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Minor Groove Recognition of the Critical Acceptor Helix Base Pair by an Appended Module of a Class II tRNA Synthetase†

Douglas D. Buechter‡ and Paul Schimmel*

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT: The class-defining active site domain of the 10 class II tRNA synthetases is well conserved and, based on the crystal structure of aspartyl-tRNA synthetase, approaches the end of the tRNA acceptor stem from the major groove side of the helix. Paradoxically, for the class II alanyl-tRNA synthetase (AlaRS), aminoacylation is dependent on minor groove recognition of an acceptor helix G3•U70 base pair. Additional contributions to aminoacylation efficiency are made by the A73 "discriminator" base and G2•C71 base pair located at the end of the acceptor stem. Using microhelix substrates containing only the first four base pairs of the alanine tRNA acceptor helix, we demonstrated that the catalytic center of AlaRS with the three class-defining sequence motifs contains determinants for recognition of A73 and G2•C71. However, this structural unit does not discriminate between different base pairs at the critical 3•70 position. Discrimination at G3•U70 was mapped to a 76 amino acid polypeptide outside the catalytic center. We propose that the G3•U70 recognition motif is a structural appendage that folds back to the catalytic center to make contact with the bound acceptor stem. A "fold-back" appendage provides a specific mechanism for minor groove recognition of the acceptor helix by a class II tRNA synthetase.

The genetic code is based on highly differentiated interactions of aminoacyl-tRNA synthetases with amino acids and transfer RNAs. The connection between amino acids and specific tRNA anticodon trinucleotides is established through the aminoacylation reaction in which amino acids are joined to specific tRNAs. The L-shaped tRNA molecule is made up of two domains; one contains the anticodon trinucleotide of the genetic code and the other the amino acid acceptor stem that terminates in the single-stranded $^{73}NCCA_{3'OH}$ sequence (Schimmel & Söll, 1979; Normanly & Abelson, 1989). Although many tRNA synthetases make direct contacts with anticodon sequences in cognate tRNAs, contacts also occur with the acceptor stem, and these contacts alone are often sufficient to confer specific aminoacylation [reviewed in Martinis and Schimmel (1995)]. Acceptor stem-directed aminoacylation reactions constitute an operational RNA code for amino acids. The "code" consists of sequences/structures in tRNA acceptor stems [in the form of RNA microhelices terminating in $^{73}NCCA_{3'OH}$ (Figure 1)] that provide signals for aminoacylation with specific amino acids (de Duve, 1988; Möller & Janssen, 1990; Schimmel, 1991; Schimmel et al., 1993). The operational RNA code possibly predated the genetic code and was combined with it in the two-domain tRNA structure (Schimmel et al., 1993).

To a first approximation, tRNA synthetases are also comprised of two major domains. The domain containing the catalytic center is believed to be the historical enzyme, and the structure of this domain is the basis for dividing the synthetases into two classes (Eriani et al., 1990; Cusack et al., 1991; Moras, 1992). In the class I enzymes this domain is built around a conserved Rossmann nucleotide-binding fold (Brick et al., 1988; Rould et al., 1989; Brunie et al., 1990). The class II enzymes are characterized by three conserved sequence/structural motifs, designated motifs 1, 2, and 3 (Eriani et al., 1990; Cusack et al., 1991). These elements contribute to a conserved active site domain constructed around an eight-stranded antiparallel β-sheet with three flanking α-helices (Cusack et al., 1990; Ruff et al., 1991). For both classes of synthetases, insertions into these domains provide for specific interactions with the tRNA acceptor stem (Rould et al., 1989; Ruff et al., 1991; Moras, 1992) and are the basis for the aminoacylation specificity of the operational RNA code (Buechter & Schimmel, 1993a; Schimmel et al., 1993). The second major synthetase domain provides for tRNA interactions more distal to the acceptor stem, including in some instances anticodon contacts. This domain is not conserved among enzymes of the same class and, even for a particular synthetase, is more variable than the class-defining domain (Hou et al., 1991).

The class II *Escherichia coli* alanyl-tRNA synthetase (AlaRS)[1] makes no contacts with the alanine tRNA anticodon (Park & Schimmel, 1988), showing that the relationship between alanine and the NGC alanine anticodons is indirect. Instead, a single G3•U70 base pair at the third position of

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* To whom correspondence should be addressed [telephone, (617) 253-6739; FAX, (617) 253-6636].

