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Activation of Methionine by *Escherichia coli* Methionyl-tRNA Synthetase†

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ABSTRACT: In the present work, we have examined the function of three amino acid residues in the active site of *Escherichia coli* methionyl-tRNA synthetase (MetRS) in substrate binding and catalysis using site-directed mutagenesis. Conversion of Asp52 to Ala resulted in a 10000-fold decrease in the rate of ATP–PP_i exchange catalyzed by MetRS with little or no effect on the K_m's for methionine or ATP or on the K_m for the cognate tRNA in the aminoacylation reaction. Substitution of the side chain of Arg233 with that of Gln resulted in a 25-fold increase in the K_m for methionine and a 2000-fold decrease in k_{cat} for ATP–PP_i exchange, with no change in the K_m for ATP or tRNA. These results indicate that Asp52 and Arg233 play important roles in stabilization of the transition state for methionyl adenylate formation, possibly directly interacting with complementary charged groups (ammonium and carboxyl) on the bound amino acid. Primary sequence comparisons of class I aminoacyl-tRNA synthetases show that all but one member of this group of enzymes has an aspartic acid residue at the site corresponding to Asp52 in MetRS. The synthetases most closely related to MetRS (including those specific for Ile, Leu, and Val) also have a conserved arginine residue at the position corresponding to Arg233, suggesting that these conserved amino acids may play analogous roles in the activation reaction catalyzed by each of these enzymes. Trp305 is located in a pocket deep within the active site of MetRS that has been postulated to form the binding cleft for the methionine side chain. Consistent with this, substitution of Ala for Trp305 resulted in a specific loss of affinity for methionine and an overall 100-fold decrease in k_{cat}/K_m for ATP–PP_i exchange. Comparison of the sequences in this domain of the Met, Ile, Leu, and Val synthetases shows that there is little homology between all members of this group but strong homology between enzymes specific for the same amino acids. This supports the idea that the region close to and including Trp305 forms part of the domain involved in discrimination of the methionine side chain by MetRS and suggests that corresponding sequences in the other enzymes play a similar role in distinguishing between the closely related side chains of this group of amino acids.

Accurate aminoacylation of tRNAs by aminoacyl-tRNA synthetases plays a crucial role in insuring the overall fidelity of protein synthesis. This accuracy is achieved by preferential binding of the enzymes to cognate substrates (amino acid and tRNA), by more rapid transfer of activated amino acid to cognate than noncognate tRNA species, and, in many cases, by additional proofreading steps which correct errors made in the initial amino acid activation and transfer reactions (eqs 1 and 2).

$$E + AA + ATP \rightleftarrows E{\cdot}AA{-}AMP + PP_i \quad (1)$$

$$E{\cdot}AA{-}AMP + tRNA \rightleftarrows AA{-}tRNA + AMP + E \quad (2)$$

The structural basis for selection of the cognate amino acid by an aminoacyl-tRNA synthetase has been determined for only one synthetase to date. *Bacillus stearothermophilus*

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tyrosyl-tRNA synthetase (TyrRS)[1] has been cocrystallized with both tyrosine and tyrosyl adenylate, and the side chains of amino acids within hydrogen-bonding distance of substrates in the active site have been identified (Bhat el al., 1982; Brick & Blow, 1987; Brick et al., 1989). Site-directed mutagenesis studies have confirmed the role of specific enzyme residues in discrimination between tyrosine and the structurally related amino acid, phenylalanine (Fersht, 1987; Fersht et al., 1988). These residues directly bind to the OH group of the tyrosine substrate.

Unlike tyrosine, many amino acids lack hydrophilic side chains that can be used by synthetases to discriminate cognate and noncognate substrates in initial binding. These enzymes misactivate noncognate amino acids with a measurable frequency; however, such synthetases have evolved efficient pretransfer or posttransfer editing functions to prevent accumulation of mischarged tRNAs [reviewed in Freist (1989)]. Little is known about the structural basis for discrimination between related amino acids in the initial binding or proofreading reactions catalyzed by these enzymes.

Escherichia coli methionyl-tRNA synthetase (α_2 = 2 × 76 000 molecular weight) is one of the enzymes in the group having the capacity to edit errors in the activation step (Fersht & Dingwall, 1979; Jakubowski & Fersht, 1981; Smith & Cohn, 1981; Jakubowski, 1990). The X-ray crystal structure of a fully biologically active monomeric form of the enzyme complexed with ATP has recently been refined to an *R* factor of 0.220 at 2.5-Å resolution (Brunie et al., 1990). Although no complex with methionine has yet been analyzed, the available structure has revealed the location of a pocket within the active site containing residues potentially capable of interacting with methionine. In this paper, we have examined the effect of site-directed changes at several of these sites and have identified residues that specifically affect methionine binding as well as functional groups that stabilize the transition state for methionyl adenylate formation.

