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Aminoacylation of RNA Minihelices: Implications for tRNA Synthetase Structural Design and Evolution

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ABSTRACT: The genetic code is based on the aminoacylation of tRNA with amino acids catalyzed by the aminoacyl-tRNA synthetases. The synthetases are constructed from discrete domains and all synthetases possess a core catalytic domain that catalyzes amino acid activation, binds the acceptor stem of tRNA, and transfers the amino acid to tRNA. Fused to the core domain are additional domains that mediate RNA interactions distal to the acceptor stem. Several synthetases catalyze the aminoacylation of RNA oligonucleotide substrates that recreate only the tRNA acceptor stems. In one case, a relatively small catalytic domain catalyzes the aminoacylation of these substrates independent of the rest of the protein. Thus, the active site domain may represent a primordial synthetase in which polypeptide insertions that mediate RNA acceptor stem interactions are tightly integrated with determinants for aminoacyl adenylate synthesis. The relationship between nucleotide sequences in small RNA oligonucleotides and the specific amino acids that are attached to these oligonucleotides could constitute a second genetic code.

KEY WORDS: genetic code, transfer RNA, acceptor stem nucleotides, aminoacyl-tRNA synthetase evolution.

I. INTRODUCTION

The aminoacylation of transfer RNA is catalyzed by the aminoacyl-tRNA synthetase family of enzymes (for earlier reviews, see References 11, 58, 60, 81, 85, and 95). The fidelity of the translation machinery is dependent on the insertion of the correct amino acid in a growing polypeptide chain. This is achieved in part through precise base pairing between the mRNA codon and tRNA anticodon. The tRNA synthetases, by virtue of catalyzing the specific aminoacylation of only their cognate tRNAs, provide the critical link between amino acids and the anticodon trinucleotide. The matching of tRNAs with amino acids by synthetases biochemically establishes the genetic code.

The synthetase-catalyzed aminoacylation of tRNA is a two-step reaction:

$$\text{Enzyme} + \text{ATP} + \text{amino acid} \rightleftharpoons \text{Enzyme(aminoacyl)-AMP} + PP_i \quad \text{[Adenylate synthesis]}$$

$$\text{Enzyme(aminoacyl)-AMP} + \text{tRNA} \rightleftharpoons \text{Aminoacyl-tRNA} + \text{AMP} + \text{Enzyme} \quad \text{[Aminoacylation]}$$

In the first step, the amino acid is activated by reaction with ATP to form the enzyme-bound aminoacyl adenylate (adenylate synthesis). In the second step, the tRNA-dependent step, either the 2′- or 3′-hydroxyl group at the 3′-end of the tRNA is esterified with the amino acid. With one or two exceptions, adenylate synthesis does not require the presence of the tRNA and can be assayed separately. Thus, aminoacyl adenylate synthesis is, to some degree, functionally independent from transfer of the activated amino acid to tRNA.

There is generally only one synthetase for each amino acid; however, because of the degeneracy of the genetic code, there can be several tRNA isoacceptors for an amino acid. All tRNAs adopt a similar L-shaped tertiary structure (Figure 1).[45,63,76,77,85] Two domains are present in this structure: one consists of the acceptor-TΨC stem and loop and the other is comprised of the D-anticodon stem and loop. Structural features within these domains provide the basis for discriminating a set of isoacceptors from noncognate tRNAs.

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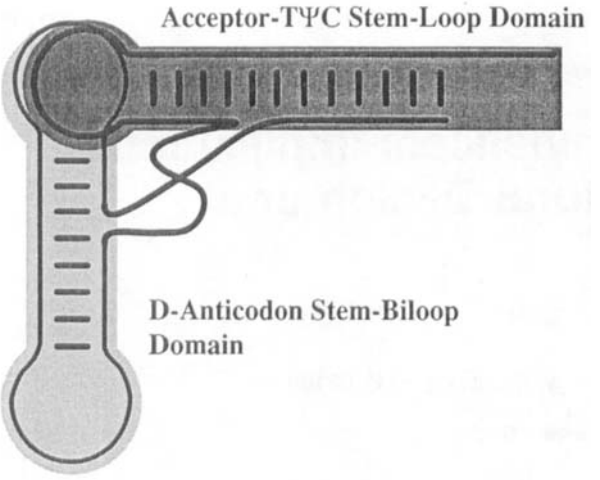

FIGURE 1. The L-shaped tertiary structure of transfer RNA with two domains formed from the four stems of the cloverleaf secondary structure. The domain comprising the acceptor stem and TΨC stem and loop is darkly shaded and is designated as the minihelix (see Figure 2); the domain comprising the D-stem-loop and anticodon stem loop is lightly shaded.