‡ Present address: Biochemistry Division, United States Surgical Corporation, 195 McDermott Road, North Haven, CT 06473.

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[1] Abbreviations: AlaRS, alanyl-tRNA synthetase; FPLC, fast protein liquid chromatography; DEAE, diethylaminoethyl.

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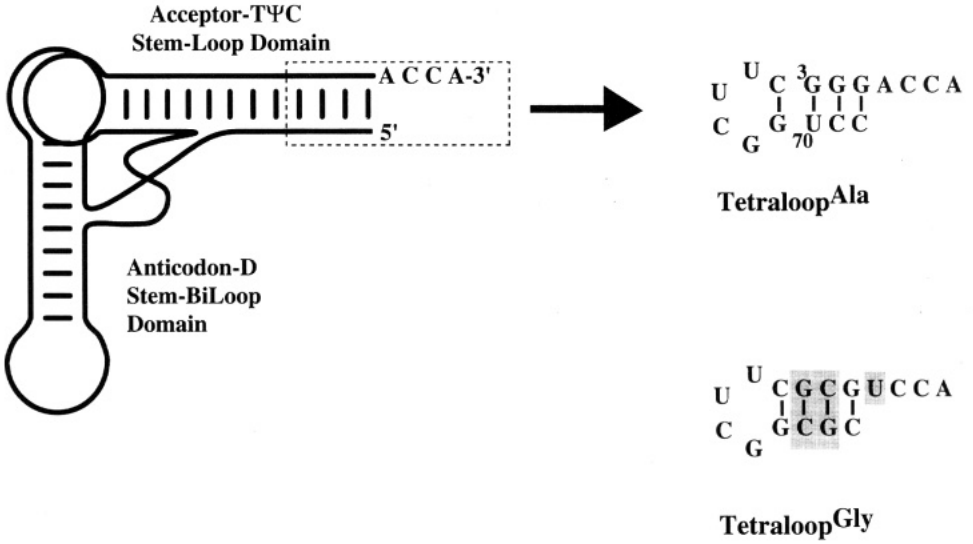

FIGURE 1: Schematic representation of the three-dimensional L-shaped structure of a tRNA molecule and the sequences of the four base pair RNA tetraloops based on acceptor stem sequences of *E. coli* $tRNA^{Ala}$ (top) and *E. coli* $tRNA^{Gly}$ (bottom). The two domains of the tRNA are labeled, and the acceptor stem region from which the microhelices are derived is enclosed with a dotted line. The positions in the glycine-based microhelix that differ in sequence from the alanine-based one are shaded.

the $tRNA^{Ala}$ acceptor helix is required for aminoacylation (Hou & Schimmel, 1988; McClain et al., 1988; Park et al., 1989). Transfer of this base pair into the 3•70 position of non-alanine tRNAs confers alanine acceptance (Hou & Schimmel, 1988, 1989; McClain & Foss, 1988). Microhelix substrates with as few as four base pairs are efficiently aminoacylated by AlaRS, provided they contain G3•U70 (Figure 1) (Francklyn & Schimmel, 1989; Shi et al., 1990, 1992). Other acceptor stem nucleotides that influence aminoacylation efficiency include the A73 "discriminator" base and G2•C71 base pair (Shi et al., 1990; McClain et al., 1991; Shi & Schimmel, 1991; Musier-Forsyth & Schimmel, 1992). These nucleotides and the G3•U70 pair are conserved in both prokaryote and cytoplasmic eukaryote alanine tRNAs.

The G•U base pair is in the wobble configuration in an RNA minihelix substrate whose sequence is based on the acceptor stem of $tRNA^{Ala}$, and in this configuration the exocyclic 2-amino group of G3 is unpaired and projects into the minor groove (Kim et al., 1974; Robertus et al., 1974). Ablation of the 2-amino group by an I3•U70 substitution into an RNA duplex inactivates aminoacylation with alanine (Musier-Forsyth et al., 1991). Aminoacylation is not rescued by other substitutions such as G•C or 2-aminopurine•U. These alternative base pairs engage the 2-amino group in a Watson−Crick hydrogen bond with complementary atoms of the nucleotide at position 70. Collectively, these data showed that the unpaired exocylic 2-amino group in the minor groove is the major signal for aminoacylation with alanine. The demonstrated importance for efficient aminoacylation of the 2′-OH groups of G4, U70, and C71, which flank the critical 2-amino group, confirmed minor groove recognition of the 3•70 region of the acceptor helix by AlaRS (Musier-Forsyth & Schimmel, 1992).