Materials and Methods

Construction, Purification, and Assay of Mutant Proteins. Site-directed mutagenesis of MetRS547 was carried out as described before (Ghosh et al., 1990) using the following deoxyoligonucleotide primers (mutagenic sites underlined): Asp52 → Ala, 5′CCGTGGGCAGCGTCGGC3′; Arg233 → Gln, 5′AGGGGCGTCCTGGGAGA3′; Trp305 → Ala, 5′ATGGCAGGCGCGAACAG3′. The R233Q and W305A mutants were overproduced from the high copy number plasmid pGG3 and were purified as described elsewhere (Ghosh et al., 1990). The D52A mutant was produced in a lower amount and was purified by polymin P precipitation of an S-100 extract prepared from 10 L of culture followed by 30–70% ammonium sulfate fractionation of the supernatant. Successive chromatography was then carried out by step elution from DEAE-Sepharose in buffer A (20 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride, 10% glycerol) plus 85 mM KCl followed by gradient elution from the same resin (20–250 mM KCl in buffer A), gradient elution from hydroxyapatite (Bio-Gel HTP; 5–200 mM potassium phosphate, pH 6.8, 10% glycerol), and Sephadex G-100 (superfine) chromatography in buffer A plus 10 mM KCl. Mono Q (Pharmacia) ion exchange and Superose-12 gel filtration were then carried out as described before (Ghosh et al., 1990). All of the proteins 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 were performed as detailed elsewhere (Ghosh et al., 1990).

Equilibrium Dialysis. Equilibrium dialysis was carried out in 20 mM imidazole–hydrochloride, pH 7.0/7 mM $MgCl_2$/1 mM dithiothreitol at 4 °C in rotating lucite cells. The two cell compartments were separated by a dialysis membrane and were filled with 32 μL of the ligand or the enzyme solution. Concentrations of 10 μM to 3.0 mM [^{35}S]methionine (300–9200 cpm/pmol) and 18–160 μM enzyme were used for the experiments. Samples were dialyzed for 5–8 h, at which time equilibration had been achieved. Duplicate aliquots were withdrawn from each chamber with a microsyringe and counted in a liquid scintillation counter.

Results

Isolation of MetRS547 Mutant Proteins. The MetRS gene has been cloned in a high copy number plasmid (pGG3) and mutagenized to produce a truncated monomeric protein (MetRS547) corresponding to the crystallized biologically active trypsin fragment of the enzyme, by insertion of a stop codon at amino acid position 548 (Ghosh et al., 1990). Single-stranded DNA generated from the phagemid vector has been used for oligonucleotide-directed mutagenesis of three amino acid residues in the active site of the enzyme. In two cases, the potential hydrogen-bonding capability of the residues has been completely eliminated by conversion of the wild-type amino acids, Asp52 and Trp305, to Ala. Arg233 has been converted to Gln, a hydrophilic amino acid of similar size but one that lacks the positive charge of the wild-type residue. The engineered mutations were confirmed, and the possiblity of unexpected mutations was eliminated, by completely sequencing the DNA of each mutagenized gene. The mutant proteins were then purified for biochemical characterization as described under Materials and Methods.

Mutagenesis of Trp305 Affects Methionine Binding. Trp305 is located on one side of a 7-Å-wide cavity in the crystal structure of the MetRS·ATP complex that has been postulated to be the binding site for methionine (Brunie et al., 1990). The role of this residue in the synthesis of methionyl adenylate by MetRS was investigated by examining the rate of exchange of radioactive inorganic pyrophosphate into ATP (eq 1) catalyzed by the W305A mutant enzyme at different substrate concentrations. A 30-fold increase in the K_m for methionine was observed in the reaction, with no change in the K_m for ATP (Table I). A small decrease (3-fold) was also seen in the rate of catalysis of ATP–PP_i exchange. Measurement of kinetic parameters for aminoacylation (eq 2) catalyzed by the mutant enzyme showed that there was no effect of the mutation on the K_m for $tRNA^{fMet}$ (Table II).

An attempt was made to directly determine the affinity of the W305A mutant for methionine by equilibrium dialysis. Measurement of the dissociation constant for wild-type MetRS547 (18.5 μM) by this method using 10–80 μM methionine gave a value of K_d = 50 ± 10 μM, in good agreement with published values obtained by fluorescence titration equilibrium (Blanquet et al., 1974). A reliable dissociation constant for the W305A mutant enzyme could not be measured using 106 μM enzyme and methionine concentrations up to 3 mM. The data obtained indicate that the K_d is >1 mM.

[1] Abbreviations: TyrRS, tyrosyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase; MetRS, methionyl-tRNA synthetase; MetRS547, monomeric MetRS truncated at amino acid residue 547; D52A, MetRS547 with aspartic acid 52 replaced by alanine; R233Q, MetRS547 with arginine 233 replaced by glutamine; W305A, MetRS547 with tryptophan 305 replaced by alanine; $tRNA^{fMet}$, the initiator methionine tRNA; Met-AMP, methionyl adenylate.