The anticodon is a logical choice for this discrimination because, for all but $tRNA^{Ser}$, at least one anticodon nucleotide is unique to the isoacceptor tRNAs for each amino acid.[83] There are no anticodon nucleotides common to all five $tRNA^{Ser}$ isoacceptors. In the complex with $tRNA^{Ser}$, serine tRNA synthetase (SerRS*) does not contact the anticodon,[17,64] so that the relationship between the amino acid and the six serine-trinucleotide codons is indirect. The two $tRNA^{Ala}$ isoacceptors that read the four GCX codons for alanine, however, share a unique GC anticodon dinucleotide that distinguishes these tRNAs from all others. In spite of the potential to discriminate alanine from other tRNAs based on the GC dinucleotide, AlaRS does not contact the anticodon of $tRNA^{Ala}$.[68] Thus, the amino acid-trinucleotide relationship for alanine also is not a direct one. These observations and considerations suggest that nucleotide sequence-amino acid relationships different from the ones specified by the genetic code may, in general, underlie the molecular recognition of tRNAs by aminoacyl-tRNA synthetases. These relationships have been referred to as the second genetic code.[20,59,83]

The discernment of relatively weak, but significant, sequence homologies between the synthetases, together with detailed three-dimensional structural information, is bringing clarification to synthetase active site and domain organization. We discuss here some of the implications of these data for the aminoacylation of acceptor stem oligonucleotides and the concept of a second genetic code. In particular, our focus is on whether synthetases evolved from primitive systems in which core catalytic domains activated amino acids and charged small RNA molecules that subsequently became the acceptor stems of contemporary tRNAs. In such systems, interactions with acceptor stem nucleotides would be integrated as part of the active site and, as explained later, this design appears to have been achieved in part by the insertion of specific amino acid sequences into the catalytic domain. As the synthetases and tRNAs evolved, additional domains, perhaps idiosyncratic to each enzyme, were apparently added to enable interactions with more distal parts of the tRNA structure.

II. RNA MINIHELICES

Nucleotide sequences that specify particular amino acids are located in the tRNA acceptor stems, proximal to the amino acid attachment site. For alanine tRNAs, a single G3:U70 base pair in the acceptor stem is a major determinant of aminoacylation with alanine,[34,67] and the adjacent G2:C71 base pair and A73 "discriminator" nucleotide have strong influence on aminoacylation efficiency.[52,88,90] The G3:U70 base pair is unique to alanine tRNAs[83,93] and, therefore, can provide the basis for distinguishing $tRNA^{Ala}$ from other tRNAs.[83] Transfer of this base pair into other tRNA frameworks, including *Escherichia coli* $tRNA^{Cys}$,[34] $tRNA^{Phe}$,[34,51] and $tRNA^{Tyr}$,[35] confers alanine acceptance.

The importance of the G3:U70 base pair in the acceptor stem of $tRNA^{Ala}$ suggested the construction of small RNA helices incorporating only the acceptor-TΨC stem and loop of $tRNA^{Ala}$ (Figure 2).[24,88] This minihelix, as well as a 7-bp hairpin acceptor stem microhelix[24] and RNA duplexes

* The aminoacyl-tRNA synthetase for a particular amino acid is abbreviated by the three letter abbreviation for the amino acid followed by the letters RS, for example, serine tRNA synthetase is abbreviated SerRS.

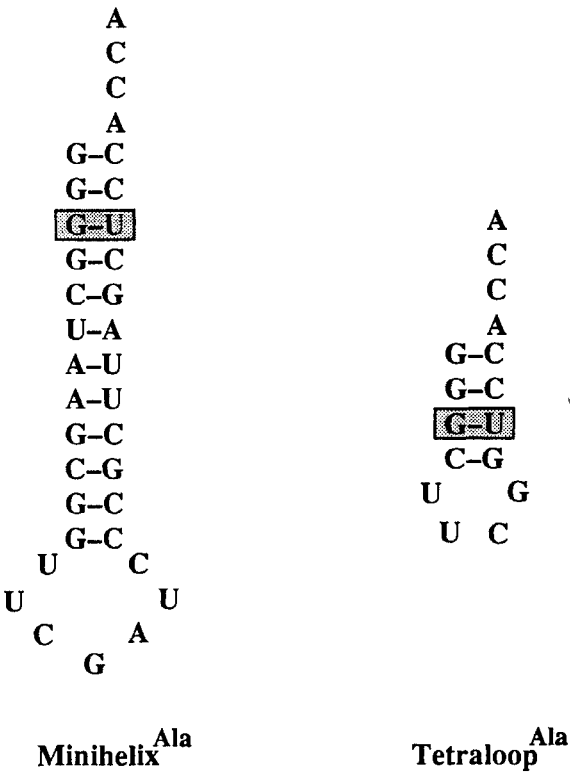

FIGURE 2. Sequences of an RNA minihelix and tetraloop that are based on the sequence of the acceptor stem of $tRNA^{Ala}$. The G3:U70 base pair that is essential for aminoacylation with alanine is boxed and shaded.

