Region of a Conserved Sequence Motif in a Class II tRNA Synthetase Needed for Transfer of an Activated Amino Acid to an RNA Substrate†

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Received November 29, 1993; Revised Manuscript Received February 23, 1994®

ABSTRACT: The class II *Escherichia coli* alanine tRNA synthetase aminoacylates RNA miniduplexes, which reconstruct the acceptor end of alanine tRNA with the critical G3:U70 base pair. A benzophenone photoaffinity label attached adjacent to G3:U70 in a miniduplex substrate was previously cross-linked to a long enzyme peptide that begins at Gly161 between the class-defining motifs 2 and 3 [Musier-Forsyth, K., & Schimmel, P. (1994) *Biochemistry 33*, 773–779]. To identify side chains in this peptide that potentially contribute hydrogen bonding or catalytic determinants for the RNA-dependent step of the aminoacylation reaction, peptide functional side chains that are conserved among sequenced alanine enzymes (Asp, Asn, Arg, Glu, Gln, and Tyr) were individually replaced. Of the 21 mutant proteins so generated, one was identified that was not viable even though it accumulated *in vivo*. This Asp235 → Ala mutant enzyme is defective in the rate of transfer of the activated amino acid to the 3′-end of the RNA substrate. The conserved Asp235 is at the beginning of motif 3. By comparison with the crystal structure of the related class II yeast aspartate tRNA synthetase complexed with $tRNA^{Asp}$ (Cavarelli et al., 1993), we suggest that D235 is not in direct contact with acceptor helix base pairs such as G3:U70. Instead, we propose that D235 contributes to transfer-step interactions at the 3′-end of alanine tRNA. Because D235 in alanine tRNA synthetase is at the beginning of one of the conserved motifs that define class II tRNA synthetases, this region of the structure may in general be important for the transfer step.

Recent analysis of aminoacyl tRNA synthetases has concentrated on understanding the specific functional roles of sequence motifs that are the basis for dividing the enzymes into two distinct classes of 10 each (Webster et al., 1984; Schimmel, 1987; Eriani et al., 1990; Cusack et al., 1990). In both classes, conserved sequence elements form parts of the active site domain for amino acid activation and for transfer of the aminoacyl adenylate to the 3′-end of the tRNA (Cusack et al., 1990, 1993; Ruff et al., 1991; Cavarelli et al., 1993). The synthetases roughly comprise two major structures, which appear to interact, respectively, with the two domains of the L-shaped tRNA molecule (Schimmel et al., 1993). Interactions with the acceptor helix-containing domain of the tRNA substrate occur via sequence elements recruited as insertions into the synthetase active site domain, and interactions with more distal parts of the tRNA are generally through a second, nonconserved structure that can be unique to the particular synthetase. Because at least eight aminoacyl tRNA synthetases catalyze sequence-specific aminoacylation of RNA hairpin oligonucleotides based on tRNA acceptor stems (Schimmel et al., 1993) (cf. Figure 1), acceptor helix interactions can be considered as an operational RNA code for amino acids and a primitive system of protein synthesis, which possibly was the progenitor of the genetic code. The class-defining conserved domains of synthetases were probably the primordial enzymes that arose early in evolution as some of the earliest proteins and were needed to interpret the operational RNA code (Buechter & Schimmel, 1993).

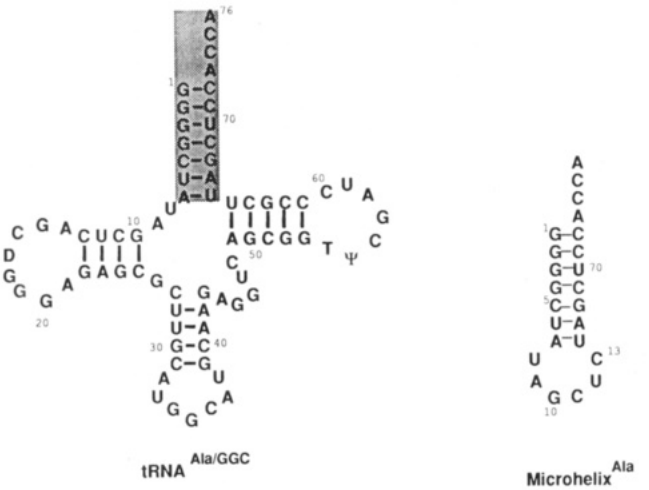

FIGURE 1: Sequence and cloverleaf structure of *E. coli* alanine tRNA and a seven base pair microhelix that is based on the acceptor stem (shaded) of $tRNA^{Ala}$.

