Evidence that Specificity of Microhelix Charging by a Class I tRNA Synthetase Occurs in the Transition State of Catalysis†

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ABSTRACT: Determinants for the identities of tRNAs are located in the acceptor stem and, commonly, in the anticodon as well. Although the anticodon is an important determinant for the identity of methionine tRNA, RNA microhelices whose sequences are based on the acceptor stem alone can be aminoacylated by the class I methionyl-tRNA synthetase. We show here that specific nucleotide substitutions in a microhelix significantly reduced its rate of aminoacylation. In contrast, affinity coelectrophoresis analysis showed that microhelix binding to the enzyme was not significantly affected by the same substitutions. These and additional experiments and considerations imply that specific determinants for microhelix aminoacylation are needed for orientation of the acceptor stem in the transition state of catalysis rather than for enhanced binding interactions. The effect of linking together acceptor stem interactions with those in the anticodon, as occurs in the whole tRNA molecule, was also evaluated. This analysis showed that linkage results in some of the favorable acceptor stem and anticodon interactions being used to offset the free energy cost of straining the structure of the enzyme−tRNA complex.

Specific recognition of tRNAs and amino acids by the aminoacyl tRNA synthetase family of enzymes provides the enzymatic basis for the genetic code. All tRNAs fold into a similar L-shaped tertiary structure that has two domains, with the 3′ amino acid acceptor end and the anticodon at opposite ends (Rich & RajBhandary, 1976). Within this L-shaped framework, most of the identity elements needed for specific and efficient aminoacylation are in either the acceptor stem or the anticodon of the tRNA (Schimmel, 1989; Giegé *et al.*, 1993; Saks *et al.*, 1994; McClain, 1995).

Synthetase−tRNA complexes are relatively loose, having dissociation constants (at pH 7.5) on the order of 1 μM (Hélène *et al.*, 1971; Blanquet *et al.*, 1973; Lam & Schimmel, 1975; Schimmel & Söll, 1979; Meinnel *et al.*, 1991). The loose association of synthetases with their cognate tRNAs enables the enzymes to turn over rapidly during protein synthesis. The relative weakness of binding interactions associated with highly specific aminoacylation reactions suggests that additional interactions are required to achieve high specificity. This expectation has been demonstrated for the class II *Escherichia coli* alanyl-tRNA synthetase, which discriminates tRNA substrates in large part on the basis of an acceptor stem G3•U70 base pair. This discrimination occurs at both the binding step and, additionally, during the transition state of catalysis, with the relative contribution of each part being dependent on pH (Park *et al.*, 1989).

In this work, we focused on the class I *E. coli* methionyl-tRNA synthetase (MetRS)[1] and the nature of the contribution of acceptor helix interactions to aminoacylation specificity and efficiency. Unlike alanyl-tRNA synthetase, which makes no contact with the anticodon, the methionine enzyme has a strong interaction with the CAU anticodon of $tRNA^{Met}$. This interaction is a major determinant of the identity of methionine tRNAs (Schulman & Pelka, 1983, 1988; Meinnel *et al.*, 1991).

The *E. coli* enzyme is a homodimer of two 676 amino acid polypeptides (Dardel *et al.*, 1984). Trypsin digestion produces an N-terminal fragment of about 550 amino acids that is stable and active as a monomer (Cassio & Waller, 1971). The 2.5 Å crystal structure of this truncated monomer showed that the protein is divided into two domains (Zelwer *et al.*, 1982; Brunie *et al.*, 1990). The N-terminal domain (residues 1−366) contains the characteristic class I catalytic site built around a Rossman nucleotide binding fold (Rossmann *et al.*, 1974). Within this domain are the class-defining conserved sequence motifs which provide for interactions with ATP and which are believed to stabilize the transition state for (aminoacyl)adenylate synthesis (Webster *et al.*, 1984; Hountondji *et al.*, 1986; Fersht, 1987; Rould *et al.*, 1989; Mechulam *et al.*, 1991). The C-terminal domain (residues 367−519) is primarily α-helical and interacts with the anticodon of $tRNA^{fMet}$ (Valenzuela & Schulman, 1986; Brunie *et al.*, 1990; Ghosh *et al.*, 1990, 1991; Gale & Schimmel, 1995b). Extending from this C-terminal domain is a peptide appendix (residues 520−547) that folds back to the active site in the N-terminal domain. This peptide appendix contains residues that appear to interact with the acceptor stem of $tRNA^{Met}$ (Mellot *et al.*, 1989; Kim *et al.*, 1993). In the full-length enzyme, this appendix is followed

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[1] Abbreviations: MetRS, methionyl-tRNA synthetase; AlaRS, alanyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; β-ME, β-mercaptoethanol; BSA, bovine serum albumin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Ni−NTA, Ni^{2+}-nitrilotriacetic acid; IPTG, isopropylthio β-D-thiogalactopyranoside; ACE, affinity coelectrophoresis.

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by another 120 amino acids whose unknown tertiary structure is needed for dimerization (Cassio & Waller, 1971).

At least ten different synthetases specifically aminoacylate small RNA substrates that recapitulate the acceptor stem (microhelix) or the acceptor and TΨC stems (minihelix) of their cognate tRNAs (Frugier *et al.*, 1994; Hamann & Hou, 1995; Martinis & Schimmel, 1995; Quinn *et al.*, 1995). These include several that are known to interact with the anticodons of their cognate tRNAs. Notably the class I methionyl- (Martinis & Schimmel, 1992, 1993, 1995), valyl- (Frugier *et al.*, 1992), and isoleucyl-tRNA synthetases (Nureki *et al.*, 1993) specifically aminoacylate minihelix substrates, even though the anticodon is a major identity determinant for all of these enzymes (Stern & Schulman, 1977; Schulman & Pelka, 1983, 1988; Muramatsu *et al.*, 1988; Meinnel *et al.*, 1991).