Table I: 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})	k_{cat}/K_m[c] ($s^{-1}\ \mu M^{-1}$)	relative k_{cat}/K_m[c]
wild type	21 ± 4	528 ± 91	74 ± 8	3.5	1.0
D52A	34 ± 4	187 ± 52	(7 ± 1) × 10^{-3}	2.1 × 10^{-4}	5.9 × 10^{-5}
R233Q	525 ± 101	531 ± 217	(3.1 ± 0.3) × 10^{-2}	5.9 × 10^{-5}	1.7 × 10^{-5}
W305A	659 ± 120	667 ± 210	24 ± 2	3.6 × 10^{-2}	1.0 × 10^{-2}

[a] ATP–PPi 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 for D52A, 50–1000 μM for R233Q, and 100–1000 μM for W305A. K_m values for ATP were determined at 2 mM methionine and ATP concentrations of 50–400 μM for D52A, 100–1000 μM for R233Q, and 250–2000 μM for W305A. [b] k_{cat} is the average of the values determined for methionine and ATP. [c] K_m here is the K_m for methionine.

Table II: Aminoacylation Activity of MetRS547 Mutants[a]

enzyme	K_m^{tRNA} (μM)	k_{cat} (s^{-1})
wild type	1.2 ± 0.2	3.2 ± 0.2
D52A	1.9 ± 0.5	(4 ± 0.5) × 10^{-4}
R233Q	2.0 ± 0.1	(2 ± 0.4) × 10^{-3}
W305A	0.8 ± 0.1	0.4 ± 0.1

[a] Aminoacylation assays were carried out using 17 μM methionine, 2 mM ATP, and 0.5–6 μM native $tRNA^{fMet}$. Saturating amounts of methionine could not be used for practical reasons; thus, k_{cat} values are underestimated.

The Asp52 to Ala Mutation Affects the Rate of Methionyl Adenylate Formation. Asp52 is located in the active site of MetRS at a position corresponding to Asp78 in *B. stearothermophilus* TyrRS. Asp78 in the tyrosine enzyme is bound to the positively charged amino group of tyrosine in the TyrRS·Tyr and TyrRS·Tyr–AMP complexes (Brick & Blow, 1987; Brick et al., 1989). In order to explore the role of Asp52 in MetRS, this residue was converted to Ala and the effect on the ATP–PP_i exchange and aminoacylation activities of the enzyme was examined (Tables I and II). The D52A mutant showed less than a 2-fold increase in the K_m for methionine in the ATP–PP_i exchange reaction; however, k_{cat} was decreased by 4 orders of magnitude. A small decrease (3-fold) in the K_m for ATP was observed in the exchange assay and no significant change was seen in the K_m for $tRNA^{fMet}$ in the aminoacylation reaction. These results indicate that the major impact of removal of the Asp52 side chain is on the catalytic step in formation of methionyl adenylate.

Conversion of Arg233 to Gln Affects both Methionine Binding and Catalysis. The crystal structure of the MetRS·ATP complex shows that the basic residue Arg233 lies near ATP but makes no contact with this substrate (Brunie et al., 1990). A potential role for this residue in the catalytic mechanism has been suggested. Consistent with this, characterization of the R233Q enzyme showed that the mutation has no effect on the K_m for ATP in the ATP–PP_i exchange reaction but reduces k_{cat} 2000-fold (Table I). In addition, the K_m for methionine was increased 25-fold, leading to an overall change in k_{cat}/K_m of almost 60 000-fold. Examination of the kinetic parameters for aminoacylation revealed less than a 2-fold change in the K_m for $tRNA^{fMet}$ (Table II), indicating no significant effect of the mutation on interaction with this substrate. Equilibrium dialysis experiments carried out as described above confirmed that the R233Q enzyme has greatly reduced affinity for methionine, with a K_d close to 1 mM.

Discussion

Aminoacyl-tRNA synthetases can be broadly divided into two major classes on the basis of the presence (class I) or absence (class II) of a nucleotide binding fold (Rossmann fold) in their N-terminal domains (Eriani et al., 1990; Cusack et al., 1990). *E. coli* MetRS belongs to the group of synthetases that contain this structure (Risler et al., 1981), consisting of a six-stranded parallel β-sheet and five intervening α-helices (Rossmann et al., 1974). Comparisons of the X-ray crystal structures of MetRS and two other class I enzymes (*E. coli* GlnRS and *B. stearothermophilus* TyrRS) have shown that significant portions of the Cα backbone within this domain of the enzymes are superimposable, despite the presence of only very limited primary sequence homology (Blow et al., 1983; Perona et al., 1991). A short consensus sequence, HIGH, is found near the N-terminus of this group of enzymes (Burbaum et al., 1990). The first His residue of this sequence is seen to interact with ATP in the crystal structures of MetRS and GlnRS complexed with this substrate (Brunie et al., 1990; Rould et al., 1989), and site-directed mutagenesis experiments have indicated that both His residues participate in activation of tyrosine by *B. stearothermophilus* TyrRS (Lowe et al., 1985; Leatherbarrow & Fersht, 1987).