The three class-defining sequence motifs of class II enzymes are degenerate and generally comprise 10% or less of the amino acid residues in the active site domain. These motifs are incorporated into parts of the eight-stranded antiparallel β-structure and three α-helices that make up the core of the conserved structure characteristic of this group of synthetases (Cusack et al., 1991; Moras, 1992). In the crystal structure of the class II yeast aspartate tRNA synthetase in complex with $tRNA^{Asp}$, motifs 2 and 3 form portions of the ATP binding site, and a variable loop in motif 2 and a segment of motif 1 have contacts with the end of the acceptor helix (Ruff et al., 1991; Cavarelli et al., 1993). Sequences idiosyncratic to the synthetase are inserted between motifs 2 and 3, and these sequences also contribute to acceptor helix interactions in the aspartate tRNA synthetase–$tRNA^{Asp}$ complex.

† This work was supported by Grant No. GM23562 from the National Institutes of Health.

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® Abstract published in *Advance ACS Abstracts,* April 1, 1994.

0006-2960/94/0433-5312$04.50/0

Little is known about the detailed molecular chemistry at the active site for the two-step aminoacylation reaction, which consists of activation of the amino acid by condensation with ATP to form an enzyme-bound aminoacyl adenylate followed by transfer of the aminoacyl moiety from the adenylate to the 3′-end of the tRNA. The transfer reaction is commonly rate-limiting for aminoacylation. Previous work established that discrimination of tRNAs is achieved at both the binding and catalytic steps (Schimmel & Söll, 1979; Lapointe & Giege, 1991; Giege et al., 1993). The K_m's for tRNAs in the aminoacylation reaction are on the order of 0.1–1 μM. Because they are relatively high for a protein–nucleic acid complex, the K_m's are limited in how much discrimination they can provide to assure the accuracy of the aminoacylation reaction. Substantial sensitivity to the substrate RNA sequence occurs kinetically at the transition state of catalysis. For example, an A3:U70 mutant $tRNA^{Ala}$ is not aminoacylated *in vitro*, but conditions can be found where, even though the mutant tRNA binds to the enzyme about as well as its wild-type counterpart, it still is not aminoacylated (Park et al., 1989). In a related study using acceptor stem oligonucleotide minisubstrates for alanine tRNA synthetase, RNA base substitutions, which prevented efficient aminoacylation, were shown to reduce severely the efficiency of transfer of the aminoacyl moiety from the adenylate to the 3′-end of the RNA substrate, even under conditions where the mutant RNA was saturating (Shi & Schimmel, 1991). Because misrecognition complexes are not aminoacylated, k_{cat} discrimination can be of greater significance than binding or K_m discrimination.

To investigate the poorly understood interactions at the acceptor helix of a class II tRNA synthetase, we previously attached a benzophenone affinity label adjacent to the G3:U70 base pair of an RNA duplex substrate for alanine tRNA synthetase (Musier-Forsyth & Schimmel, 1994). The enzyme makes an essential functional contact with the free exocylic 2-amino group in the minor groove of the wobble-paired G3 (Musier-Forsyth et al., 1991). Additional functional contacts occur with the 2′-OH's of G4, U70, and C71 and the 2-amino group of G2 (Musier-Forsyth & Schimmel, 1992). The benzophenone was attached to the internucleotide backbone between U70 and C69 so as not to interfere with essential functional contacts. As a consequence, the labeled substrate was efficiently aminoacylated, suggesting that binding contacts with the synthetase were largely intact. While these circumstances are favorable for obtaining an enzyme–RNA complex for affinity labeling, the location of the probe on the complexed RNA obviously is skewed away from the binding axis on the protein. The consequence is that affinity labeling with this probe is apt to indicate a general region rather than a specific residue or residues needed for acceptor helix interactions.

The photo-cross-linked peptide isolated from the enzyme is between motifs 2 and 3, a location consistent with acceptor helix interactions contributed by a region between these two motifs in the known structure of aspartate tRNA synthetase complexed with $tRNA^{Asp}$ (Cavarelli et al., 1993). Recognizing that the isolated peptide was long (60–70 amino acids), and that the structural resolution of the affinity label substrate we had designed (see above) was inherently limited, we investigated the contribution to activity by each of the conserved functional groups in this peptide and also extended the analysis through the entirety of motif 3. These functional groups were substituted individually with alanine and then tested for enzyme activity through an *in vivo* complementation assay. The effect of substitutions on protein stability was also monitored by determining whether the substituted proteins accumulated *in vivo*. These initial studies were then extended by *in vitro* analysis of those mutant proteins that showed phenotypes consistent with the substituted residue being important for aminoacylation. In this way, we identified a conserved aspartic acid at the beginning of motif 3, whose replacement severely reduced the efficiency of the transfer reaction.