Class I synthetases initially acylate the 2′-OH of the 3′-terminal nucleotide, while class II enzymes catalyze attachment to the 3′-OH (Eriani et al., 1990). This difference appears to be due to a basic distinction in the way that the enzymes of the two classes bind tRNA (Cavarelli et al., 1993). Because the major groove of an A-form RNA helix is deep and narrow, it is not as accessible to protein structural elements as is the major groove of B-form DNA (Seeman et al., 1976). For this reason, the wide, shallow, and more accessible minor groove is thought to provide the primary surface of atoms for specific protein−RNA contacts. However, the major groove is accessible at the end of an RNA helix, such as the acceptor stem helix of a tRNA (Weeks & Crothers, 1993). The class II aspartyl-tRNA synthetase interacts with the major groove side of $tRNA^{Asp}$ (Ruff et al., 1991), where residues in the loop of the β-strand−loop−β-strand structure of motif 2 contact the single-stranded G73 discriminator base, C74, C75, and the major groove O4 and N6 atomic groups of the U1•A72 base pair at the end of the $tRNA^{Asp}$ acceptor stem (Ruff et al., 1991; Cavarelli et al., 1993). In contrast, for the complex of the class I glutaminyl-tRNA synthetase with $tRNA^{Gln}$, the enzyme interacts with the minor groove side of the acceptor stem (Rould et al., 1989).

Alanyl-tRNA synthetase is a class II enzyme, and we imagined that, analogous to the aspartyl system, major groove interactions would be of importance. Considering the tight conservation between the active site regions of class II enzymes, we were thus interested in how alanyl-tRNA synthetase achieved minor groove interactions at the critical G3•U70 base pair. To investigate this question, we took advantage of fragments of the 875 amino acid *E. coli* enzyme. The predicted class-defining motifs are contained within the first 250 amino acids (Ribas de Pouplana et al., 1993; Shi et al., 1994) of AlaRS, and an amino-terminal fragment of 461 amino acids (fragment 461N) has full activity for aminoacylation of microhelix substrates (Buechter & Schimmel, 1993b). Amino-terminal fragments of 368 and 385 amino acids (fragments 368N and 385N) have severely diminished aminoacylation activity but retain full activity for synthesis of alanyl adenylate (Putney et al., 1981; Jasin et al., 1983). We imagined (by analogy to the class II aspartyl-tRNA synthetase) that because fragments 368N and 385N contain the conserved active site domain, they might retain contacts with the A73 discriminator base and adjacent nucleotides. These interactions would include major groove interactions with at least the end of the acceptor helix. How minor

groove contacts were achieved was an open question, but, among other possibilities, we thought that the deficiency in aminoacylation of fragments 368N and 385N might be caused by the absence of a contact in the minor groove at the 3•70 position. These possibilities were investigated by determining the activities of the three fragments with substrates containing substitutions at N73, 2•71, and 3•70.

MATERIALS AND METHODS

Fragment 368N was prepared by digestion of 5 mg of *E. coli* AlaRS (10 mg/mL in 90 mM NH_4OAc, pH 8.3) with 50 μg of trypsin for either 3 or 16 h at 37 °C (Herlihy et al., 1980). The crude digest was twice purified on a Superose 12 FPLC column (Pharmacia, Piscataway, NJ) using a buffer of 40 mM Bis-Tris•HCl (pH 6.5)/100 mM NaCl at 0.4 mL/min. Monomeric fragment 368N and tetrameric native enzyme differ in molecular mass by greater than 340 kDa and are readily resolved from each other under these conditions. Fractions were assessed for protein by SDS−PAGE and fragment 368N-containing fractions were pooled, concentrated, and stored at −20 °C in 40% glycerol. Immunoblotting with polyclonal anti-AlaRS antibodies (Regan et al., 1986) of purified fragment 368N resolved by SDS−PAGE did not show a band for native AlaRS under conditions where more than an ~1/1200 level of AlaRS would have been detectable.

