserved and allows the CCA arm to be correctly positioned in the active site.

While substitution in the $tRNA^{Met}$ anticodon of the base U_{36} with cytosine dramatically reduces the affinity of the $tRNA^{Met}$ for the wild-type enzyme, the affinity increases for a MetRS containing an Asn391→Asp mutation (Table IV). This suggests that the interaction between Asn391 and $tRNA^{Met}$ occurs at the 3′ end of the anticodon, possibly with the U_{36} base directly. In the wild-type enzyme, the side chain of Asn391 might interact with U_{36} by forming a hydrogen bond with the 4-keto group of uracil. In the same way, the aspartic acid side chain might form a hydrogen bond with the 4-amino group of cytosine, which might explain the increased affinity of the Asn391→Asp mutant for the $tRNA^{Met}$(CAC) derivative. Since the side chain of alanine is unlikely to form hydrogen bonds with an amino group, this might explain why the Asn391→Ala mutant exhibits a low affinity for the $tRNA^{Met}$(CAC) derivative. The Asn391→Ala mutant would have a relatively higher affinity for the wild-type $tRNA^{Met}$ anticodon than the Asn391→Asp mutant because there may be an unfavorable electrostatic interaction between the negatively charged Asp side chain and the 4-keto group of uracil.

In support of a specific function for Asn391 in tRNA selection, it should be underlined that an asparagine residue is conserved at the same site in yeast cytoplasmic MetRS (Fasiolo et al., 1985), in *Thermus thermophilus* MetRS (Nureki et al., 1991), and in *B. stearothermophilus* MetRS- (Mechulam et al., 1991).

In order to analyze the anticodon binding domain, similar experiments have been performed on the MetRS from yeast (Despons et al., 1992). However, although Asn391 is well conserved in most known MetRS enzymes, the results obtained from both enzymes, *E. coli* (this work) and yeast (Despons et al., 1992), are rather different. The N391D mutant from *E. coli* led to a 2-fold decrease in k_{cat} for aminoacylation and a 40-fold increase in the K_m for tRNA. In yeast, the corresponding N584D mutant showed a 150-fold decrease in k_{cat}; K_m is unchanged. This means that the role of this conserved Asn is not the same in the two synthetases. It could be argued that since the sequence of the peptide Asn391–Arg395 is well conserved in the two synthetases, the 3-D structure of this peptide can also be expected to be preserved. While this is probably true, the sequence of the peptide Val455–Glu467 in *E. coli* MetRS is not conserved in yeast. A possible correspondence could be Leu653–Ser666, with an inserted Thr in position 661 (Despons et al., 1992). However, there is no evidence that the peptide Leu653–Ser666 in the yeast enzyme has the same 3-D conformation as the observed X-ray structure of the Val455–Glu467 peptide in the *E. coli* enzyme. In *E. coli* MetRS, Trp461 has been shown to be a crucial residue in the recognition of the $tRNA^{Met}$ anticodon. Asp659 in yeast synthetase corresponds to Trp461 in *E. coli* MetRS. Site directed mutagenesis has shown that Asp659 is probably not a key residue in the $tRNA^{Met}$ binding to yeast synthetase. Thus, it is likely that recognition of the CAU anticodon is different in the two synthetases, even if they involve the same two regions of the molecules. We believe that, in both cases, it is not reasonable to speculate on the detailed interaction between the anticodon loop and the MetRS molecule.

From the profile of protein fluorescence quenching upon $tRNA^{fMet}$ binding, the 391 and 461 positions play a major

role in initial complex formation with the tRNA. The distance between the C_α carbons of Trp461 and Asn391 is 10 Å, rendering the possibility that these residues simultaneously interact with both ends of the anticodon. In addition, the significant effect of mutation Asn387→Ala on aminoacylation and tRNA binding (fluorescence titration) by MetRS suggests that Asn387 also plays an important role in the interaction with $tRNA^{Met}$. The side chain of Asn387 is located between Asn391 and Trp461 in the crystal structure, further supporting its possible role in anticodon recognition.

Attempts to construct a model of the interaction of the anticodon loop with the static enzyme structure using molecular graphics indicates that the anticodon might be grasped on each side by Asn391 and Trp461 (Figure 3). However, this requires an opening of the two separate peptides. Molecular dynamics simulation on the MetRS structure revealed a possible significant change in the conformation of the loop containing amino acid residues 460–477 (Ghosh et al., 1991b). The conformation of the anticodon loop of $tRNA^{Met}$ might also undergo significant changes upon complex formation with the protein, on the basis of direct observations of the GlnRS-$tRNA^{Gln}$ and AspRS-$tRNA^{Asp}$ complexes (Rould et al., 1991; Cavarelli et al., 1993). Taken together, the results presented here suggest that tRNA recognition by MetRS allows simultaneous contacts between the anticodon loop and the two peptides Asn387–Arg395 and Val460–Glu467 with a significant conformational change occurring in the anticodon binding domain of the enzyme on interaction with $tRNA^{Met}$.

Unlike the mutations at Asn391 and Trp461, mutations at Arg395 mainly affect the rate of aminoacylation by MetRS as well as initial tRNA binding, suggesting that this residue plays a role in the transition state for the transfer reaction. Changes of Arg395 are coupled to changes at the active site of the enzyme, reducing the efficiency of the transfer step. Such changes could involve long-range effects on the conformation of reactive groups at the active site. We believe that a direct effect on the correct positioning of the 3′ terminus of the tRNA in the active site is more likely. Clearly, Trp461, Asn387, and Asn391 are residues involved in the anticodon recognition. In addition, the integrity of the side chain of Arg395 is required for an accurate alignment of the tRNA molecule on the enzyme. So far, no other residue has been identified to possess such a role.

There is considerable diversity in the way that tRNAs interact with their cognate synthetases. 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 understand the specificity and efficiency of aminoacylation reactions.

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