Although MetRS specifically aminoacylates microhelix^{fMet} substrates, the rate of charging of these substrates is so low that k_{cat} and K_m can not be reliably determined. Nonetheless, consistent with the observed sensitivity of aminoacylation of tRNA^{fMet} to acceptor helix sequence alterations (Lee *et al.*, 1992; Meinnel *et al.*, 1993), the aminoacylation of microhelix^{fMet} is sequence-specific (Martinis & Schimmel, 1992, 1993). To investigate further the question of whether this specificity derived from the binding step or, additionally or alternatively, from the transition state of catalysis, we sought to obtain an independent measure of the association of wild type and mutant microhelices with MetRS.

For these studies, RNA microhelix substrates were synthesized having sequences which reconstruct the acceptor stem of tRNA^{fMet} and which gave variants of that stem. An adaption of the conventional aminoacylation assay was used to achieve a more sensitive detection of the charging of these substrates. To study microhelix binding to methionyl-tRNA synthetase in isolation from kinetic phenomena, we utilized an affinity coelectrophoresis (ACE) procedure as adapted for protein−RNA interactions (Lee & Lander, 1991; Kim *et al.*, 1993). In an earlier study, we used this method to investigate the interaction of the methionine enzyme with RNA hairpins which reconstructed the anticodon stem−loop of tRNA^{fMet} (Gale & Schimmel, 1995b). Thus, in addition to providing an approach to investigating the basis for specificity of microhelix aminoacylation by methionyl-tRNA synthetase, these affinity coelectrophoresis studies enabled us to assess separately the relative contribution of binding interactions involving each of the two domains of the tRNA structure. The interaction free energies for the individual domains calculated from these data can be compared with the free energy of interaction of whole tRNA^{fMet} with MetRS. This comparison, in turn, enabled us to estimate the free energy of distortion of the complex that occurs as a result of linking the two domains together.

MATERIALS AND METHODS

Synthesis and Radioactive Labeling of RNA Oligonucleotides. RNA oligonucleotides were chemically synthesized on a Gene Assembler Plus synthesizer (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) as previously described (Usman *et al.*, 1987; Scaringe *et al.*, 1990; Musier-Forsyth *et al.*, 1991). The RNA substrate was 5′-[^{32}P]-labeled according to (Silberklang *et al.*, 1977; Park & Schimmel, 1988).

Aminoacylation of tRNA^{fMet} and Microhelix RNA Substrates. All aminoacylation assays were carried out at 37 °C, in 50 mM HEPES, pH 7.5, 0.1 mM EDTA, 150 mM NH_4Cl, 4 mM $MgCl_2$, and 100 μg of BSA/mL. These assays were carried out using a modification of described methods (Francklyn & Schimmel, 1989, 1990; Musier-Forsyth *et al.*, 1991). This modification was necessary to study the charging of inefficient substrates and is described in Francklyn *et al.* (1992). Briefly, aminoacylation reaction products were digested with RNase A (Boehringer Mannheim, Indianapolis, IN) until quenched with 0.7 M HOAc. After centrifugation to remove protein, the hydrolysate was fractionated on a Vydac 401 TP HPLC column to isolate radioactive (aminoacyl)adenosine. This product was then quantitatively measured by placing a sample in Hydrofluor (National Diagnostics, Manville, NJ) to measure radioactivity on an LKB Wallac model 1211 liquid scintillation counter (Gaithersburg, MD). Because of the low rates of aminoacylation, it was not practical to exploit a wide concentration range. For that reason, reported k_{cat}/K_m values should be viewed as "operational" or "apparent" kinetic parameters. However, in all cases the measured velocities were directly proportional either to total substrate or to total enzyme, depending on which was varied in the analysis of a particular microhelix. Either three different enzyme or three different (at least) substrate concentrations were investigated in each instance. These concentrations varied from 2-fold to more than 8-fold, depending on the microhelix being investigated.

Protein Purifications. MetRS$_{1-547}$ and MetRS$_{1-676}$ were expressed from plasmid pAG112 (Gale & Schimmel, 1995b) and plasmid pJB103 (Burbaum & Schimmel, 1991), respectively. The purification was performed as described earlier (Gale & Schimmel, 1995b) with some modifications. Both plasmids were transformed into *E. coli* strain TG1 (*K12, SupE hsd*Δ5 thi Δ(lac-proAB) F′[traD36 proAB+ lacI^q lacZΔM15]) (Sambrook *et al.*, 1989). Expression of MetRS and preparation of crude lysates were done as described previously (Gale & Schimmel, 1995b). The crude extract was then dialyzed into 25 mM Tris-HCl (pH 7.5) containing 1 mM β-mercaptoethanol (buffer A) and loaded onto a 500 mL DEAE-TSK column. The column was then washed with 600 mL of buffer A, and the protein was eluted with a linear NaCl gradient from 0 to 400 mM in 1600 mL total volume. Fractions containing MetRS$_{1-547}$ or MetRS$_{1-676}$ were identified by SDS−PAGE, concentrated to about 5−10 mg/mL, and then purified over a 100 cm × 2.4 cm Sephacryl 100HR column (Pharmacia LKB Biotechnology, Uppsala, Sweden) in buffer A mixed with 200 mM NaCl. Protein concentration was determined by absorbance at 280 nm (Cassio & Waller, 1971). For the aminoacylation assays, MetRS$_{1-676}$ was purified as described previously (Burbaum & Schimmel, 1991) and subsequently was stored at 4 °C in buffer A mixed with 200 mM NaCl. Samples were removed from time to time, and enzyme concentrations were determined by active site titrations as described by Fersht *et al.* (1975).