Fragment 461N was expressed from plasmid pT461 (Jasin et al., 1983; Regan, 1986) and purified from *alaS* null strain W3110 (*lacI*^{q} *rec A* Δ1 Kan^r *alaS*Δ2). This strain does not contain a chromosomal copy of *alaS* (Jasin & Schimmel, 1984). Purification followed previous methods (Hill & Schimmel, 1989; Buechter & Schimmel, 1993b). Native *E. coli* AlaRS was expressed from plasmid pT875 (Regan, 1986) and purified from the *alaS* null strain in the same manner. Fragment 385N was expressed from plasmid pT385 (Jasin et al., 1983; Regan, 1986) in *E. coli* strain TG1 (*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F′ [*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) and purified by DEAE-cellulose batch chromatography followed by Mono Q and Superose 12 FPLC (Buechter & Schimmel, 1993b). Fragment 385N was cleanly resolved from native AlaRS by both Mono Q and Superose 12 chromatography. Purified proteins were concentrated and stored at −20 °C in 40% glycerol.

Aminoacylation assays were carried out at 37 °C in 50 mM Hepes•Na (pH 7.4), 20 mM KCl, 10 mM $MgCl_2$, 20 mM β-mercaptoethanol, 0.1 mg/mL BSA, 22 μM [2,3-^{3}H]-alanine (~5 mCi/μmol), and 4 mM ATP according to published procedures (Schreier & Schimmel, 1972). RNA substrates were heated at 80 °C for 3 min followed by cooling on ice prior to addition to the aminoacylation reaction mixture. All rates were corrected for the precipitation efficiency of the 16-nucleotide tetraloop substrate (Shi et al., 1992).

RNA synthesis was carried out on a Pharmacia Gene Assembler Plus. Synthetic RNAs were deprotected and purified as previously described (Shi et al., 1992). All RNA phosphoramidites were from CPG, Inc. (Lincoln Park, NJ), except for the inosine phosphoramidite which was from ChemGenes (Waltham, MA).

RESULTS

In previous studies, monomeric fragments 368N and 385N were reported to lack aminoacylation activity (Putney et al., 1981; Jasin et al., 1983; Regan et al., 1987). This deficiency in activity was demonstrated directly *in vitro* and also *in vivo* by the failure of fragment 385N to complement an *alaS* null strain which has an ablation of *alaS* from the chromosome (Jasin & Schimmel, 1984). Monomeric fragment 461N has aminoacylation activity of about 1% of that of the native tetrameric enzyme, when $tRNA^{Ala}$ is the substrate for aminoacylation (Ho et al., 1985; Regan et al., 1987; Buechter & Schimmel, 1993b). When expressed from a multicopy plasmid, this fragment provides sufficient activity to sustain the *alaS* null strain (Ho et al., 1985; Regan et al., 1987). In addition, fragment 461N has the same activity as that of native AlaRS for aminoacylation of RNA hairpin substrates that reconstruct the acceptor stem of $tRNA^{Ala}$ (Buechter & Schimmel, 1993b). Thus, fragment 461N has all of the protein determinants needed for contacts with the acceptor stem of $tRNA^{Ala}$, including the critical G3•U70 base pair. Additional determinants, presumably for contacts outside the acceptor stem, are believed responsible for the 100-fold higher activity of the native enzyme on $tRNA^{Ala}$.