that comprise complementary single strands containing only acceptor stem nucleotides,[61] is specifically aminoacylated with alanine, provided it encodes the G3:U70 base pair. In an attempt to obtain the minimal structure for aminoacylation, the stabilizing RNA tetraloop motif found in ribosomal RNAs[104] was incorporated into helices consisting of only the first 4 bp of the acceptor stem (Figure 2). These helices, which terminate at the 3′-end in the single-stranded ACCA, demonstrated G3:U70-dependent aminoacylation with alanine.[89]

Activity for sequence-specific aminoacylation of RNA acceptor stem oligonucleotides, in addition to *E. coli* AlaRS, has now been reported for *E. coli* glycine,[26] histidine,[25] methionine,[50] and yeast valine[27] tRNA synthetases; additional reports are anticipated. For *E. coli* GlyRS, the C2:G71 base pair and U73 are essential for aminoacylation,[26] and these three nucleotides are unique to *E. coli* glycine tRNAs.[93] Histidine tRNAs have an extra nucleotide at the 5′-end that is unique, and this nucleotide (a G in *E. coli* $tRNA^{His}$) is essential for aminoacylation.[25,26,31] In all of these examples, aminoacylation of the full-length tRNA is more efficient than that of the acceptor stem model substrate but, at least for alanine, glycine, and histidine aminoacylation, the acceptor stem contributes more than the rest of the tRNA structure to the overall efficiency of aminoacylation.[26,89] For methionine and valine tRNA synthetases, the anticodon is an important determinant of aminoacylation specificity.[86] However, the sequence-specificity of aminoacylation of acceptor stem substrates by these enzymes[27,50] demonstrates that, even when anticodon interactions are important for aminoacylation efficiency, there is still a relationship between acceptor stem sequences and specific amino acids.

III. SYNTHETASE CLASSIFICATION AND DOMAIN STRUCTURE

The aminoacyl tRNA synthetases are widely diverse in primary structure, oligomeric state, and subunit size. Quaternary structures are α, α_2, $\alpha_2\beta_2$, and α_4, and subunit sizes in *E. coli* range from 329 to 951 amino acids.[58,81,85] Relatively little sequence homology between the synthetases is superficially apparent, but careful primary sequence comparisons have placed the synthetases into two distinct classes of ten enzymes each.[19,21] This classification is based on relatively short sequence motifs held in common by the members of each class and by the assignment of these motifs to specific secondary structures within the enzymes. It has not been possible to locate regions of similarity across the two classes, and there is no example of a "class-switch" in evolution. These observations indicate that the separation into two classes occurred early.

A. Class I Synthetases

Class I enzymes (ArgRS, CysRS, GluRS, GlnRS, IleRS, LeuRS, MetRS, TyrRS, TrpRS, and ValRS) are principally monomeric, appear to catalyze the attachment of the amino acid to the 2′-hydroxyl at the 3′-end of the tRNA,[21] and share

both an 11 amino acid signature sequence that ends in the HIGH tetrapeptide[48,81,103] and a conserved KMSKS pentapeptide.[11,37] Both sequence motifs are found within the N-terminal region, and the HIGH signature sequence always precedes the KMSKS pentapeptide in the sequence.[11,60]

Crystal structures of three class I enzymes have been determined: TyrRS,[5] GlnRS,[79] and a large, active N-terminal tryptic fragment of MetRS.[8] In each of these structures, the active site is in the N-terminal region that contains the class I sequence motifs and a topologically conserved domain built around a classic Rossmann nucleotide-binding fold with alternating α-helices and β-structure that forms a five-stranded parallel β-sheet. The HIGH signature sequence is located in a loop that follows the first β-strand of the dinucleotide fold. The conserved histidines in this motif make contact with the phosphates of bound ATP[79] and the aminoacyl adenylate intermediate.[5] The KMSKS motif is in a loop following a β-strand in the second half of the nucleotide fold. The second invariant lysine of this motif has been proposed to be involved in stabilization of the transition state leading to the aminoacyl adenylate intermediate.[23,79] The importance of active site residues in discriminating between amino acids has been evaluated for TyrRS.[5,23]

The class I enzymes can be divided into three subclasses.[11] All of the class I synthetases contain an insertion of variable size (connective polypeptide 1 (CP1)) between the two halves of the nucleotide-binding fold. In GlnRS, this insertion (amino acids 100 to 210) occurs at a break between the $\alpha_2\beta_3$ and $\alpha_2\beta_2$ halves of the nucleotide-binding fold. Sequence-specific contacts with the acceptor stem of bound $tRNA^{Gln}$ in the GlnRS-$tRNA^{Gln}$ complex are mediated by this insertion (see the following).[79] The MetRS subclass (MetRS, LeuRS, IleRS, ValRS, and CysRS) contains a second connective polypeptide (CP2) after the first β-strand of the second half of the nucleotide-binding fold.[11] In the GlnRS subclass (GlnRS, GluRS, and TrpRS), CP2 is found between the second and third β-strands of the second half of the active site fold, and in the TyrRS subclass (TyrRS and ArgRS), CP2 is not present. In the TyrRS subclass, amino acids at the N-terminal end of the active site domain form the outermost β-strand of the second half of the nucleotide-binding fold.[11] In the two other subclasses, the outermost β-strand of the fold is contributed by the sequence at the C-terminal end of the fold.