MATERIALS AND METHODS

Materials. Alanine transfer RNA was a product of Subriden RNA (Rolling Bay, WA). Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA) or Boehringer Mannheim (Indianapolis, IN). $Microhelix^{Ala}$ was synthesized chemically according to Scaringe et al. (1990) on a Gene Assembler Plus (Pharmacia, Piscataway, NJ). Deoxyoligonucleotides for mutagenesis were synthesized on an Applied Biosystems 380B DNA synthesizer at the MIT Biopolymers Laboratory in the Center for Cancer Research.

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out by using the Amersham (Arlington Heights, IL) mutagenesis system that is based on the methods of Nakamaye and Eckstein (1986) and Sayers et al. (1988). DNA manipulations were according to Sambrook et al. (1989). The entire gene *alaS* for alanine tRNA synthetase was cloned into the *Eco*RI site of phagemid pBluescript KS(+) (Stratagene, La Jolla, CA) to give phagemid KS/*alaS*. This phagemid also encodes a gene for Amp^r. Single-stranded template DNA was prepared from MV1184 (*ara* Δ(*lac–proAB*) *rpsL thi*(*ϕ80 lacZ*ΔM15) Δ(*srl–recA*)306::TN10(tet^r) F′(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15) cells coinfected with phage M13K07 (Viera & Messing, 1987) and phagemid KS/*alaS*. Mutation of specific codons between G161 and D257 was accomplished with synthetic 20–22-nucleotide primers that spanned the codon of interest and that were complementary to the sense strand of the DNA, except at the site of the desired change. Mutant DNA sequences were determined by application of the chain-termination method of Sanger et al. (1977) with the sequenase enzyme of United States Biochemical Corporation (Cleveland, OH). In general, a 250-nucleotide region encompassing the mutation of interest was sequenced.

In Vivo Complementation Assays. These analyses were performed in *E. coli* strain W3110(*laqI*^{q} Δ*recA1 Kan*^{r} Δ*alaS2*)/pMJ901. This strain contains an ablation of the chromosomal copy of *alaS* and is maintained by plasmid pMJ901, which has a wild-type copy of *alaS*, a gene for Tet^r, and a temperature-sensitive replicon that cannot direct replication at 42 °C (Jasin & Schimmel, 1984; Regan, 1986). As a consequence, W3110/pMJ901 grows at 30 but not at 42 °C. Phagemid-encoded mutant genes for alanine tRNA synthetase were introduced into W3110/pMJ901 by selection for Amp^r and Tet^r transformants at 30 °C on LB plates, and then these transformants were tested for growth at 42 °C on LB/ampicillin (50 μg/mL) plates. Elimination of the pMJ901 maintenance plasmid was confirmed by the sensitivity of the growth to tetracycline (20 μg/mL).

Western Blot Analysis. Cells were grown at 30 °C in LB broth containing ampicillin (50 μg/mL) to an OD_{600} of approximately 1.2, harvested, resuspended in SDS[1] sample

[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AlaRS, alanine tRNA synthetase.

buffer consisting of 5.8 mM $NaPO_4$, 8% glycerol, and 1% SDS, and then heated to 95 °C for 20 min. Aliquots (3–5 μL) of the lysed extract were applied to the top of an SDS–polyacrylamide (8%) gel and subjected to electrophoresis (Hoefer Scientific Instruments Model SE200, San Francisco, CA) at 10 mA constant current until the tracking dye entered the separating gel. The current was then increased to 15 mA (18 V/cm) for 45 min. Proteins on the gel were transferred onto Immobilon PVDF membranes (Millipore Corp., Bedford, MA) using a Milliblot-SDE apparatus (Millipore). Membranes were treated with anti-*Escherichia coli* alanine tRNA synthetase polyclonal antibodies, washed with phosphate buffer (5.8 mM (pH 7.4)/saline (0.8% NaCl)), incubated with horse radish peroxidase-linked donkey anti-rabbit whole antibody, and visualized with the Amersham ECL detection system (Roswell & White, 1978).