AlaRS$_{1-461}$-6H was expressed from the plasmid pQE-461 which was cotransformed with plasmid pREP4 (which carries the *lacI* gene (Farabaugh, 1978)) into strain TG1 to express high levels of the *lac* repressor. Plasmid pQE-461 contains the gene for AlaRS$_{1-461}$-6H which encodes residues 1−461 of AlaRS joined to a C-terminal extension of six His codons (courtesy of L. Ribas, unpublished results) to facilitate purification of the expressed protein by affinity chromatog-

raphy on a Ni^{2+}−nitrilotriacetic acid (Ni−NTA) resin column (Hochuli, 1989; Janknecht *et al.*, 1991) (Qiagen, Chatsworth, CA). Expression of $AlaRS_{1-461}$-6H was induced with 1 mM IPTG, and the cells were harvested in late log phase. The cells were resuspended in 50 mM sodium phosphate (pH 7.8), 300 mM NaCl, and 0.5 mM phenylmethanesulfonyl fluoride and lysed in a French press at 15 000 lb/in^2. The lysate was centrifuged at 30 000 rpm for 1 h in a Beckman ultracentrifuge (Palo Alto, CA). $AlaRS_{1-461}$-6H was then purified on a 4 mL Ni−NTA agarose column according to the manufacturer's instructions (Qiagen, Chatsworth, CA), except that none of the buffers used contained glycerol. Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Affinity Coelectrophoresis. Affinity coelectrophoresis (Lee & Lander, 1991) was used to investigate the binding of the proteins to the RNA substrates and was performed as described previously (Kim *et al.*, 1993; Gale & Schimmel, 1995b). The standard buffer was 50 mM HEPES (pH 7.5), 0.1 mM EDTA, 4 mM $MgCl_2$, 1 mM β-mercaptoethanol, and 100 μg of BSA/mL. This buffer was used in all ACE gel experiments except where specifically indicated. All experiments were done in a thermostated circulating gel box (Hoefer Super Sub model HE100, Hoefer Scientific, San Fransisco, CA) at 25 °C. Although the aminoacylation assays were performed at 37 °C, which provided a basis for comparison with related work (Lee *et al.*, 1992), affinity coelectrophoresis was not practical at 37 °C because low-melting point agarose was used to prevent excessive heating of the protein when it was mixed with liquid agarose to pour the gel.

For the affinity coelectrophoresis competition experiments, an ACE gel was prepared as described previously except that an unlabeled competitor tRNA (either $tRNA^{fMet}$ or $tRNA^{Val}$) was added to the gel along with the protein ($MetRS_{1-676}$) in one lane. $MetRS_{1-676}$ was added to the gel in two lanes at a final concentration of 10 μM. The competitor tRNA sample was also added to one of these lanes at a final concentration of 12 μM. The 5′-[^{32}P]-labeled microhelix RNA substrate was then electrophoresed through these lanes and the shift of 5′-[^{32}P]RNA was compared between the lane with and the one without competitor tRNA.

RESULTS

Aminoacylation of $tRNA^{fMet}$ and Microhelix RNA Substrates. Mutations in the acceptor stem of $tRNA^{fMet}$ can substantially decrease V_{max}/K_m for aminoacylation. For example, for the "discriminator base" G73 mutant, V_{max}/K_m for aminoacylation was decreased 30-fold relative to wild type $tRNA^{fMet}$. While the G72 mutant was reduced only 2.3-fold in its rate of aminoacylation, a double G72, G73 mutant had V_{max}/K_m reduced 475-fold, apparently due to a strong synergistic coupling of the two mutations in the double mutant. This double mutant was also demonstrated to be defective *in vivo*. Interestingly, the apparent K_m for aminoacylation was only decreased 2.3-fold, showing that the acceptor helix interactions in the full tRNA have a strong influence on V_{max} (Lee *et al.*, 1992). Other acceptor stem mutations also strongly affect the kinetic parameters (Meinnel *et al.*, 1993).

We were interested to see whether the effects of mutations in the acceptor stem of full-length $tRNA^{fMet}$ would be reproduced in microhelices based on the acceptor stem alone or whether the effects of these mutations in the whole tRNA were influenced by the part of the structure which is missing from microhelices. In particular, we wanted to determine if much of the discrimination of $microhelix^{fMet}$ was due to interactions in the transition state of catalysis, as suggested for acceptor stem interactions of $tRNA^{fMet}$. In previous work, Martinis and Schimmel (1992) used a gel electrophoresis assay to detect directly the charging of $microhelix^{fMet}$ with [^{35}S]methionine. This assay was not sensitive enough for measuring k_{cat}/K_m. Alternatively, the sensitive HPLC assay developed by Francklyn *et al.* (1992) was adopted and used to determine the rate of aminoacylation under conditions where the rate is linear with respect to total enzyme or total substrate concentration.