An additional short region of homology consisting of the consensus sequence KMSKS is found near the end of the Rossmann fold in class I synthetases (Hountondji et al., 1986). This conserved pattern was first identified in *E. coli* MetRS through affinity labeling studies (Hountondji et al., 1985). A derivative of $tRNA^{fMet}$ containing an oxidized 3′-terminal adenosine was cross-linked to Lys335, the second lysine of the KMSKS sequence. More recent site-directed mutagenesis studies have shown that Lys335 makes no direct contact with the cognate tRNA during the aminoacylation reaction but rather binds to the pyrophosphate group of ATP in the transition state for methionyl adenylate formation (Mechulam et al., 1991). The crystal structure of MetRS complexed with ATP shows that Lys335 lies near the γ-phosphate (Brunie et al., 1990), and the analogous lysine in *E. coli* GlnRS is seen to interact with the phosphates of ATP in the crystal of the enzyme complexed with ATP and $tRNA^{Gln}$ (Rould et al., 1989). No complex of *B. stearothermophilus* TyrRS with ATP is available; however, site-directed mutagenesis experiments have shown that the two lysine residues of the corresponding KFGKT sequence function in a manner similar to Lys335 in MetRS, stabilizing the transition state for tyrosyl adenylate formation by strongly interacting with the pyrophosphate moiety of ATP (Fersht et al., 1988). Examination of the crystal structure of TyrRS complexed with Tyr–AMP shows that these lysines are too distant from the active site to interact with ATP unless the enzyme undergoes a significant conformational change, and it has been postulated that the flexible peptide loop containing the KFGKT sequence moves at least 8 Å during the course of the reaction to bring the lysine side chains in contact with the γ-phosphate of ATP (Fersht et al., 1988). Reorientation of key residues within the active site of MetRS during methionyl adenylate formation must also be postulated to explain the results obtained in the present studies.

Figure 1 shows the location of Asp52, Arg233, Trp305, and Lys335 within the overall structure of MetRS, and Figure 2 gives a stereoview of the active site in the crystal structure of

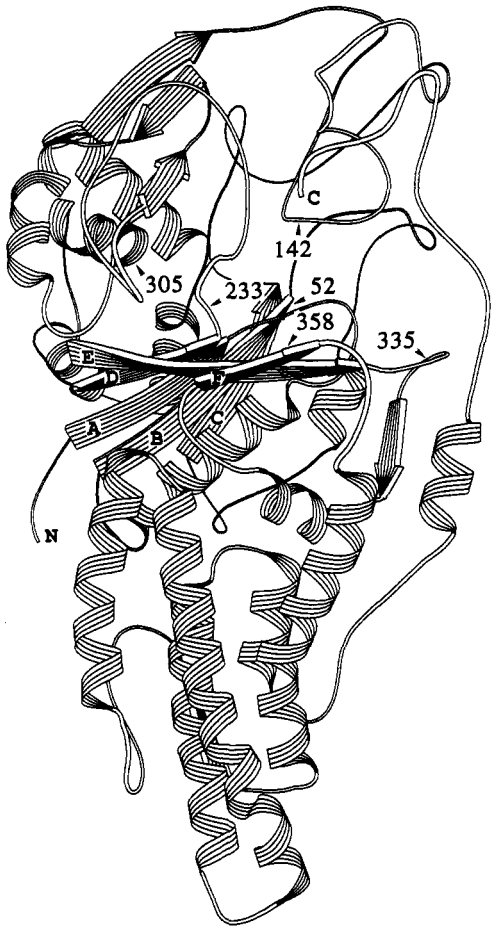

FIGURE 1: Schematic drawing of the structure of MetRS. Residues studied in this work are highlighted. In addition, the positions of Lys335, studied by Mechulam et al. (1991), and Lys142 and Tyr358, studied by Ghosh et al. (1991), are shown.

the MetRS·ATP complex (Brunie et al., 1990). Asp52 is located at the C-terminal end of β-strand B (residues 44–52) within the Rossmann fold, and Arg233 is found at the C-terminal end of βD (224–232). Trp305 is located in a pocket deep within the active site and Lys335 lies on the periphery of the catalytic center in a peptide loop (loop V) that joins βE (318–328) and α-helix HE (341–348). Substitution of the side chain of Asp52 with that of Ala resulted in about a 20000-fold decrease in k_{cat}/K_m for ATP–PP_i exchange catalyzed by MetRS. No significant change in the K_m for $tRNA^{fMet}$ and only a small changes in the K_m's for ATP and methionine were observed. Recent equilibrium binding studies have shown that the affinity of the Ala52 mutant for methionine is the same as that seen with the wild type enzyme.[2] The data indicate that the major role played by Asp52 is in the catalytic step of methionine activation. The apparent change in free energy resulting from the D52A mutation is 5.8 kcal/mol.[3] The position of Asp52 at the end of βB within the Rossmann fold of MetRS corresponds to the position of Asp78 at the end of βC in *B. stearothermophilus* TyrRS. Asp78 interacts with the positively charged amino group of tyrosine in the TyrRS·Tyr and TyrRS·Tyr–AMP complexes (Brick & Blow, 1987; Brick et al., 1989), and we propose a similar role for Asp52 in stabilizing the transition state for Met–AMP formation.