Purification and Assays of Mutant Enzyme. The noncomplementing D235A mutant enzyme was purified by a special procedure, which afforded the nearly inactive mutant enzyme free from contamination with the active enzyme needed to sustain cell growth. A fragment spanning codons Glu209–Arg574 was released by the cleavage of phagemid DNA with *Bgl*II and *Pfl*MI and transferred into plasmid pT875. Plasmid pT875 encodes wild-type *alaS* behind a *tac* promoter (Regan, 1986). The pT875-based plasmid encoding the mutant enzyme was introduced into strain W3110/pLR461, where the null allele of W3110 (see above) is maintained by enzyme activity expressed from plasmid pLR461 (Regan, 1986). This plasmid encodes a monomeric N-terminal fragment of 461 amino acids that has sufficient activity to sustain cell growth as the only source of alanine tRNA synthetase. The full-length enzyme of 875 amino acids, which assembles into a tetramer, is easily separated from the 461-mer N-terminal fragment. W3110/pLR461 cells harboring the pT875 mutant enzyme-encoding plasmid were grown at 30 °C to an OD_{600} = 0.5 and then induced with 1 mM isopropyl 1-β-D-thiogalactopyranoside (IPTG) in midlog phase for 6 h (Barkley & Bourgeois, 1978) and harvested. The cells were disrupted in a French press (SLM Instruments, Inc., Urbana, IL), and enzyme purification used DEAE–cellulose chromatography, Mono Q FPLC (Pharmacia, Piscataway, NJ), and Superose 6 (Pharmacia) size exclusion chromatography [an adaptation of the procedures of Hill and Schimmel (1989) and Buechter and Schimmel (1993)]. Enzyme concentrations were determined by active site titration (Fersht et al., 1975).

Aminoacylation assays were carried out at 37 °C in a reaction mixture containing 50 mM Hepes (pH 7.5), 2 mM ATP, 20 μM [^{3}H]alanine (6 Ci/mmol, New England Nuclear, Boston, MA), 10 mM $MgCl_2$, 20 mM KCl, 20 mM β-mercaptoethanol, and the concentrations of enzyme indicated (Schreier & Schimmel, 1972). The ATP concentration is saturating (K_m = 83 μM; Hill & Schimmel, 1989). Because the aminoacylation rates are linear with alanine concentration in this range (K_m = 240 μM for alanine; Hill & Schimmel, 1989), and because we wished to compare the aminoacylation rates with those for amino acid activation carried out at an alanine concentration of 50 μM, the measured aminoacylation rates were multiplied by a factor of 2.5. Similarly, because of the large difference in K_m for the microhelix versus the tRNA substrate (Shi et al., 1992), it was not possible to measure aminoacylation rates at the same RNA concentration for each. However, by taking measurements for each substrate at a concentration below its respective K_m, so that initial velocities were linear with RNA concentration, we were able to convert the rate with each substrate to the same apparent concentration [0.5 μM, which is below the K_m for tRNA or the microhelix (Shi et al., 1992)]. Amino acid activation rates were determined at 37 °C by the ATP–PP_i exchange assay adapted from Calendar and Berg (1966). The assay mixture contained 100 mM Hepes (pH 7.5), 2 mM ATP, 2 mM [^{32}P]$NaPP_i$ (0.2 mCi/mmol, New England Nuclear), 10 mM KF, 50 μM alanine, 10 mM β-mercaptoethanol, 5 mM $MgCl_2$, and enzyme concentrations as indicated. The preparation of the enzyme–adenylate complex and the conditions for studying the single-turnover transfer reaction were according to Shi and Schimmel (1991).

RESULTS

Experimental Strategy. The aforementioned benzophenone-labeled RNA was cross-linked to a 60–70 amino acid peptide in *E. coli* alanine tRNA synthetase that starts at Gly161 (Musier-Forsyth & Schimmel, 1994). In the predicted secondary structure of the conserved domain of the enzyme, Gly161 is located between strands S5 and S6 of the eight-stranded antiparallel β-structure (Ribas de Pouplana et al., 1993). We examined the aligned sequences of six alanine tRNA synthetases and identified conserved side chains with functional chemical groups that were located in the region G161–D257. Although D257 is well beyond the estimated C-terminus of the cross-linked peptide, it was convenient to extend the analysis so as to encompass all of motif 3 (which approximately includes the segment from T236 to H249). Altogether, 21 amino acids with conserved chemical groups were identified in the G161–D257 segment. Following the rationale for alanine-scanning mutagenesis described by Cunningham and Wells (1989), the 21 aforementioned residues were individually substituted with alanine, and the mutant proteins were then expressed in the W3110/pMJ901 *alaS* null strain. This tester strain has an ablation from the chromosome of the gene *alaS* for alanine tRNA synthetase and is maintained by the *alaS*-containing maintenance plasmid pMJ901, whose replication is directed by a temperature-sensitive replicon (Jasin & Schimmel, 1984). As a consequence, strain W3110/pMJ901 is temperature-sensitive, growing at the permissive temperature of 30 °C, but not at 42 °C where plasmid pMJ901 is lost. When a second, compatible plasmid encoding alanine tRNA synthetase activity is introduced, the temperature-sensitive phenotype is rescued. We first tested our mutant proteins, therefore, for their ability to complement the null strain by introducing plasmids that encode the mutant enzymes.