Table 1: Kinetic Parameters for Aminoacylation of RNA Substrates by *E. coli* MetRS at pH 7.5, 37 °C

RNA	k_{cat}/K_m (M^{-1} s^{-1})[a]	relative $k_{cat}/K_m \times 10^6$ [b]
$tRNA^{fMet}$	2.8×10^6	1.0×10^6
$microhelix^{fMet}$	9.4×10^{-1}	0.34 (1)[c]
G73 $microhelix^{fMet}$	1.3×10^{-1}	0.046 (0.14)
G72, G73 $microhelix^{fMet}$	7.0×10^{-2}	0.025 (0.074)
$microhelix^{Ala}$	8.3×10^{-2}	0.030 (0.088)

[a] The estimate error in k_{cat}/K_m values is ±15%. All data are for full-length native enzyme. [b] Relative k_{cat}/K_m is the ratio of k_{cat}/K_m of each RNA substrate to the k_{cat}/K_m of $tRNA^{fMet}$. [c] The values in parentheses are k_{cat}/K_m values for the microhelices relative to wild type $microhelix^{fMet}$.

The k_{cat}/K_m for the aminoacylation of $tRNA^{fMet}$ is 2.8×10^6 M^{-1} s^{-1}, at pH 7.5, 37 °C (Table 1). [This value is comparable to a value of 1.43×10^6 M^{-1} s^{-1} (at pH 7.6, 25 °C) determined by Meinnel *et al.* (1993).] In contrast, k_{cat}/K_m for the aminoacylation of $microhelix^{fMet}$ (Figure 1) is 9.4×10^{-1} M^{-1} s^{-1}. Thus, k_{cat}/K_m is decreased (3.0×10^6)-fold relative to the k_{cat}/K_m for the aminoacylation of $tRNA^{fMet}$ (Table 1). Because of the large reduction in rate, we could not accurately measure individual k_{cat} and K_m parameters.

Two mutant $microhelix^{fMet}$ substrates were also characterized. Mutation of the discriminator base from A73 to G73 decreases the k_{cat}/K_m for aminoacylation 7-fold below that of wild type $microhelix^{fMet}$. The k_{cat}/K_m for the aminoacylation of the double mutant G72, G73 (Figure 1) is 13-fold less than that of wild type $microhelix^{fMet}$ (Table 1). Thus, the single and double mutants are defective for aminoacylation but less so relative to the wild type counterpart than are the same mutations when placed in the full tRNA (see above).

In the acid gel techniques used by Martinis & Schimmel (1992), trace levels of charging of $microhelix^{Ala}$ by MetRS were detectable. The k_{cat}/K_m of the noncognate substrate $microhelix^{Ala}$ (Figure 1) was measured in this work and found to be 8.3×10^{-2} M^{-1} s^{-1} (Table 1), which is a decrease of about 11-fold relative to that of wild type $microhelix^{fMet}$. Earlier work showed that no aminoacylation was detected in the cases of $microhelix^{His}$, $microhelix^{Gly}$, $microhelix^{Glu}$, or $microhelix^{Phe}$ or in the cases of several mutant variants based on the $tRNA^{Met}$ or $tRNA^{fMet}$ acceptor stem (Martinis & Schimmel, 1992, 1993). Therefore, with the exception of a weak aminoacylation of $microhelix^{Ala}$, $microhelix^{fMet}$ charging is highly specific.

Binding of MetRS to $Microhelix^{fMet}$ Investigated by Affinity Coelectrophoresis. To determine whether the effect

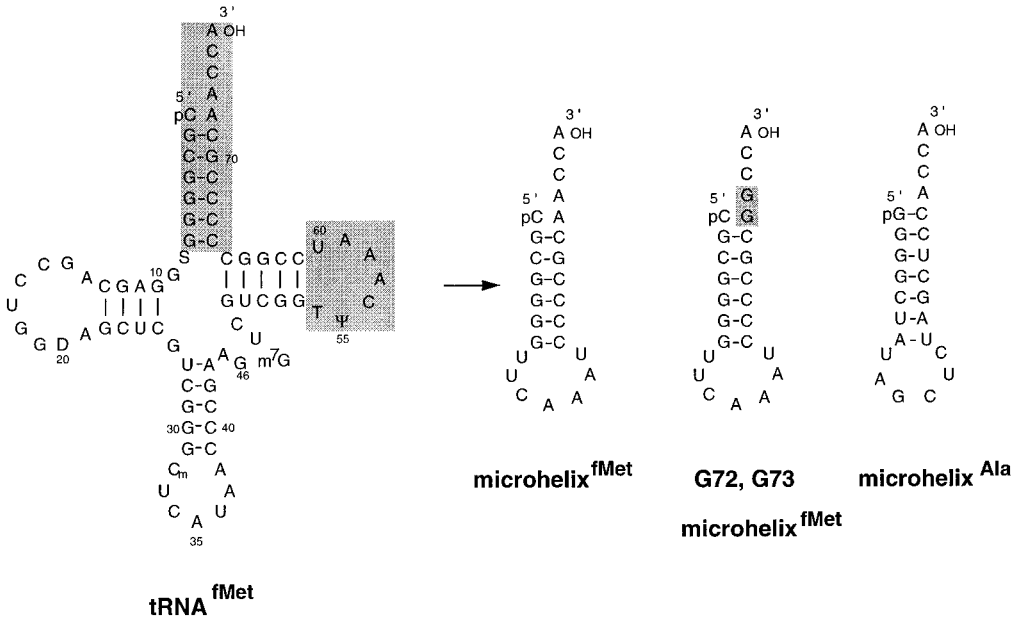

FIGURE 1: Sequence and cloverleaf structure of *E. coli* tRNA^{fMet} (left) (Sprinzl *et al.*, 1989). The acceptor stem and TΨC loop are highlighted to indicate portions of the tRNA recapitulated in microhelix RNA substrates shown on the right. From left to right are wild type microhelix^{fMet}, double mutant G72, G73 microhelix^{fMet}, and wild type microhelix^{Ala}.