In this work, we confirmed the lack of aminoacylation activity of fragments 368N and 385N. In order to detect any residual activity that might be encoded by these fragments, we raised their respective concentrations to higher amounts than used in previous studies and also used higher RNA substrate concentrations. Under these conditions, a low activity for aminoacylation of $tRNA^{Ala}$ by either of these fragments could be detected that amounted to about 0.3% of that of fragment 461N. This result suggested that contacts with specific parts of the acceptor stem of $tRNA^{Ala}$ might be mapped on AlaRS by comparing the activities of the three fragments (368N, 385N, and 461N) on microhelix substrates. Because previous work showed that hairpin helices based on the first four base pairs of the $tRNA^{Ala}$ acceptor stem were aminoacylated about as efficiently as those that contained the entire seven base pairs of the acceptor helix (Shi et al., 1992; Buechter & Schimmel, 1993b), we used the smaller substrates. These four base pair helices are stabilized by the well-characterized UUCG tetraloop (Tuerk et al., 1988; Cheong et al., 1990; Woese et al., 1990). Previous work established that these substrates have melting temperatures of greater than 70 °C and that their melting profiles are concentration independent, suggesting that they remained monomeric under the conditions of the aminoacylation experiments (Shi et al., 1992). In the present work, the concentration range over which the aminoacylation activity of the AlaRS fragments was linear with substrate concentration overlapped with the range used previously where the substrates were monommeric (Shi et al., 1992).

Fragment 368N catalyzes the specific aminoacylation of the four base pair microhelix substrate based on the acceptor stem sequence of *E. coli* $tRNA^{Ala}$ (Figure 2). The activity of fragment 368N with $tetraloop^{Ala}$ as substrate is decreased by greater than ~300-fold compared to native AlaRS. The activity of fragment 368N was linear over a 5-fold range of enzyme and of substrate concentration. The observed aminoacylation activity of fragment 368N was not due to contamination with wild-type AlaRS because (1) no full-length AlaRS was detected in purified preparations of 368N by immunoblotting under conditions capable of detecting a greater than 1/1200 level of contamination (data not shown) (2) tryptic digestion of AlaRS for either 3 or 16 h at 37 °C resulted in purified 368N of identical activity.

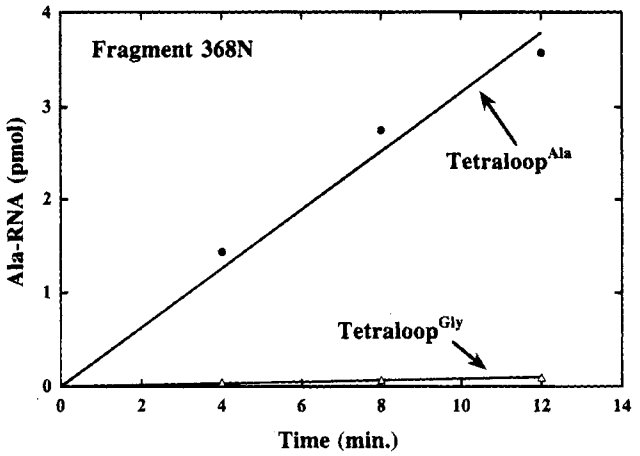

FIGURE 2: Aminoacylation by fragment 368N of tetraloop^{Ala} and tetraloop^{Gly} at pH 7.4, 37 °C. The number of picomoles of charged RNA in a 10-μL aliquot of the aminoacylation mixture is plotted on the ordinate axis. Enzyme and RNA concentrations were 5 and 100 μM, respectively.

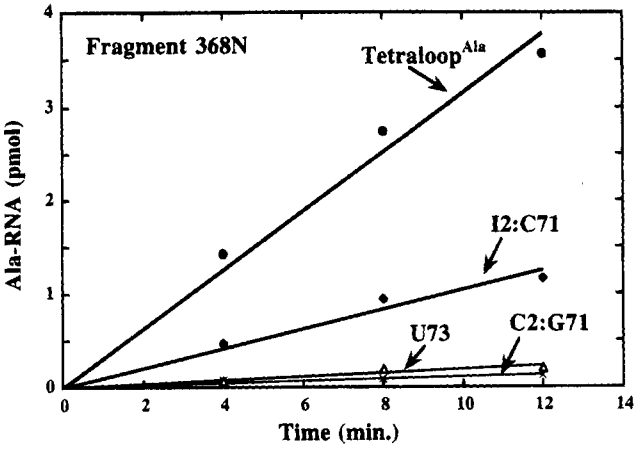

FIGURE 3: Aminoacylation by fragment 368N of tetraloop^{Ala} and substituted RNA tetraloops at pH 7.4, 37 °C. The number of picomoles of charged RNA in a 10-μL aliquot of the aminoacylation mixture is plotted on the ordinate axis. Enzyme and RNA concentrations were 5 and 100 μM, respectively.