B. Class II Synthetases

The class II synthetases (AlaRS, AsnRS, AspRS, GlyRS, HisRS, LysRS, PheRS, ProRS, SerRS, and ThrRS) have greater variability in size and quaternary structure than the class I enzymes. They appear to catalyze aminoacylation at the 3′-hydroxyl and are characterized by three highly degenerate sequence motifs (Figure 3).[19,21] In the dimeric class II enzymes, motif 1 is involved in formation of the dimer interface and consists of an α-helix followed by a short β-strand.[80] The α-helix of one subunit makes contact with the corresponding helix in the other subunit. A comparable motif in the tetrameric glycine, alanine, and phenylalanine enzymes has

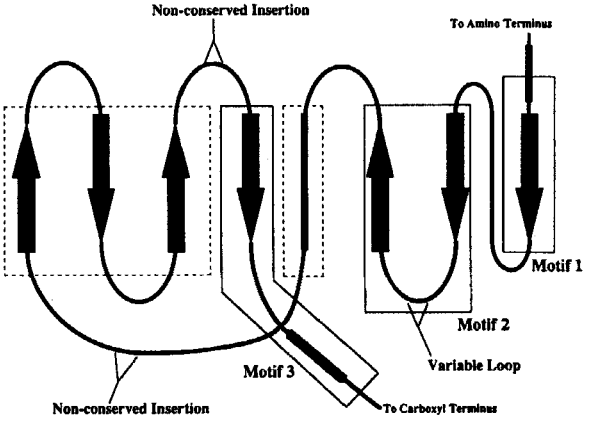

FIGURE 3. Schematic representation of the seven-strand antiparallel β-sheet motif as found in the active site domains of class II yeast AspRS[80] and *E. coli* SerRS.[18] Dark arrows indicate β-strands, and the approximate positions of three helices are indicated by heavy lines. The locations of the conserved class II sequence motifs are boxed and shaded. The three β-strands and the α-helix of the polypeptide insertion between motifs 2 and 3, which are topologically conserved in AspRS and SerRS, are enclosed by dotted lines, and the locations of two topologically non-conserved regions within this insertion also are indicated. In addition, the variable loop between the two β-strands of motif 2 that contacts the acceptor stem in the AspRS-$tRNA^{Asp}$ complex is labeled.

not yet been identified. Motifs 2 and 3 are located in the C-terminal region of the class II enzymes (with the exception of GlyRS and AlaRS, where they are in the N-terminal domain), and motif 2 is always on the N-terminal side of motif 3.

In the crystal structures of AspRS[80] and SerRS,[18] the active site is formed by a seven-stranded antiparallel β-sheet structure that is flanked by three α-helices (Figure 3). This active site architecture is conserved among the class II synthetases. Motif 2 contributes two of the active-site antiparallel β-strands, whereas motif 3 contributes a β-strand and an α-helix. The conserved arginine of motif 2 contacts bound ATP.[17] A variable loop of 10 to 18 amino acids located between the two β-strands of motif 2 makes sequence-specific contacts with the acceptor stem of bound $tRNA^{Asp}$ in the AspRS-$tRNA^{Asp}$ complex (see the following).[60,80] Additional insertions that are of varying size are found between motifs 1 and 2 and between motifs 2 and 3 (Figure 3).

The class II synthetases have been divided further into two subclasses whose members are more closely related in primary sequence to each other than to the enzymes of the other subclass.[19,60] Subclass 2a contains the enzymes for serine, threonine, proline, and histidine. These enzymes are likely to be structurally most closely related to SerRS.[19] Subclass 2b, composed of the aspartic acid, asparagine, and lysine enzymes, is represented by the structure of yeast AspRS. Sequence similarities between the members of each subclass extend beyond the conserved motifs and include insertions within, as well as extensions on, both the N- and C-terminal sides of the active site domain. These insertions may be related to similar modes of tRNA binding.[19] The tetrameric class II enzymes do not fall cleanly into either subclass. They include AlaRS (α_4), which is suggested to be more related to subclass 2a than to 2b, and the $\alpha_2\beta_2$ PheRS and GlyRS, which are suggested as likely members of subclass 2b.[19]