The mutant proteins were divided into two groups—those that complemented the null strain and those that did not. In each case, plasmid DNA was reisolated from the test strain and sequenced in the region of the mutation for verification. The production of mutant alanine tRNA synthetase in the complemented null strain was verified by Western blot analysis of cellular proteins resolved by SDS gel electrophoresis and probed with anti-*E. coli* alanine tRNA synthetase antibodies. With the noncomplementing mutants, we checked for protein production in a different way. A plasmid encoding a noncomplementing mutant was introduced into strain W3110/pLR461. This null strain was maintained by plasmid pLR461, which encodes a truncated N-terminal 461 amino acid active fragment of alanine tRNA synthetase (Ho et al., 1985; Regan et al., 1987, 1988). By introducing the second plasmid encoding the full-length mutant protein and growing the cells at 30 °C, expression of the mutant protein could be determined by Western blot analysis because it completely separates from the maintenance protein fragment (461 amino acids) on SDS gel electrophoresis.

Table 1: Mutagenesis of Alanine tRNA Synthetase

mutation	complementation[a]	mutant protein detected by Western blot analysis[b]
D167A	+	+
N168A	+	+
E183A	–	–
E183D	+/–	[+]
D187A	+	+
D190A	(–)[c]	(–)[c]
D203A	+	+
D205A	+	+
E209A	+	+
N212A	+	+
Q217A	+	+
N219A	+	+
R220A	+	+
D223A	+	+
D235A	–	+
E241A	+	+
R242A	–	–
Q248A	+	+
N253A	+	+
Y254A	+	+
D255A	+	+
D257A	+	+

[a] +, complementation at 42 °C of the Δ*alaS2* null allele. –, no complementation of the Δ*alaS2* null allele. +/–, marginal complementation (poor growth) of the Δ*alaS2* null allele. [b] +, full-length protein detected. [+], a reduced amount of full-length protein (relative to other complementing mutants) was detected. –, full-length protein not detected. [c] DNA sequence had a single deletion at D187 so that the reading frame was shifted. The non- or weak-complementing mutants with detectable proteins, E183D and D235, are boxed.

Mutant proteins that failed to complement and that showed no enzyme production were not considered further. Mutants that failed to complement but produced stable enzymes were of the most interest for *in vitro* analysis. These mutants were studied for their amino acid activation and aminoacylation activities and, as will be explained here, for their activity in the transfer reaction.

Active and Inactive Mutant Enzymes. The complementation tests and Western blot analyses with proteins having substitutions at each of 21 positions show that 17 of these alanine-substituted mutants complemented the null strain (Table 1). These 17 complementing mutants are in positions that encompass the entire region from D167 to D257, and collectively they replace a conserved Asp, Asn, Glu, Gln, Arg, or Tyr with alanine. Not surprisingly, all 17 of these plasmid-encoded mutants produced enzyme proteins that could be detected by anti-alanine tRNA synthetase antibodies.

Three mutants did not complement the null strain (E183A, D235A, and R242A). (One additional mutant (at position 190) was subsequently shown to have a deletion and frameshift and was not pursued further.) In addition to rechecking the DNA sequence in the region of each of these mutations (see above), we also checked the possibility that a spurious, unintended secondary mutation caused the noncomplementing phenotype. For this purpose, a DNA fragment encompassing each of the four noncomplementing mutations was recloned into plasmid pT875, and the resulting plasmid was retested for its complementation phenotype. Of the remaining three mutants (E183A, D235A, and R242A), only the D235A mutant protein accumulated *in vivo* (Table 1). The remaining two mutants—E183A and R242A—possibly failed to complement because the protein could not fold into a stable structure. The sensitivity to the substitution of position 183 was tested further by construction of an E183D mutant. This enzyme weakly complemented the null strain (viz., the cells grew slowly), and a small amount of protein could be detected by Western blot analysis.

Figure 2 presents an idiographic representation of the predicted, conserved domain of alanine tRNA synthetase and a linear diagramatic representation of secondary structure elements and the locations of the three class-defining conserved motifs. The predicted secondary structures resulted from an application of the neural network algorithm of Rost and Sander (1992), which uses alignments of multiple sequences for the same protein, and from the knowledge that the conserved sequence motifs are associated with specific structural elements (Ribas de Pouplana et al., 1993). In the three-helix, eight-stranded β-structure, motif 1 comprises helix H1 and strand S1, motif 2 strands S2 and S3, and motif 3 strand S8 and helix H3.