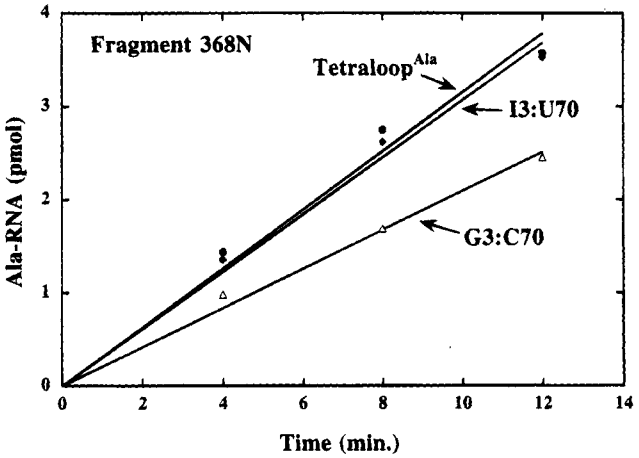

FIGURE 4: Aminoacylation by fragment 368N of tetraloop^{Ala} and substituted RNA tetraloops at pH 7.4, 37 °C. The number of picomoles of charged RNA in a 10-μL aliquot of the aminoacylation mixture is plotted on the ordinate axis. Enzyme and RNA concentrations were 5 and 100 μM, respectively.

The aminoacylation catalyzed by fragment 368N is specific because fragment 368N does not aminoacylate a microhelix based on the sequence of *E. coli* tRNA^{Gly} (Figure 2). These results mimic the discrimination shown by native AlaRS, which also does not aminoacylate tetraloop^{Gly} [data not shown and Shi et al. (1992)]. AlaRS is known from previous work to be sensitive to the nucleotides at specific positions where tetraloop^{Gly} differs in sequence from tetraloop^{Ala}. For example, the "glycine" substrate differs from the "alanine" substrate at the discriminator base (A73 → U73), the second base pair (G2•C71 → C2•G71), and the third base pair (G3•U70 → G3•C70) (Figure 1). Thus, mutation of G3•U70 to G•C results in a tRNA^{Ala} that is nonfunctional both *in vivo* (Hou & Schimmel, 1988; McClain et al., 1988) and *in vitro* (Hou & Schimmel, 1988), while point mutations at positions 73 and 2•71 are weakly active *in vivo* (Hou & Schimmel, 1988). The corresponding U73, C2•G71, and dI2•G71 single mutations in an RNA microhelix substrate severely decrease aminoacylation efficiency *in vitro* (Shi et al., 1990; Francklyn et al., 1992; Musier-Forsyth & Schimmel, 1992).

In the cocrystal of *Saccharomyces cerevisiae* aspartyl-tRNA synthetase complexed with tRNA^{Asp}, contacts with the end of the acceptor stem are made by the residues in the variable loop of the strand−loop−strand structure of motif 2 (Ruff et al., 1991; Cavarelli et al., 1993). The analogous structure of *E. coli* AlaRS is contained within fragment 368N. Thus, we sought to determine whether comparable contacts in the microhelix substrate were important for the specificity of fragment 368N by comparing its activity with microhelix substrates individually substituted at the discriminator base and at the second and third base pairs.

Substitution with U73 decreased aminoacylation efficiency ~20-fold, while C2•G71 and I2•C71 substitutions resulted in activity decreases of 25-fold and 3.5-fold, respectively (Figure 3). In contrast, substitution of G3•U70 with either G•C or I•U had little effect on aminoacylation rates (Figure 4). In particular, the I3•U70 microhelix is aminoacylated by fragment 368N at the same rate as the G3•U70 substrate, while aminoacylation of the G3•C70 substrate is reduced only 1.4-fold. Identical results were obtained with fragment 385N for all substrates (data not shown). Thus, both fragments 368N and 385N discriminate between microhelix substrates at the second base pair of the acceptor stem and at the A73 discriminator base but not at the 3•70 position of the acceptor stem.