Table 2: Dissociation Constants of $MetRS_{1-676}$ and $AlaRS_{1-461}$-6H for tRNA^{fMet} and Small RNA Substrates at pH 7.5 and 25 °C[a]

	dissociation constants (K_d, μM)	
	$MetRS_{1-676}$	$AlaRS_{1-461}$-6H
tRNA^{fMet}	0.51 ± 0.14[b]	not tested
anticodon S−L	22 ± 6	not tested
microhelix^{fMet}	12 ± 3	not detectable
G72, G73 microhelix^{fMet}	12 ± 1	not tested
microhelix^{Ala}	5.6 ± 1.6	12 ± 5
C70 microhelix^{Ala}	4.6	not detectable

[a] The K_d values were determined from Scatchard plots of the data obtained from affinity coelectrophoresis. [b] Result previously reported in Gale and Schimmel (1995a).

of mutations on k_{cat}/K_m was due to a change in binding affinity, we chose to investigate the association of these microhelix substrates to native $MetRS_{1-676}$, using the technique of affinity coelectrophoresis. Affinity coelectrophoresis is a gel retardation method in which the protein of interest is imbedded directly in an agarose gel at defined concentrations. The labeled RNA substrate is then electrophoresed through the protein in the gel, allowing the measurement of binding affinities under equilibrium conditions (Lee & Lander, 1991; Lim *et al.*, 1991; Kim *et al.*, 1993). Using this technique we determined that MetRS and the C-terminal domain of MetRS bind specifically to an RNA substrate that recapitulates the anticodon stem−loop of tRNA^{fMet}. For both proteins, binding was not detectable when a single base of the anticodon was substituted within the anticodon stem−loop substrate (Gale & Schimmel, 1995b).

As measured by affinity coelectrophoresis, full length *E. coli* $MetRS_{1-676}$ binds to tRNA^{fMet} with a K_d of 0.51 ± 0.14 μM (Table 2) (Gale & Schimmel, 1995a). We then found that $MetRS_{1-676}$ binds to the wild type microhelix^{fMet} substrate with a K_d of 12 ± 3 μM. A K_d of 12 μM suggests a significant amount of binding energy for acceptor stem interactions with $MetRS_{1-676}$.

To further analyze binding of MetRS to the acceptor stem of tRNA^{fMet}, we investigated binding to G72, G73 microhelix^{fMet}. We also tested binding of the noncognate microhelix^{Ala} to $MetRS_{1-676}$. In contrast to the aminoacylation results (Table 1), we found that the double mutation G72, G73 had little or no effect on binding to $MetRS_{1-676}$. $MetRS_{1-676}$ bound to both the wild type microhelix^{fMet} and the G72, G73 microhelix^{fMet} with a K_d of 12 μM (Table 2, Figure 2A,B). In addition, microhelix^{Ala} binds more tightly to $MetRS_{1-676}$ than to either of the microhelix^{fMet} substrates, with a K_d of 5.6 μM (Table 2, Figure 2C). Finally, a mutant C70 microhelix^{Ala} also bound to $MetRS_{1-676}$ (with a K_d of 4.6 μM) (Table 2).

The affinity coelectrophoresis experiments are carried out in the absence of added monovalent salt. In order to confirm that our results could be directly related to the aminoacylation experiments, NH_4Cl (150 mM) was added to the standard affinity coelectrophoresis buffer and the experiments were repeated. The resulting composition is identical to the buffer conditions used in the aminoacylation assays. Under these conditions, the binding of $MetRS_{1-676}$ to microhelix substrates was weakened 5−10-fold, but for all three substrates the relative decrease was about the same. Therefore, the relative binding constants for the three microhelix substrates were unchanged (data not shown).

These experiments collectively suggest that synthetase−microhelix interactions in the transition state rather than at the binding step are the major contributor to the specificity of microhelix aminoacylation.

Binding of AlaRS to Microhelix^{Ala} Is Sensitive to the 3·70 Base Pair. In recent work, we showed that the affinity coelectrophoresis method could easily demonstrate the marked difference in affinity for methionyl-tRNA synthetase of a wild type and mutant RNA hairpin mimicking the anticodon stem−loop of tRNA^{fMet}. In particular, a hairpin with a CAU anticodon bound with a dissociation constant of about 30 μM, while binding of a GAU-containing hairpin could not be detected (Gale & Schimmel, 1995b). These and other experiments support the idea that highly specific interactions can be monitored by the gel electrophoresis method and suggest that the lack of specificity of binding