In addition to the core active site domain, synthetases in each class contain discrete domains that bear little or no structural similarity to each other. For example, following the nucleotide-binding fold domain of class I GlnRS, a helical subdomain and then a large β-barrel domain occur in the C-terminal region of the enzyme.[79] The β-barrel domain makes sequence-specific contacts with the $tRNA^{Gln}$ anticodon.[79] In class I MetRS, however, an α-helical domain on the C-terminal side of the active site domain interacts with the anticodon.[8,28,55,99] Similar examples can be found in the class II synthetases: yeast AspRS has an N-terminal β-barrel-like domain,[80] but SerRS has an unusual antiparallel coiled-coil fused to the active site domain.[18] These additional domains contribute to protein-tRNA interactions at sites removed from the acceptor stem.[17,60,80]

IV. BIOCHEMICAL EVIDENCE FOR DISCRETE DOMAINS

In addition to structural data, there also are biochemical results that demonstrate discrete synthetase domains. These results can be interpreted in the context of the class-defining sequence motifs and the overall domain organization of the synthetases.

Proteolysis of the class II *E. coli* alanine tRNA synthetase (an α_4 tetramer of 875 amino acid subunits) results in a monomeric amino terminal 368 amino acid fragment (368N).[73] This fragment has full adenylate synthesis activity but does not catalyze aminoacylation of either an acceptor stem minihelix or full-length tRNA.[9,72,73,75] Subsequently, a series of N-terminal fragments of the enzyme were expressed from plasmids with truncated recombinant genes.[41] All fragments of 699 or fewer amino acids were monomeric, whereas those with 808 amino acids or more were tetrameric, suggesting that the oligomerization domain lies between Gly699 and Glu808.[41,75] Amino-terminal fragments larger than fragment 368N catalyze aminoacyl adenylate synthesis as efficiently as the native enzyme, implying that the domain for amino acid activation is on the N-terminal side of Arg368. In between these extremes are sequences that are critical for tRNA binding and aminoacylation. For example, extension of the 368 amino acid N-terminal proteolytic fragment by 17 amino acids gives fragment 385N, which binds tRNA, although with an affinity reduced compared with the wild-type protein. Fragment 385N has no aminoacylation activity. Fusion of an additional 76 amino acids to the C-terminus of fragment 385N gives fragment 461N, which is active for aminoacylation.[33,41,75]

These results suggest a linear arrangement of functional domains along the AlaRS polypeptide sequence.[41,75,82] The N-terminal domain containing the active site for aminoacyl adenylate synthesis extends to Arg368 and contains both motif 2 (Thr62 to His104) and motif 3 (Pro230 to Val266). Following the active site domain in the sequence, a region occurs (Thr368 to Asp461) that is essential for tRNA and minihelix aminoacylation (see the following). A separate oligomerization domain is at the extreme C-terminus (Gly699 to Glu808).

In an analogous manner, mild proteolysis of the homodimeric class I *E. coli* methionine tRNA synthetase (an α_2 dimer of 676 amino acid subunits) results in a stable N-terminal fragment.[13,46] Proteolysis occurs at Lys551 and results in the loss of the oligomerization domain. This fragment (551 amino acids) is monomeric and has slightly decreased adenylate synthesis and aminoacylation activity compared with the full-length enzyme.[3,4,13,39] In the 2.5-Å resolution crystal structure of the tryptic fragment of MetRS, two domains are evident.[7,8] The N-terminal domain, which extends to Thr360, contains the conserved class I motifs and the intact active site. The C-terminal domain (Thr360 to Pro526) is responsible for contacting the $tRNA^{Met}$ anticodon. Two fragments of MetRS, where the protein has been "split" at the interface of these two domains, can be expressed separately from recombinant plasmids and can associate into enzymatically active species both *in vivo* and *in vitro*.[10]

A stable N-terminal fragment of *B. stearothermophilus* tyrosine tRNA synthetase (an α_2 dimer of 419 amino acid subunits) has been expressed from a recombinant plasmid.[102] This fragment of 319 amino acids is missing a C-terminal domain (amino acids 320 to 419) that is disordered in the structure of the native enzyme.[2] It remains dimeric and has wild-type adenylate synthesis activity but is unable to either catalyze the aminoacylation of or bind to $tRNA^{Tyr}$.[102] The 2.5-Å resolution crystal structure of this truncation mutant has been solved.[6] The stable N-terminal domain contains the conserved motifs and the active site. Mutagenesis[1] and analysis of heterodimers of the truncated and native TyrRS[12] suggest an explanation for the loss of tRNA binding in the fragment: $tRNA^{Tyr}$ binds across the subunit interface with residues in the disordered domain contacting the anticodon region.