Recent sequence comparisons of class I synthetases based on alignment of peptides corresponding to the β-strands and α-helices of the Rossmann fold show that all but one of the enzymes in this group have a conserved aspartic acid residue at the site corresponding to Asp52 in MetRS.[4] Figure 3 shows the sequences around this site in the enzymes most closely related to MetRS. Two highly conserved glycine residues are also found in this domain, and mutation of one of these (Gly93) in IleRS to Arg leads to a 6000-fold increase in the K_m for isoleucine in the ATP–PP_i exchange reaction (Clarke et al., 1988), consistent with its location at the amino acid binding site of this enzyme.

Another region of homology, containing the consensus sequence DWCISRQ, has previously been noted in the synthetases specific for Met, Ile, Val, and Leu (Härtlein & Madern, 1987; Heck & Hatfield, 1988). The arginine residue of this consensus sequence is absolutely conserved in all members of this group of enzymes and corresponds to Arg233 in MetRS (Figure 4). Modification of the cysteine residue of the consensus sequence in *E. coli* IleRS (Cys462) with *N*-ethylmaleimide inactivates the enzyme (Iaccarino & Berg, 1969; Kula, 1974), and affinity labeling of IleRS with isoleucyl bromoethyl ketone leads to covalent reaction at the same cysteine (Rainey et al., 1977; Webster et al., 1984), indicating that this residue lies in close proximity to the isoleucine binding site of the enzyme.

The data presented here show that modification of Arg233 to Gln produces almost a 60000-fold decrease in k_{cat}/K_m for ATP–PP_i exchange, indicating a critical role for this residue in activation of methionine. A 25-fold increase in the K_m for methionine is observed, with no change in the K_m's for ATP or $tRNA^{fMet}$. Equilibrium binding studies show that the R233Q mutant has a greatly reduced affinity for methionine in the ground state, and we postulate that the mutation also reduces the stability of the methionyl adenylate reaction intermediate by elimination of important contacts with the methionine moiety in the transition state.

Arg233 at the end of βD in the Rossmann fold of MetRS is located in a position close to that of Gln195 at the end of βE in *B. stearothermophilus* TyrRS. The 3-D structures of βD and βE are superimposable through residue 231 in the methionine enzyme and residue 192 in the tyrosine synthetase (Brunie et al., 1990). The two β-strands also appear to be functionally homologous. The main chain carbonyl of Ile231 is hydrogen bonded to the 2′-OH group of the ribose of ATP in the MetRS·ATP complex (Brunie et al., 1990), and a corresponding contact is made between the main chain NH group of Gly192 and the 2′-O of adenosine in the TyrRS·Tyr–AMP complex (Brick et al., 1989). The two 3-D structures begin to diverge at the C-terminal end of the β-strand, such that Arg233 in MetRS and Gln195 in TyrRS assume different orientations within the active sites of the two enzymes.

Conversion of Gln195 to Gly affects both the K_m for tyrosine and the k_{cat} for ATP–PP_i exchange catalyzed by TyrRS, and Gln195 has been postulated to interact with the carboxyl group of tyrosine in the activation reaction (Fersht et al., 1985; Brick et al., 1989). We propose that a similar, possibly even stronger, interaction occurs between Arg233 and the carboxyl group of methionine in the transition state for methionyl adenylate formation. The apparent loss of free energy resulting from the R233Q mutation is 6.5 kcal/mol[3]; however, this value may

[2] H. Y. Kim and L. H. Schulman, unpublished results.

[3] Calculated from the equation $\Delta G_{app} = -RT \ln [(k_{cat}/K_m)_{mutant}/(k_{cat}/K_m)_{wildtype}]$ (Wilkinson et al., 1983).

[4] C. Landès, J. J. Perona, S. Brunie, M. A. Rould, C. Zelwer, T. A. Steitz, and J. L. Risler, submitted for publication.