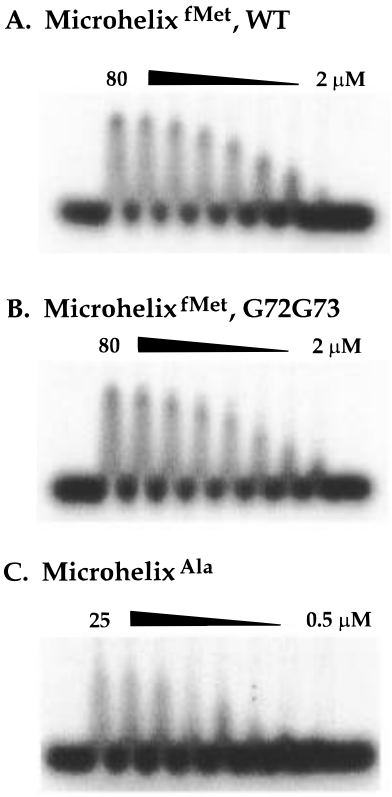

FIGURE 2: Affinity coelectrophoresis analysis of the binding of $MetRS_{1-676}$ to microhelix RNA substrates. (A) ACE gel of $MetRS_{1-676}$ with microhelix^{fMet}. $MetRS_{1-676}$ concentration ranged from 2 to 80 μM. (B) ACE gel of $MetRS_{1-676}$ with G72, G73 microhelix^{fMet}. $MetRS_{1-676}$ concentration ranged from 2 to 80 μM. (C) ACE gel of $MetRS_{1-676}$ with microhelix^{Ala}. $MetRS_{1-676}$ concentration ranged from 0.5 to 25 μM.

of microhelix^{fMet} substrates to methionyl-tRNA synthetase truly reflects a greater emphasis on transition state interactions.

To demonstrate further the detection of specific interactions, however, we turned to alanyl-tRNA synthetase, where acceptor helix interactions have been well studied by other methods. The main identity element of $tRNA^{Ala}$ is a G3·U70 base pair in the acceptor stem (Hou & Schimmel, 1988; McClain & Foss, 1988). Mutation of this base pair to G·C or to A·U eliminates aminoacylation *in vivo* and *in vitro* (Hou & Schimmel, 1988). Furthermore, a $tRNA^{Ala}$ with a substituted 3·70 base pair does not inhibit charging of wild type $tRNA^{Ala}$ (at pH 7.5), suggesting that binding is weakened by a factor of at least 30 (Park *et al.*, 1989). Therefore, we would expect that the binding of AlaRS to microhelix^{Ala} substrates would be sensitive to substitutions of the G3·U70 base pair.

We used a fragment of AlaRS that contains residues 1−461 ($AlaRS_{1-461}$-6H), because a fragment of the N-terminal 461 amino acids aminoacylates microhelix^{Ala} with the same efficiency as full-length AlaRS (Buechter & Schimmel, 1993). We found by affinity coelectrophoresis that $AlaRS_{1-461}$-6H bound to microhelix^{Ala} with a K_d of 12 ± 5 μM. However, we could not detect binding to $AlaRS_{1-461}$-6H of either G3·C70 microhelix^{Ala} or microhelix^{fMet} (Table 2). These results confirm that interactions with acceptor helices which differ by a single base pair can be discriminated by the ACE method.

Competition Between Binding of Microhelix ^{fMet} and $tRNA^{fMet}$. In order to prove that binding of microhelix substrates to MetRS was at part of the same site as that occupied by $tRNA^{fMet}$, we performed an affinity coelectrophoresis gel competition assay. $MetRS_{1-676}$ and $tRNA^{fMet}$ were placed together into the ACE gel at concentrations of 10 and 12 μM, respectively, in one lane. In a parallel lane, $MetRS_{1-676}$ was added alone at a concentration of 10 μM. The three 5′-[^{32}P]-labeled microhelix substrates of interest were then separately electrophoresed through these lanes (Figure 3). Presumably, $tRNA^{fMet}$ should compete for the binding sites on $MetRS_{1-676}$ and inhibit the binding of the microhelix substrates. Therefore, in the lane containing $tRNA^{fMet}$, the microhelix substrate should have a decreased shift relative to the lane without $tRNA^{fMet}$. This expectation was fulfilled for all three microhelix substrates (Figure 3). (The inhibition of shift was most pronounced for wild type microhelix^{fMet}.) As a negative control, $tRNA^{Val}$ was used as the competitor RNA for microhelix^{fMet}. In this case, the shift of microhelix^{fMet} was only slightly inhibited (Figure 3). We conclude that wild type and G72, G73 microhelix^{fMet} and noncognate microhelix^{Ala} each bind to the same site as the one occupied by $tRNA^{fMet}$.

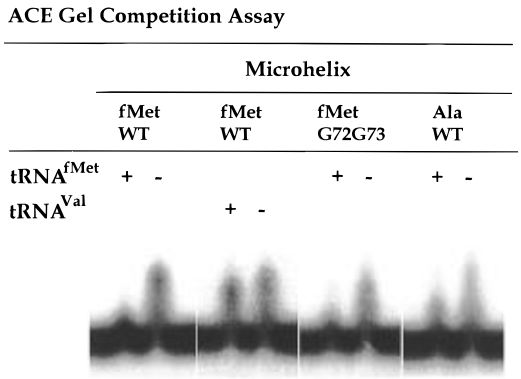

FIGURE 3: ACE gel competition assay: For each 5′-[^{32}P]-labeled microhelix RNA substrate, $MetRS_{1-676}$ at a concentration of 10 μM was placed in each of two lanes. Transfer RNA^{fMet} or $tRNA^{Val}$ at a concentration of 12 μM was added to the left lane of each pair. The 5′-[^{32}P]-labeled microhelices were electrophoresed through the paired lanes (+ −) and unlabeled tRNA inhibited the shift of the labeled microhelix substrate, if the two RNA molecules competed for the same binding site on $MetRS_{1-676}$.