Fragment 461N aminoacylates the G3•U70 microhelix at a rate 300-fold greater than the rate catalyzed by fragment 368N (Figure 5). This increment in activity could result from additional positive interactions of fragment 461N with one or more specific nucleotides in the microhelix. We sought to identify these nucleotides by comparing the activities of fragments 461N and 368N with the substituted microhelices as substrates. The G3•C70-substituted microhelix retains the wild-type A73 discriminator base and G2•C71 base pair. However, it is aminoacylated at a 300-fold lower rate by fragment 461N than is the G3•U70-substituted microhelix. With this reduction, the G3•C70 microhelix is no better a substrate for fragment 461N than it is for fragment 368N (Figure 5, inset). Thus, if there are interactions at either A73 or G2•C71 by the region between Arg368 and Asp461, then these interactions do not contribute to the increase in aminoacylation seen with fragment 461N.

The identical activities of fragments 368N and 461N for the G3•C70-substituted microhelix show that the enhanced

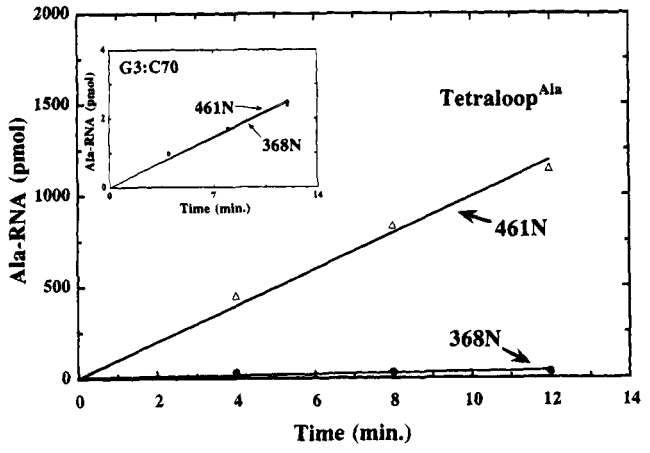

FIGURE 5: Aminoacylation with alanine of tetraloop^{Ala} (100 μM) by fragment 368N (5 μM) and fragment 461N (30 nM) at pH 7.4, 37 °C. The number of picomoles of charged RNA in a 10-μL aliquot of the aminoacylation mixture is plotted on the ordinate axis, where the number of picomoles for fragment 461N was corrected to a 5 μM enzyme concentration. The inset shows the aminoacylation of the G3•C70-substituted tetraloop^{Ala} (100 μM) by fragment 368N (5 μM) and fragment 461N (5 μM).

activity of fragment 461N on the G3•U70 "wild-type" substrate is due to a positive interaction of the segment between Arg368 and Asp461 with G3•U70. The majority of the postive interaction is localized to an interaction with the 2-amino group of G3. We found that the I3•U70-substituted microhelix is also a poor substrate for fragment 461N (reduced in rate by ~35−40-fold relative to the G3•U70 wild-type substrate; data not shown). The I3•U70 substitution selectively removes the minor groove free exocyclic 2-amino group of G3 that has previously been shown to be a critical determinant of aminoacylation (Musier-Forsyth et al., 1991). Thus, the region between Arg368 and Asp461 appears to make a direct contact with the exocylic 2-amino group of G3. This segment (Arg368−Asp461) is appended to the catalytic domain which contains the three motifs that define AlaRS as a class II enzyme.

DISCUSSION

Previously described contacts with the tRNA acceptor helix in the class II synthetases are through polypeptide insertions into the class-defining catalytic domain. In the crystal structure of class II yeast aspartyl-tRNA synthetase complexed with tRNA^{Asp}, the interaction of the side-chain hydroxyls of the motif 2 variable loop Ser339 and Thr341 with O-1 and N-6 of G73, respectively, contributes to aminoacylation efficiency (Ruff et al., 1991; Cavarelli et al., 1993, 1994). Mutation of residues in the motif 2 loop of AspRS that contact the discriminator base of tRNA^{Asp} affects both the k_{cat} for aminoacylation and the K_D for tRNA^{Asp} (Cavarelli et al., 1994). Furthermore, RNA microhelix substrates are aminoacylated with aspartate, provided that they contain G73 (Frugier et al., 1994). Residues in motif 2 also provide for interaction with bound ATP and the aminoacyl adenylate. We imagined by analogy that motif 2 of *E. coli* alanyl-tRNA synthetase facilitated interactions with ATP and alanyl adenylate and that the variable loop of motif 2 made contact with the A73 discriminator base of tRNA^{Ala}. (Because the motif 2 variable-sized loops lack sequence similarities among the 10 class II enzymes, the counterparts to Ser339 and Thr341 of aspartyl-tRNA synthetase cannot be assigned in AlaRS.) Consistent with this notion, tRNA^{Ala} with a periodate-oxidized 3′-terminus cross-links to Lys73, which is at the beginning of the variable loop of motif 2 in AlaRS (Hill & Schimmel, 1989). However, while a mutation analysis demonstrated that motif 2 of alanyl-tRNA synthetase provided interactions for the adenylate transfer reaction, no contacts with tRNA^{Ala} were unequivocally identified (Davis et al., 1994; Lu & Hill, 1994). The genetic screen used in these studies may have been insensitive to some protein−tRNA contacts. It is also possible that discriminator base contacts are different in AlaRS than in AspRS. However, our data show that, whatever the nature of these differences, discriminator base contacts are made in AlaRS by a fragment (368N) which, like the aspartyl-tRNA synthetase, encompasses the catalytic core.