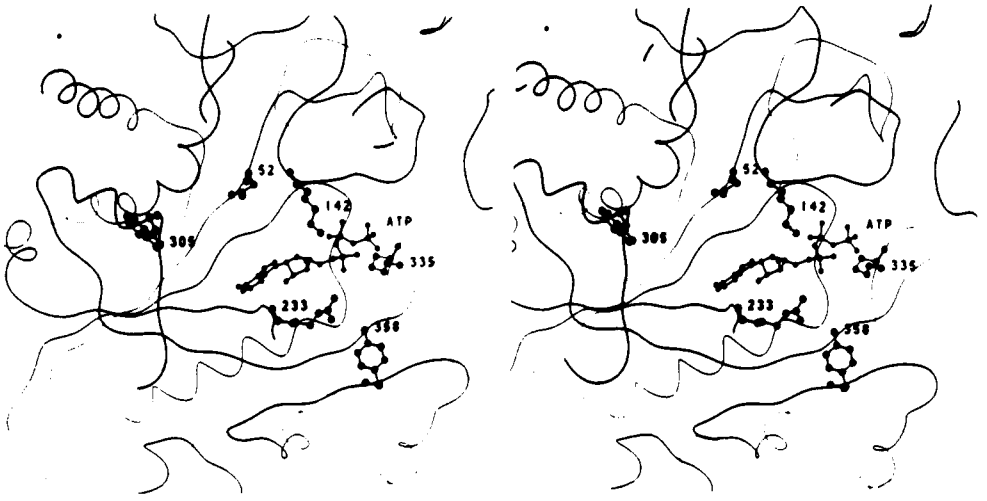

FIGURE 2: Stereoview of the active site of MetRS.

		52									
MetRS (Ec)	50	A	D	D	A	H	G	T	P	I	
MetRS (Sc)	241	G	T	D	E	Y	G	T	A	T	
MetRS (Scm)	57	G	T	D	E	H	G	L	K	I	
IleRS (Ec)	93	G	W	D	C	H	G	L	P	I	
IleRS (Sc)	84	G	W	D	T	H	G	V	P	I	
LeuRS (Ec)	77	G	W	D	A	F	G	L	P	A	
LeuRS (Scm)	91	G	W	D	A	F	G	L	P	A	
ValRS (Ec)	77	G	T	D	H	A	G	I	A	T	
ValRS (Bst)	84	G	M	D	H	A	G	I	A	T	
ValRS (Sc)	225	G	F	D	H	A	G	I	A	T	

FIGURE 3: Conservation of the aspartic acid residue corresponding to Asp52 in *E. coli* MetRS in class I synthetases. Primary sequence data are taken from Barker et al. (1982) and Dardel et al. (1984) (*E. coli* MetRS), Fasiolo et al. (1985) (yeast MetRS), Tzagoloff et al. (1989) (yeast mitochondrial MetRS), Webster et al. (1984) (*E. coli* IleRS), Englisch et al. (1987), and Martindale et al. (1989) (yeast IleRS), Härtlein and Madern (1987) (*E. coli* LeuRS), Tzagoloff et al. (1988) (yeast mitochondrial LeuRS), Heck and Hatfield (1988) (*E. coli* ValRS), Borgford et al. (1987) (*B. stearothermophilus* ValRS), and Chatton et al. (1988) (yeast ValRS). Sequence comparisons are based on alignments derived by Landès et al.[4] Although only the synthetases most closely related to MetRS are shown here, all class I synthetases except ArgRS have a conserved Asp residue at the same site.[4] The synthetases have been numbered such that amino acid 1 corresponds to the second codon of the structural gene. Sites of sequence identity in all or nearly all of the synthetases listed are boxed. The circled residues (Asp52 in *E. coli* MetRS and Gly93 in *E. coli* IleRS) have been shown to be at or near the amino acid binding site of the respective enzymes by site-directed and cassette mutagenesis studies [this work and Clarke et al. (1988)]. Ec, *E. coli*; Sc, *S. cerevisiae*; Scm, *S. cerevisiae* mitochondria; Bst, *B. stearothermophilus*.

		233														
MetRS (Ec)	226	L	Q	Q	W	D	I	S	R	D	A	P	Y	F	G	F	E	I	P
MetRS (Sc)	421	L	K	P	R	C	I	T	R	D	L	-	V	W	G	T	P	V	P
MetRS (Scm)	217	L	P	D	L	S	I	S	R	P	SA	RL	K	W	G	I	P	T	P
IleRS (Ec)	458	R	P	D	W	G	I	S	R	Q	R	-	T	W	G	V	P	M	S
IleRS (Sc)	452	A	R	D	W	N	V	S	R	N	R	-	Y	W	G	T	P	I	P
LeuRS (Ec)	416	L	R	D	W	G	V	S	R	Q	R	-	Y	W	G	A	P	I	P
LeuRS (Scm)	441	I	R	D	W	L	I	S	R	Q	R	-	Y	W	G	T	P	I	P
ValRS (Ec)	419	I	Q	D	W	C	I	S	R	Q	L	-	W	W	G	H	R	I	P
ValRS (Bst)	402	I	R	D	W	C	I	S	R	Q	L	-	W	W	G	H	R	I	P
ValRS (Sc)	561	I	Q	D	W	C	I	S	R	Q	L	-	W	W	G	H	R	C	P