Influence of the C-Terminus on Microhelix ^{fMet} Binding to the N-Terminal Domain. The dissociation constant for the complex of $MetRS_{1-676}$ with $tRNA^{fMet}$ is 7-fold smaller than that for the complex between the truncated $MetRS_{1-547}$ and $tRNA^{fMet}$ (Table 3) (Gale & Schimmel, 1995b). The relative difference in affinity for $tRNA^{fMet}$ is comparable to that seen by Blanquet *et al.* (1973), as measured by fluorescence quenching. We speculated that the difference in affinity (for $tRNA^{fMet}$) between the native and truncated forms of methionyl-tRNA synthetase was related to the role in acceptor helix binding of the peptide appendix located at the C-terminal end of $MetRS_{1-547}$. This peptide appendix is critical for the stability of the truncated synthetase (Mellot *et al.*, 1989), and mutations within a conserved portion of the appendix (Y531−D535) have a marked affect on binding of $tRNA^{fMet}$ or of microhelix^{fMet} (Mellot *et al.*, 1989; Kim *et al.*, 1993). In particular, an Arg533Ala substitution abolished detectable binding of microhelix^{fMet} (Table 3) (Kim *et al.*, 1993).

We reasoned that the conformational stability of this appendix might be weakened in $MetRS_{1-547}$ compared with $MetRS_{1-676}$ and, additionally or alternatively, that the C-

Table 3: Dissociation Constants of $MetRS_{1-547}$ Wild Type and the R533A Mutant for $tRNA^{fMet}$ and Small RNA Substrates at pH 7.5 and 25 °C[a]

	dissociation constants (K_d, μM)	
	$MetRS_{1-547}$	$MetRS_{1-547}$ (R533A)[b]
$tRNA^{fMet}$	3.6,[c] (0.51)[d]	not tested
anticodon S−L	31,[c] (22)	5.4 ± 1.0
$microhelix^{fMet}$	330 (12)	not detectable
G72, G73 $microhelix^{fMet}$	520 (12)	not tested
$microhelix^{Ala}$	210 (5.6)	not tested

[a] The K_d values were determined from Scatchard plots of the data obtained from affinity coelectrophoresis. The values given had errors ranging from 16% to 28%. [b] Results for this protein previously reported in Kim *et al.* (1993). [c] Results previously reported in Gale and Schimmel (1995b). [d] The numbers in parentheses are the equivalent values for $MetRS_{1-676}$ shown in Table 2.

terminal side of residue 547 might also contribute to acceptor helix interactions. In either case, weakened interactions with the acceptor stem could explain why $MetRS_{1-547}$ has a reduced affinity for $tRNA^{fMet}$. Alternatively, the strength of anticodon interactions could be different with the two forms of MetRS, especially because the C-terminal domain contains all of the determinants for binding to the anticodon of $tRNA^{fMet}$.

We found that $MetRS_{1-676}$ binds to an RNA hairpin that recreates the anticodon stem−loop (of $tRNA^{fMet}$) with a dissociation constant of 22 μM (Table 2). This value is similar to that observed for the complex of the RNA hairpin with $MetRS_{1-547}$ (31 μM) (Table 3) (Gale & Schimmel, 1995b). Therefore, the difference in affinity (for $tRNA^{fMet}$) between $MeRS_{1-676}$ and $MetRS_{1-547}$ is not due to a difference in binding to the anticodon stem−loop of $tRNA^{fMet}$. This result is consistent with anticodon interactions occurring entirely within the domain Ile367-Lys547 (Gale & Schimmel, 1995b) and suggests that the integrity of this structural unit for anticodon binding is not influenced by the region from residues 548−676.

In contrast, we found a large difference in binding of $microhelix^{fMet}$ to $MetRS_{1-547}$ compared to $MetRS_{1-676}$. $MetRS_{1-676}$ binds to $microhelix^{fMet}$ with an apparent K_d of 12 μM, while $MetRS_{1-547}$ binds with an apparent K_d of 330 μM (Tables 2 and 3). A lack of binding discrimination between wild type, mutant, and noncognate microhelix substrates was also seen with $MetRS_{1-547}$ (Table 3). Therefore, the difference in binding affinity for $tRNA^{fMet}$ of native and truncated MetRS is mirrored in the affinity for $microhelix^{fMet}$. This result suggests that, within the portion of MetRS that is unique to the full-length synthetase (residues 548−676), there are residues that directly or indirectly influence the interaction of MetRS with the acceptor stem of $tRNA^{fMet}$.

DISCUSSION

The structure of the cocrystal of class I GlnRS with $tRNA^{Gln}$ shows that, in order for the site of aminoacylation to fit into the active site, a large conformational change in the tRNA is required. Some of these changes are at the end of the acceptor stem where, for example, the U1·A70 base pair of $tRNA^{Gln}$ is disrupted. Also, the 2-amino group of G73 is hydrogen bonded to the phosphate group of A72, thereby stabilizing a hairpin loop conformation for bound $tRNA^{Gln}$ (Rould *et al.*, 1989). The sensitivity of aminoacylation of $tRNA^{Gln}$ and $microhelix^{Gln}$ to specific sequences in the acceptor stem has been investigated (Jahn *et al.*, 1991; Wright *et al.*, 1993). Mutation of G73, or of the G2·C71 and G3·C70 base pairs of $tRNA^{Gln}$ has effects on both k_{cat} and K_m, possibly through effects on the conformational change at the acceptor end of $tRNA^{Gln}$.

Comparison of the structure of $MetRS_{1-547}$ with the structure of the GlnRS:$tRNA^{Gln}$ cocrystal led to a model for binding of $tRNA^{fMet}$ to MetRS which suggested binding-induced distortion of $tRNA^{fMet}$, similar to that seen with $tRNA^{Gln}$ (Perona *et al.*, 1991). If this distortion is required, then substitution of the first base pair (C1·A72) and of the discriminator base (A73) of $tRNA^{fMet}$ would be expected to have a significant effect on this conformational change.