The positions of the alanine substitutions are marked with arrows in Figure 2 and span the region from D167 to D257. The 17 open boxes enclose those positions where an alanine substitution was tolerated, while three shaded boxes designate the locations of inactivating alanine substitutions. Five of the substitutions are located within predicted secondary structural elements of the core domain, and two of these are inactivating (E183A and R242A). A sixth substitution (D235A), which is also inactivating, is immediately adjacent to the start of the S8 β-strand. The 14 remaining substitutions are outside the predicted secondary structure elements of the core class II enzyme domain, and all of these are at positions that give active protein. Although far more mutants need to be investigated, the findings that half of the six substitutions in or immediately adjacent to predicted secondary structures give defective proteins, and that none of the remaining 14 positions outside these predicted elements are sensitive to substitution, are compatible with the proposed secondary structure model.

In Vitro Analysis of the D235A Mutant Protein. The noncomplementing D235A mutant enzyme was well-expressed and was prepared for further investigations *in vitro*. For this purpose, the noncomplementing D235A enzyme had to be expressed in cells that had an alternative source of alanine tRNA synthetase activity and then purified away from that activity. The mutant enzyme coding sequence was expressed behind an IPTG-inducible *tac* promoter in plasmid pT875, which was introduced into the null strain W3110/pLR461 which is maintained by the N-terminal 461-mer fragment of alanine tRNA synthetase. Cell cultures were grown at 30 °C, induced with IPTG, harvested, and disrupted to give cell extracts, and mutant enzyme was purified away from the active fragment by, among other steps, size exclusion chromatography. Wild-type enzyme was additionally prepared for comparative purposes. Although we also hope to investigate the poorly complementing E183D mutant enzyme, the accumulation of this protein *in vivo* was greatly reduced compared to the wild-type and D235A enzymes, so that the E183D mutant enzyme was not pursued further.

Four assays were conducted with the D235A mutant enzyme: amino acid activation as measured by the alanine-dependent ATP–PP_i exchange reaction, aminoacylation of $tRNA^{Ala}$, aminoacylation of the microhelix^{Ala} substrate, and transfer of the alanyl group from the preformed, enzyme-bound alanyl-AMP to microhelix^{Ala}. The D235A mutant had severely reduced rates of aminoacylation of $tRNA^{Ala}$ and microhelix^{Ala} and a modest reduction in the rate of amino acid activation (Table 2). That the aminoacylation of microhelix^{Ala} is sharply reduced suggests that much of the defect in the $tRNA^{Ala}$ aminoacylation activity of the D235A