The aminoacylation activity of fragment 368N is also sensitive to the identity of the nucleotides at the second base pair. Both the C2•G71 and I2•C71 substitutions decrease the aminoacylation activity of fragment 368N. In the aspartyl-tRNA synthetase−tRNA^{Asp} crystal structure, contacts with the 2•71 position of the acceptor helix are not seen. However, the backbone NH and CO of Asn330 of the variable loop of motif 2 hydrogen bond with O-4 of U1 and N-6 of A72 in the U1•A72 base pair. Asn330 is between Ser329 and Thr331, which hydrogen bond to the G73 discriminator base (see above). In this work we have not investigated the G1•C72 base of tRNA^{Ala}, because it is semiconserved among many tRNAs (Steinberg et al., 1993) and, therefore, is not especially useful for tRNA discrimination. However, our data show that functional interactions with the G2•C71 base pair are contained within the same small fragment (368N) of AlaRS that provides for interactions with the A73 discriminator base, and we believe that interactions with G1•C72 also are contained within this fragment.

By analogy with aspartyl-tRNA synthetase, we imagine that alanyl-tRNA synthetase approaches tRNA^{Ala} from the major groove side of the acceptor helix and that this orientation can account for contacts with the 3′-single-stranded region (i.e., A73) at the end of the acceptor helix. These contacts are likely made by AlaRS-specific insertions or variable polypeptide regions that are integral parts of the active site domain. This approach to the acceptor stem would also be consistent with these two enzymes, like most class II tRNA synthetases, attaching the amino acid initially to the 3′-hydroxyl of the tRNA substrate.

The additional 76 amino acids of fragment 461N compared to fragment 385N are required, either directly or indirectly, for recognition of the G3•U70 base pair. Our data show that a critical minor groove interaction with the tRNA acceptor helix is made possible (within the framework of a class II enzyme) by the addition of an external module to the class-defining catalytic domain. This module is not an integral component of the active site domain because fragment 368N is competent for the essential catalytic functions of AlaRS. We propose that this minor groove recognition domain folds back toward the active site containing the bound acceptor helix where it makes contact with G3•U70 and, in particular, with the exocylic 2-amino group of G3. Because interactions with G3•U70 provide not only "K_M" but also "k_{cat} discrimination" (Park et al., 1989), this appended domain must communicate with the active site during the transition state of catalysis. A major challenge is to understand how this communication occurs.

There are as yet no other examples of an appended domain of a class II tRNA synthetase providing for interactions with the acceptor helix. [In the class II *E. coli* seryl-tRNA synthetase, a coiled-coil domain appended to the N-terminal side of the active site domain interacts with the large variable loop of *E. coli* $tRNA^{Ser}$ (Biou et al., 1994).] Because of the strong relatedness of the sequences of *E. coli*, *S. cerevisiae*, *Bombyx mori*, and human alanyl-tRNA synthetases in the region corresponding to fragment 461N of the *E. coli* enzyme (Ripmaster et al., 1995), and because G3•U70 is the major determinant for aminoacylation of each of the corresponding alanine tRNAs, we surmise that strong selective pressure developed and retained through evolution an appended domain for making contacts with the minor groove at the 3•70 position.

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