FIGURE 4: Conservation of the arginine residue corresponding to Arg233 in class I synthetases related to *E. coli* MetRS. See the legend to Figure 3. The circled residues have been shown to be at or near the amino acid binding site of the corresponding synthetases by site-directed mutagenesis (Arg233 in *E. coli* MetRS; this work) or affinity labeling (Cys462 in *E. coli* IleRS; Rainey et al., 1977; Webster et al., 1984).

be an overestimate of the contribution of the Arg233 side chain to stabilization of the transition state since the side chain of Gln233 could negatively impact on the activation reaction. Nevertheless, a major role for Arg233 in the catalytic step is clearly indicated.

Examination of the crystal structure of the MetRS·ATP complex shows that Arg233 lies immediately adjacent to Tyr358 (Figure 2). Recent site-directed mutagenesis studies have shown that Tyr358 also participates in transition-state stabilization of Met–AMP formation, possibly through direct interaction with an oxygen of the α-phosphate of ATP (Ghosh et al., 1991). The adenylate is formed by in-line attack of the negatively charged carboxyl group of methionine on the α-phosphate of ATP, resulting in release of pyrophosphate and inversion of configuration at Pα (Langdon & Lowe, 1979). This requires the energetically unfavorable juxtaposition of two negatively charged groups during the catalytic reaction. We propose that Arg233 facilitates this process by binding to both the methionine carboxyl and ATP α-phosphate oxygen (Figure 6).

Location of Asp52 and Arg233 in the crystal structure of the MetRS·ATP complex (Figure 2) shows that these residues are too far apart to simultaneously interact with the amino and carboxyl groups of the methionine substate without a significant shift in their relative positions in the transition state. It has been known for some time that binding of methionine to MetRS induces a conformational change in the active site as evidenced by a greatly increased affinity of the E·Met complex over the free enzyme for adenosine, a competitive inhibitor of adenylate formation, as well as for the reaction product, pyrophosphate (Blanquet et al., 1975; Fayat et al., 1977). Conversely, saturation of the enzyme with adenosine or pyrophosphate increases its affinity for methionine, and even tighter binding is observed to the enzyme saturated with both adenosine and PP_i. The synergistic coupling between the methionine and adenosine binding sites is not observed when AMP, ADP, or ATP is substituted for adenosine, presumably due to electrostatic repulsion introduced by the presence of the α-phosphate group (Hyafil et al., 1976; Fayat et al., 1977). The facilitated binding of one ligand by the other has been postulated to provide part of the driving force for adenylate formation by compensating for the unfavorable interaction between the negatively charged groups of the two substrates.

Certain changes in the active site of MetRS can be followed by measuring the intrinsic fluorescence of the protein. For example, binding of methionine produces a 26% increase in the fluorescence of the free enzyme (Blanquet et al., 1972). Addition of ATP to the E·Met complex decreases the fluorescence from 126% to a final value of 73%, reflecting formation of the enzyme-bound adenylate (Hyafil et al., 1976).

		305
MetRS (Ec)	294	G K D I V Y F H S L F W P A M L E G
MetRS (Sc)	484	G K D N V P F H T V V F P G S Q L G
MetRS (Scm)	302	G K D I A K F H T V Y W P S F L L A
IleRS (Ec)	528	D T L D V W F D S G S T H S S V V D
IleRS (Sc)	523	E V F D C W F E S G S M P Y A S Q H
LeuRS (Ec)	493	M E S S W Y Y A R Y T C P Q Y K E G
LeuRS (Scm)	520	I D S S W Y Y F R F L D P K N T S K
ValRS (Ec)	477	S S A L W T F S T L G W P E N T D A
ValRS (Bst)	453	S S A L W P F S T M G W P D T D S P
ValRS (Sc)	632	S S G L W P F S T L G W P E K T K D

FIGURE 5: Sequences proposed to be partially responsible for discrimination in binding the amino acid side chain of methionine and closely related amino acids by the corresponding cognate synthetases. See the legend to Figure 3. Sites of sequence identity in nearly all of the synthetases listed are shaded. Sites of sequence identity in synthetases specific for activation of the same amino acid are enclosed in solid lines, and conservative replacements are indicated by dashed lines. Conservative replacements included in this description are S = T, W = F, Y = F, G = A, V = I, M = L or I, and D = E. Circles indicate the sites of amino acid residues implicated in methionine binding by *E. coli* MetRS (Trp305; this work) and yeast MetRS (Gly501; Chatton et al., 1987).