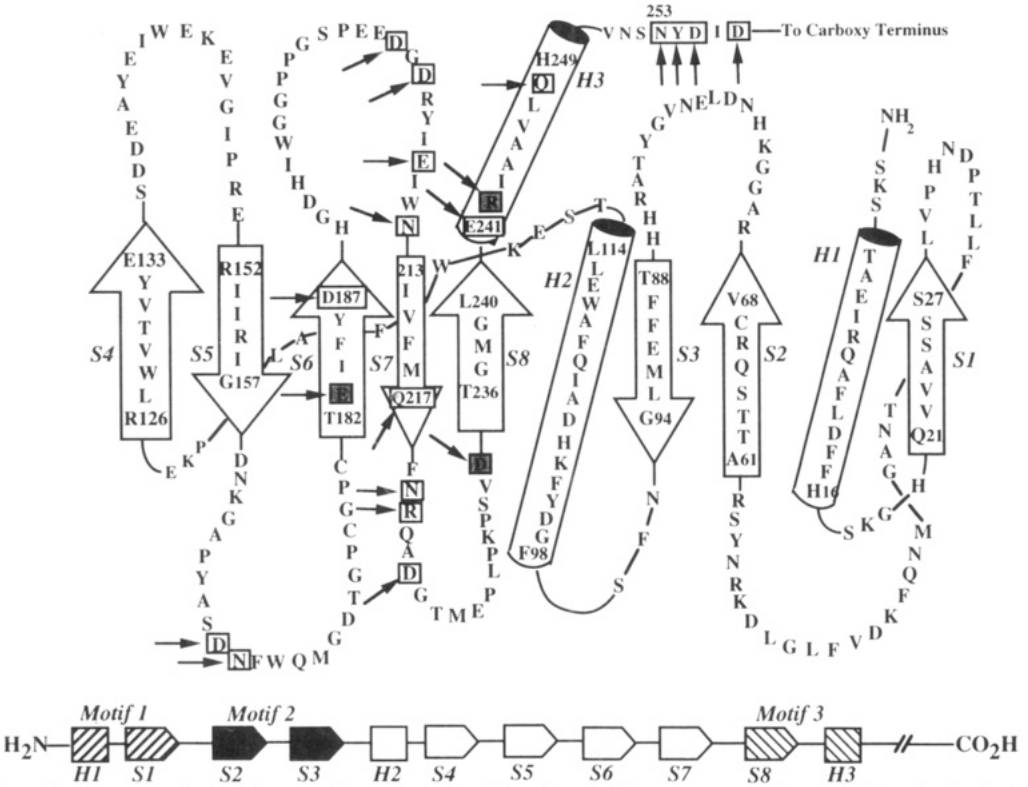

FIGURE 2: Idiographic representation of the secondary structure of the class-defining conserved domain of *E. coli* alanine tRNA synthetase, with arrows designating β-strands and cylinders α-helices (Ribas de Pouplana et al., 1993). The linear diagram beneath the idiograph indicates the locations of the three motifs shared by class II tRNA synthetases. [In addition to the predicted secondary structural elements given by Ribas de Pouplana et al. (1993), this figure includes an additional eighth strand, which was identified by taking advantage of additional sequence information (L. Ribas de Pouplana and P. Schimmel, unpublished results).] Arrows designate the positions of individual alanine substitutions made in this work. Those residues whose replacement with alanine had no effect on the complementation of the *alaS* null strain are enclosed in open boxes, while those whose replacement resulted in noncomplementation are in shaded boxes.

Table 2: Comparison of Apparent Rate Constants for Wild-Type and D235A Mutant Enzymes at pH 7.5, 37 °C

	amino acid activation (s^{-1}) [relative value]	aminoacylation (s^{-1}) [relative value]	
		$tRNA^{Ala}$	microhelix^{Ala}
wild-type[a]	9.6 [1.0]	0.76 [1.0]	4.8×10^{-3} [1.0]
D235A	1.4 [0.15]	3.6×10^{-3} [0.0048]	1.7×10^{-4} [0.036]

[a] The apparent rate parameters for both reactions were computed to pertain to conditions of 2 mM and 50 μM for the concentrations of ATP and alanine, respectively, and 0.5 μM for the RNA concentration in the aminoacylation reactions. To obtain the rate constants, initial velocities were calculated from the linear part of the time course and then divided by the enzyme concentration which, in the ATP–PP_i exchange reactions, was 50–150 (wild-type enzyme) and 150–675 nM (D235A), in the tRNA aminoacylation reactions it was 3–10 (wild-type) and 15–30 nM (D235), and in the microhelix reactions it was 25–50 (wild-type) and 66–133 nM (D235A). Additional details are given in Materials and Methods.

mutant is localized to interactions between the protein and the acceptor stem.

The rate of aminoacylation of the D235A mutant enzyme is so low that reliable k_{cat} and K_m kinetic parameters could not be determined. For the wild-type enzyme, amino acid activation rates are about 10-fold greater than aminoacylation rates, under the conditions of the assay. The higher amino acid activation rate is consistent with the transfer step being rate-limiting for aminoacylation. For the D235A mutant, the 200-fold reduction in the relative rate constant for aminoacylation is far greater than the 7-fold lowering of the rate of the amino acid activation reaction (Table 2). This divergence suggests that the defect in aminoacylation by the D235A mutant enzyme is at the transfer step.

To investigate the transfer step in a single-turnover reaction, we prepared enzyme–adenylate complexes at 0 °C (to maintain their stability; Shi & Schimmel, 1991) and determined the rate of transfer of the alanyl moiety to microhelix^{Ala}. By using RNA concentrations estimated to be below the K_m for microhelix^{Ala}, an apparent k_{cat}/K_m for the mutant and wild-type enzymes was determined at three RNA concentrations. The rate of the single-turnover transfer step is significantly reduced (about 15-fold) for the D235A mutant enzyme at 0 °C, and as anticipated, the k_{cat}/K_m parameter was concentration-independent over the limited RNA concentration range that we were able to investigate (Figure 3).

DISCUSSION

For the 10 class II enzymes, a well-conserved part of the relatively degenerate motif 3 is the GLER tetrapeptide, which includes the absolutely conserved arginine (Eriani et al., 1990; Cusack et al., 1991, 1993; Moras, 1992). In *E. coli* alanine tRNA synthetase, this tetrapeptide begins with G239 and the conserved arginine is R242 (Figure 2). In *E. coli* serine tRNA synthetase, this residue is at the active site near bound ATP; however, while the ATP binding site is formed from motifs 2 and 3, this arginine is close to, but not in contact with, bound ATP and may therefore play a role in catalysis (Cusack et al., 1993). Of the 20 mutants investigated here, the R242A enzyme is only one of two noncomplementing ones that failed to accumulate *in vivo*. Replacement of this arginine in another class II enzyme has not yet been reported. In the absence of further information, we propose that, in addition to or as an alternative to a role in catalysis, the motif 3 conserved arginine may be needed for the assembly of the common active site