The genes for the tRNA synthetases in *Sacchromyces cerevisiae* and higher eukaryotes generally encode proteins that are significantly larger than their *E. coli* counterparts by virtue of an N-terminal extension.[14,58,85] In certain cases, it is possible to remove these extensions without affecting catalytic activity (reviewed in References 49 and 58). For example, the monomeric MetRS from *S. cerevisiae* lacks the C-terminal oligomerization domain found in the *E. coli* enzyme but has an N-terminal extension of ~200 amino acids.[22,100] Up to 185 amino acids of this extension can be removed without a significant effect on enzymatic activity; however, further deletions result in diminished adenylate synthesis and aminoacylation activity.[101] A 204 amino acid N-terminal extension of *S. cerevisiae* GlnRS can be removed with only small increases in the K_ms for ATP and glutamine and no significant effect on either the K_m for tRNA or the k_{cat} for aminoacylation. Moreover, a monoclonal antibody directed against the N-terminal extension has little effect on aminoacylation activity.[49] The specific biological functions of the N-terminal extensions are not known, but they may be involved in the association of some of the eukaryotic enzymes into high-molecular-weight complexes (see Reference 58 and references therein).

V. MOTIFS FOR ACCEPTOR STEM INTERACTIONS AND MINIHELIX AMINOACYLATION

Structural and biochemical results suggest a common global organization of both class I and class II synthetases. A model of this organization is presented in Figure 4. In this model, the synthetases comprise at least two discrete domains. One of these domains contains the active site for adenylate synthesis and polypeptide insertions that are needed for acceptor stem binding. This active site domain represents the core synthetase wherein amino acid activation and acceptor stem aminoacylation activities are tightly associated. The overall arrangement of this domain is con-

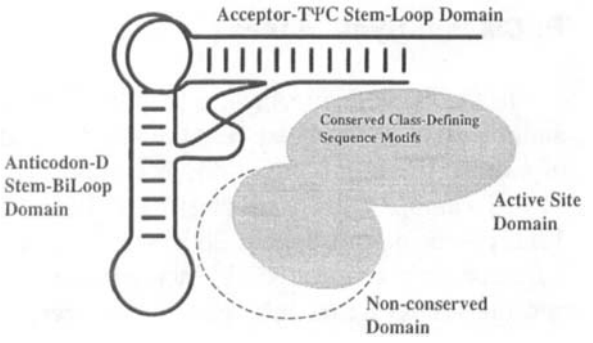

FIGURE 4. Model of the structure of an aminoacyl-tRNA synthetase-tRNA complex. The synthetase is shown as two discrete domains that interact with the two tRNA structural domains. The active site domain containing the conserved sequence motifs that define each class of synthetases contacts the 3′-end and the acceptor helix of the tRNA. The nonconserved domain is not conserved among synthetases in the same class and may (dotted line) or may not contact the anticodon.

served within each synthetase class, but the polypeptide insertions are variable in size and sequence. In at least two cases (AlaRS and MetRS, see the following), the core domain is functionally independent of an intact C-terminal domain for the aminoacylation of acceptor stem helices. This type of architecture, in which polypeptide insertions into a conserved core domain perform enzyme-specific roles (such as acceptor stem interactions), is a common theme in both the class I and class II synthetases.

The structure of the second domain is more unique to each synthetase. This domain interacts with regions of the tRNA substrate that are removed from the acceptor stem. This would include protein-anticodon interactions in those enzymes that recognize the tRNA anticodon. Examples of this arrangement for the class I and class II enzymes are discussed in the following section.

A. Class I Synthetases

An insertion of variable size (CP1) is located between the two halves of the nucleotide-binding fold in the class I synthetases. In the GlnRS-$tRNA^{Gln}$ complex, this 110 amino acid insertion binds a distorted conformation of the tRNA acceptor stem and has been termed the acceptor-binding domain.[79] Distortion of the acceptor helix of $tRNA^{Gln}$ enables specific protein-tRNA contacts between the G2:C71 and G3:C70 base pairs in the acceptor stem and residues within the active site domain. For example, Pro181 in the acceptor-binding domain makes a backbone hydrogen bond to the exocyclic N2 of G2, and the side chain carboxyl of Asp235 in the second half of the nucleotide fold makes a hydrogen bond to the exocyclic N2 of G3. These acceptor stem base pairs are identity elements of $tRNA^{Gln}$,[40,78] and mutation of Asp235 results in enzymes that mischarge tRNA.[70] Analogous insertions are present in both MetRS and TyrRS, where they may play a comparable role in acceptor stem interactions.[69]

E. coli MetRS will catalyze the specific aminoacylation of a 7-bp microhelix that recreates only the acceptor stem sequence of $tRNA^{Met}$.[50] Residues in the N-terminal active site domain of MetRS also mediate interactions with nucleotides in the acceptor stem of $tRNA^{Met}$. Cross-linking of periodate-oxidized $tRNA^{Met}$ to MetRS has identified Lys335, Lys61, Lys142, Lys147, and Lys149 as near the 3′-end of the bound tRNA.[36,38,54,55] These lysines are within the active site domain and include the second lysine of the KMSKS pentapeptide (Lys335). Comparison of the structures of MetRS and GlnRS suggests that both enzymes bind tRNA in a similar orientation.[69] In particular, a conserved α-helix-turn-β-strand motif at the beginning of the acceptor-binding insertion in GlnRS and the corresponding domain in MetRS (Asn102 to Ile124) is proposed to maintain the correct conformation of the insertion and enable interactions with the acceptor stem of the bound tRNA. Thus, acceptor stem interactions are closely integrated with determinants for amino acid activation.