Binding of ATP alone produces no change in enzyme fluorescence; however, comparison of the crystal structure of the free enzyme and the MetRS·ATP complex shows that large shifts in the positions of several peptides occur following interaction with this substrate, particularly in the regions containing amino acid residues 192–197 and 237–247 (Brunie et al., 1990). In addition, the diameter of a cavity deep within the active site changes from about 9 to 7 Å when the enzyme complexes with ATP. This cavity contains amino acid residues that are conserved in methionine synthetases from different sources and has been postulated to form the binding site for the methionine side chain (Brunie et al., 1990). Trp305 is positioned on one wall of this pocket with its side chain extended toward the cavity. Conversion of Trp305 to Ala produces a 30-fold increase in the K_m for methionine in the ATP–PP_i exchange reaction and a small (3-fold) decrease in k_{cat}, yielding an apparent change in free energy of 2.7 kcal/mol.[3] No change in the K_m for ATP or $tRNA^{fMet}$ is observed, indicating a specific effect of the mutation on interaction with the amino acid substrate. Equilibrium binding studies have also directly demonstrated that the Ala305 mutant has greatly reduced affinity for methionine in the ground state, consistent with location of Trp305 in the methionine binding site. Comparison of sequences in the region surrounding Trp305 with corresponding sequences in the closely related synthetases specific for Ile, Val, and Leu is shown in Figure 5. In contrast to the strong conservation of sequence between all members of this group of enzymes at sites corresponding to Asp52 and Arg233 in MetRS (Figures 3 and 4), little homology extends across different amino acid accepting species in this domain. On the other hand, extensive homology is found on the N-terminal side of Trp305 for enzymes from different organisms that are specific for the same amino acids. This homology is particularly striking when conservative amino acid replacements are considered and extends an additional 8–9 residues beyond those shown in Figure 5 for the enzymes specific for activation of valine and leucine. In the case of the methionine enzymes, another mutation in addition to W305A has been reported that falls in this domain. A mutant of yeast MetRS that requires a high concentration of methionine for growth has been shown to contain a single Gly to Asp change at position 501 (Chatton et al., 1987), corresponding to Gly311 in *E. coli* MetRS (Figure 5). The available data, together with the sequence comparisons, suggest that the domain close to and including Trp305 forms part of the binding pocket that

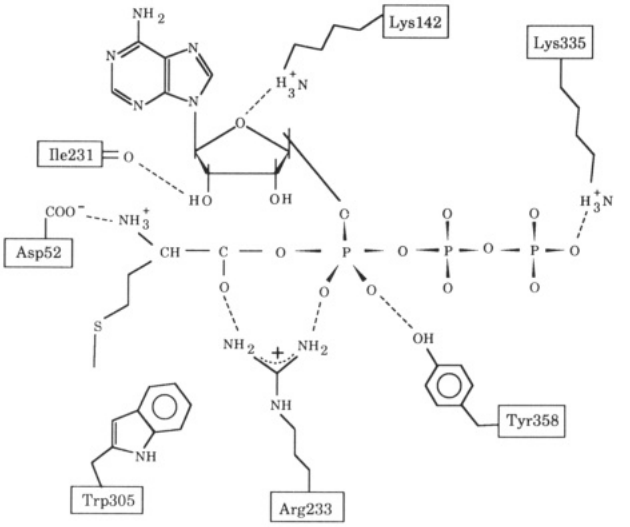

FIGURE 6: Schematic drawing of the interactions proposed to occur between MetRS and methionine and ATP in the transition state for amino acid activation. The roles of Asp52, Arg233, and Trp305 have been studied in the present work. The functions of Lys142 and Tyr358 have been investigated by Ghosh et al. (1991), and the role of Lys335 has been analyzed by Mechulam et al. (1991). The interaction with the main chain carbonyl of Ile 231 is based on analysis of the crystal structure of the MetRS·ATP complex (Brunie et al., 1990).

allows discrimination between the side chain of methionine and closely related amino acids by MetRS and that corresponding regions in the Ile, Val, and Leu enzymes may serve a similar function for these synthetases. Figure 2 shows the location of this pocket within the active site of the MetRS·ATP complex. As noted above, conformational changes induced by substrate binding are required to bring Trp305, Asp52, and Arg233 into the proper orientation for simultaneous interaction with methionine.

Figure 6 gives a schematic representation of the proposed contacts between MetRS and methionine during the activation reaction. The large effect of mutations at Asp52 and Arg233 is consistent with strong interactions of these charged residues with complementary charged groups on the amino acid. Arg233 is suggested to facilitate close approach of the carboxylate and α-phosphate of ATP by binding to both negatively charged groups in the transition state, while release of pyrophosphate is assisted by strong interactions with Lys335 in the KMSKS sequence. The side chain of Tyr358 also participates in the catalytic step (Ghosh et al., 1991), possibly by reducing the concentration of negative charge at the reaction center through a hydrogen bond to an oxygen atom of the α-phosphate (Figure 6). It will be of interest to examine the role of homologous sequences in other class I synthetases in activation of their cognate amino acids as well as to investigate possible participation of the active site residues identified here in the editing reactions carried out by this group of synthetases. Studies along these lines are in progress.

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