The effect of acceptor stem mutations on k_{cat}/K_m was qualitatively the same for $tRNA^{fMet}$ and $microhelix^{fMet}$. However, our results show that the dissociation constant for $microhelix^{fMet}$ is not affected by the G72, G73 substitution (Table 2). This observation is in parallel with that reported by Meinnel *et al.* (1993) for the whole tRNA substrate. These authors showed that acceptor stem substitutions in $tRNA^{fMet}$ had little affect on binding interactions, even in cases where the K_m for aminoacylation was affected. Thus, the K_m for aminoacylation of $tRNA^{fMet}$ is not measuring just a binding phenomenon.

The results obtained here suggest that the N73 and 1·72 determinants for aminoacylation of $microhelix^{fMet}$ are responsible for correct orientation of the substrate in the transition state of the aminoacylation reaction. In the full-length $tRNA^{fMet}$, the identity of N73 affects the stability of the end of the acceptor stem as well as the conformation of the CCA end. In a $tRNA^{fMet}$ variant with a C1·G72 bp, substitution of A73 with U or C destabilizes the C1·G72 bp, possibly due to a loss of the stacking interaction of A73 with G72 (Lee *et al.*, 1993). NMR analysis of $microhelix^{fMet}$ substrates containing a mutant G1·C72 bp and either the wild type A73 or mutant U73 discriminator base showed a large effect of the U73 substitution on the conformation of the CCA end of the microhelix. In the A73 microhelix, the CCA end remains stacked on the acceptor stem. In the U73 microhelix, the CCA end folds back such that the 3′-terminal A76 is close to G1 (Puglisi *et al.*, 1994). In related work, NMR studies of $tRNA^{Ala}$ acceptor stem duplex RNA substrates showed that the G1·C72 bp and A73 both contribute significantly to the stability of the acceptor stem helix. Substrates with a C73 substitution or a U1·A72 bp were significantly less stable (Limmer *et al.*, 1993). Thus, in light of the studies by Lee *et al.* (1993), Limmer *et al.* (1993), and Puglisi *et al.* (1994), there is a sound structural basis for proposing that N73 and 1·72 determinants for aminoacylation could affect the orientation of the acceptor stem in the transition state of catalysis.

The results reported here on binding of $microhelix^{fMet}$ to MetRS, combined with the earlier results on the binding of the anticodon stem−loop, provide sufficient information to estimate the free energy cost of distorting the enzyme−tRNA complex when the two domains are linked together and bound simultaneously to the protein. This estimate can be achieved by adding the free energy of binding of each of the two stem−loop structures and comparing this sum with that obtained from an independent measure of the dissociation constant for the MetRS−$tRNA^{fMet}$ interaction. This estimate does not include all distortion energies such as, for

example, that part of the free energy of distortion of the acceptor stem which is common to both microhelix^{fMet} and tRNA^{fMet}.

Calculating the free energy of binding ($\Delta G°$) as $RT \ln K_d$ and given the values for binding of the RNA substrates to MetRS$_{1-676}$ (Table 2), $\Delta G°$ for tRNA$^{fMet} = -11$ kcal mol^{-1}, $\Delta G°$ for the anticodon stem−loop $= -8.7$ kcal mol^{-1}, and $\Delta G°$ for microhelix$^{fMet} = -9.1$ kcal mol^{-1}. [These values have been corrected for the cratic entropy contribution ($-RT \ln 55 = -2.4$ kcal mol^{-1}) associated with a bimolecular reaction (Cantor & Schimmel, 1980).] The sum of free energies for the anticodon stem−loop and microhelix^{fMet} is -17.8 kcal mol^{-1}, thus suggesting an estimate of about 7 kcal mol^{-1} for the free energy of strain associated with binding the linked domains of the whole tRNA. Because there may be additional favorable synthetase−tRNA contacts outside of the acceptor stem and anticodon, the actual free energy of distortion may be greater than 7 kcal mol^{-1}.

Another perspective comes from comparing the difference in apparent free energy of activation [$-RT \ln(k_{cat}/K_m)$] for aminoacylation of the microhelix *versus* tRNA^{fMet}. This difference is approximately 9.2 kcal mol^{-1} [$-RT \ln(0.34 \times 10^{-6})$; Table 1]. On the other hand, the difference in binding energies (based on K_d's) of the microhelix *versus* tRNA^{fMet} is only 1.9 kcal mol^{-1} [$-RT \ln(0.5/12)$; Table 2]. Thus, some or all of the 7 kcal mol^{-1} of excess binding energy which goes into strain (see above) may be applied to raising k_{cat} by reducing the apparent free energy needed for formation of the transition state of catalysis for the synthetase−tRNA complex. This reduction could be achieved by having a conformation of the synthetase−tRNA complex which is closer to that of the transition state than is the synthetase−microhelix complex.

Our analysis of the higher affinity of MetRS$_{1-676}$ compared to MetRS$_{1-547}$ for tRNA^{fMet} suggests that the incremental binding energy is due to contacts with the acceptor helix and not with the anticodon stem−loop. By comparison of the data in Tables 2 and 3, we estimate that their incremental contribution amounts to -1.2 (tRNA^{fMet}) and -2.0 (microhelix^{fMet}) kcal mol^{-1}. The less negative value for tRNA^{fMet} (by 0.8 kcal mol^{-1}) may reflect the cost of coupling a binding-dependent distortion of the acceptor helix to the rest of the tRNA structure.

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