The MetRS-catalyzed aminoacylation of microhelices has defined further the functional relationship between the domains of MetRS. The C-terminal α-helical region of MetRS (amino acids 360 to 526) binds the anticodon of $tRNA^{Met}$.[99] Within this domain, Trp461 is essential for anticodon recognition.[28,55] Following the C-terminal domain is a peptide (Phe527 to Lys551) that has been proposed to "guide" the acceptor stem of

$tRNA^{Met}$ toward the active site and coordinate events occurring in the anticodon-binding domain with those in the active site domain.[8,54,56] Kim and Schimmel[44] created a series of MetRS mutants in which Trp461 and amino acids on either side of it were deleted. Deletions of 4 to 11 amino acids encompassing Trp461 were stable and had adenylate synthesis activity comparable to the wild-type enzyme. One of the 11 amino acid deletion mutants that was characterized further had low *in vitro* aminoacylation activity with $tRNA^{fMet}$ as the substrate, but catalyzed the specific aminoacylation with methionine of a 7-bp microhelix based on the acceptor stem of $tRNA^{fMet}$ at a rate indistinguishable from the wild-type enzyme. Thus, despite deletion of an essential region of the anticodon-binding domain, amino acid activation and acceptor stem aminoacylation are not affected, implying that these activities are functionally independent from an intact anticodon-binding domain.

Related to these studies are results reported by Khoda et al.[47] Four stable domains can be isolated after proteolysis of *Thermus thermophilus* MetRS (an α_2 dimer of 616 amino acid subunits).[47] The two N-terminal domains (Met1 to Lys503) correspond to the two domains of the N-terminal 551 amino acid tryptic fragment of *E. coli* MetRS.[47,65] The 503 amino acid fragment of the *T. thermophilus* enzyme is fully active for both adenylate synthesis and aminoacylation. The second in sequence of the two domains (Thr301 to Lys503) is not catalytically active by itself and, presumably, is the domain that binds the anticodon of $tRNA^{fMet}$. This isolated domain can be cross-linked to $tRNA^{fMet}$ in the absence of the other domain.[47] The domain at the extreme N-terminus (Met1 to Lys300) contains the class I sequence motifs, is fully active for adenylate synthesis, and, significantly, catalyzes the aminoacylation of *E. coli* $tRNA^{fMet}$, independent of the anticodon-binding domain. These observations support the idea that the catalytic domain of MetRS can function without the remainder of the enzyme and, furthermore, suggest that the anticodon-binding domain is able to bind tRNA whether or not the active site domain is present.

B. Class II Synthetases

In the class II enzymes, a loop of 10 to 18 amino acids is situated between the two β-strands of motif 2 (Figure 3). This loop is comprised of 11 to 18 amino acids in subclass 2a and of 10 to 12 amino acids in subclass 2b.[19] Sequence-specific contacts with the G73 discriminator base and the U1:A72 first base pair in the acceptor stem of $tRNA^{Asp}$ are made by this loop in the AspRS-$tRNA^{Asp}$ complex.[80] The discriminator base G73 is an identity element of both yeast and *E. coli* $tRNA^{Asp}$.[29,74] This loop probably makes acceptor stem contacts in other class II enzymes.[60,80] Polypeptide insertions of variable size also are located between motifs 1 and 2 and between motifs 2 and 3. Most of the insertion between motifs 1 and 2 in AspRS and SerRS is topologically conserved.[80] A portion of the insertion between motifs 2 and 3 corresponding to three β-strands and an α-helix of the active site fold is topologically conserved (Figure 3), but the remaining portion is not conserved and may be associated with specific recognition of tRNA acceptor stem nucleotides. As with the class I enzymes, these insertions into the catalytic domain appear to mediate synthetase-specific functions.

Bound AlaRS affords extensive protection of the acceptor stem of $tRNA^{Ala}$.[68] These acceptor stem contacts are made by the N-terminal active site domain because fragment 461N gives a similar pattern of nuclease protection.[9] Also, motifs 2 (Thr62 to His104) and 3 (Pro230 to Val266) are both located in the N-terminal domain and periodate-oxidized $tRNA^{Ala}$ cross-links to lysine73 within motif 2.[30] Within the polypeptide insertion between motifs 2 and 3 of *E. coli* AlaRS, a putative metal-binding domain occurs (Cys178-X_2-Cys-X_6-His-X_2-His). This region has been suggested by spectroscopic studies on a Co(II)-substituted enzyme[57] to be important for acceptor stem interactions.