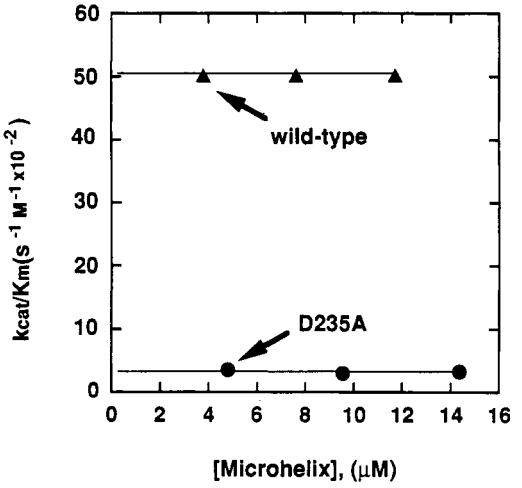

FIGURE 3: Apparent k_{cat}/K_m (with respect to RNA substrate) versus RNA microhelix concentration, for the initial velocity of the single-turnover transfer reaction at 0 °C, pH 7.5. The enzyme concentration were 40 and 33 nM, respectively, for the wild-type and D235A mutant enzymes.

structure, perhaps as a residue required for a specific step in the folding pathway.

The E183A mutant was the only other noncomplementing mutant protein that failed to accumulate *in vivo*. Glutamic acid 183 is located between possible ligands (C178 and C181 on one side and H188 and H191 on the other) for the bound zinc atom in alanine tRNA synthetase (Miller et al., 1991). Possibly, formation of the zinc binding site is impaired in E183A and in the poorly complementing E183D mutant enzyme.

The D235A enzyme was the only mutant not to complement the null strain and to accumulate in amounts comparable to the wild-type enzyme, when expressed from the same IPTG-inducible promoter. Position 235 is at the beginning of motif 3 on the N-terminal side of the GLER tetrapeptide and the conserved β-strand S8 (Figure 2). In the cocrystal of yeast aspartate tRNA synthetase with $tRNA^{Asp}$, this position is not near acceptor stem base pairs, but rather is located about 8–9 Å beyond the 3′-end of the bound tRNA (Cavarelli et al., 1993). Thus, it is unlikely that the conserved (among alanine tRNA synthetases) D235 makes contact with the acceptor helix. Instead, it probably forms a structure for, or directly participates in, the chemical step at the 3′-end of the acceptor stem. That the D235A substitution has a modest effect on amino acid activation (Table 2) is consistent with this part of the structure also being important for the formation of the site for ATP binding (and presumably adenylate synthesis) in serine tRNA synthetase (Cusack et al., 1993).

The D235A substitution lowers the aminoacylation of $tRNA^{Ala}$ by about 200-fold (Table 2). The 8-fold reduction in the rate of adenylate synthesis probably makes no contribution to the 200-fold reduction in the rate of aminoacylation, because the amino acid activation step is not rate-limiting for aminoacylation. The reduction in the rate of aminoacylation of $microhelix^{Ala}$ did not seem as great (25-fold) as that achieved with the $tRNA^{Ala}$ substrate, when the mutant was compared to the wild-type enzyme. This difference may reflect an effect of the rest of the tRNA structure on the conformation of the acceptor stem or, additionally or alternatively, of protein interactions with distal parts of the tRNA structure, which affect the acceptor end of the molecule. However, the aminoacylation rates with the $microhelix^{Ala}$ substrate are lower to begin with, even with the wild-type enzyme. The difference between the tiny rate of D235A enzyme-catalyzed aminoacylation of $tRNA^{Ala}$ compared to $microhelix^{Ala}$ may not be large enough to interpret unequivocally.

The D235A substitution is the first in a class II enzyme to demonstrate an effect on the transfer reaction. A previous study showed that substitutions of the A73 "discriminator base" in a $microhelix^{Ala}$ substrate severely affected transfer efficiency within the ternary enzyme–adenylate–microhelix complex formed with the wild-type protein. This result suggested that one role for the discriminator base was to improve RNA specificity through modulating the free energy of activation for the transition state of the transfer step (Shi & Schimmel, 1991). However, we doubt that D235 interacts directly with the discriminator base, because those interactions in the aspartate tRNA synthetase–$tRNA^{Asp}$ complex occur with the loop between strands S2 and S3 of motif 2. More likely, D235 is needed for formation of the pocket that binds the 3′-end of the tRNA or for catalysis per se, and this role is intimately associated with the efficiency of the transfer step. Because D235 is at the end of a conserved structural motif of class II enzymes, this region is probably generally involved in the reaction between an aminoacyl adenylate and the 3′-end of bound tRNA.

ACKNOWLEDGMENT

We thank Mr. Matthew Davis, Drs. Douglas Buechter, Deborah Hipps, and Sunghoon Kim, and Alyssa Shepard for helpful discussions throughout the course of this work.

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