The monomeric 461 amino acid N-terminal fragment of *E. coli* AlaRS is fully active for adenylate synthesis and has significant, but reduced (compared with native AlaRS), activity for aminoacylation of $tRNA^{Ala}$ (see earlier discussion).[41] In contrast, the fragment specifically

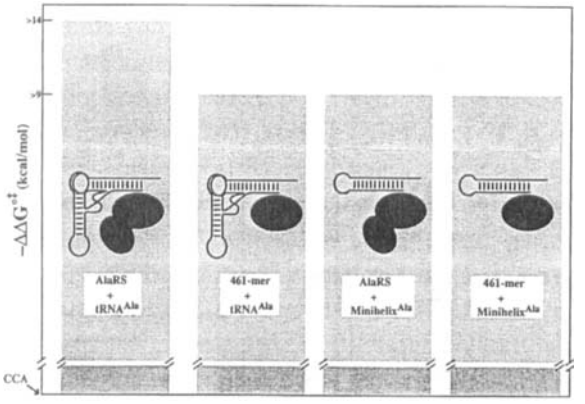

FIGURE 5. Relative aminoacylation activity of AlaRS and fragment 461N with full-length $tRNA^{Ala}$ and $minihelix^{Ala}$ as substrates at 30°C. Activity is expressed on the ordinate as the incremental lowering of the free energy of activation for aminoacylation ($\Delta\Delta G^{\circ\ddagger}$) provided by interactions with the specified substrate vs. the CCA trinucleotide common to the 3′-end of all tRNAs.[26,89] The value of $-\Delta\Delta G^{\circ\ddagger}$ for the full-length-tRNA compared with the CCA trinucleotide is greater than 14 kcal/mol at 37°C, but the exact value at either 37 or 30°C is not known because, despite extensive efforts with the native enzyme, aminoacylation of CCA could not be detected.[26] It is likely that the aminoacylation rate of CCA by fragment 461N is not greater than that of the full-length enzyme. The uncertainties in the absolute values of $-\Delta\Delta G^{\circ\ddagger}$ are indicated by breaks at the bottom of each bar.

aminoacylates with alanine a 12-bp RNA minihelix that recreates the acceptor-TΨC stem-loop of $tRNA^{Ala}$, and the rate of aminoacylation is as efficient as that catalyzed by the native enzyme (Figure 5).[9] A 4-bp RNA tetraloop substrate based solely on the acceptor stem sequence of $tRNA^{Ala}$ also is aminoacylated by the fragment at a rate comparable to that of native enzyme. Furthermore, the 461 amino acid fragment aminoacylates intact $tRNA^{Ala}$, the minihelix, and the tetraloop with comparable efficiencies. These results imply that the active site domain that is responsible for the specific recognition of the G3:U70 base pair is entirely within the N-terminal domain that contains the class-defining sequence motifs. Amino acids outside the N-terminal domain (His462 to Gln875) make neither a direct nor an indirect contribution to the aminoacylation efficiency of the acceptor stem of $tRNA^{Ala}$.

Only in the case of *E. coli* AlaRS has it been possible to isolate a relatively small domain (compared with the native protein) for the aminoacylation of acceptor stem substrates. Fragment 461N is slightly over half the size of the intact AlaRS subunit (875 amino acids) and demonstrates that a large portion of the synthetase is dispensable for activity. The results with MetRS show that for it too the domain for amino acid activation and acceptor helix interactions is functionally independent of an intact structure for the part of the protein required for tRNA contacts distal to the acceptor stem. These two examples may turn out to be representative of many of the other synthetases.

VI. SYNTHETASE EVOLUTION AND A SECOND GENETIC CODE

The concept of a second genetic code is based on the premise that there are specific interactions between the acceptor stem nucleotides of tRNAs and aminoacyl-tRNA synthetases that specify, through the aminoacylation reaction, an amino acid/nucleotide sequence relationship distinct from the classical code. The demonstration of sequence-specific aminoacylation of acceptor stem oligonucleotides devoid of the anticodon supports this concept. Because the part of contemporary synthetases associated with the class-defining motifs and active site contains the domains responsible for these interactions, it is likely that these domains represent the oldest portion of the protein and were responsible for the second genetic code.

The aminoacyl-tRNA synthetases are constructed from discrete structural/functional domains and subdomains that may have originally been associated in noncovalent complexes. At a later stage, the noncovalently associated domains may have been covalently linked and evolved into the multidomain modern synthetases.

GlyRS may be a natural example of this model of synthetase evolution. *E. coli* GlyRS is an $\alpha_2\beta_2$ tetramer with an α-subunit size of 303 amino acids and a β-subunit size of 689 amino acids. Neither subunit is active by itself for either amino acid activation or aminoacylation.[53,66,97] The class II conserved sequence motifs are found in the

α-subunit[19] and a point mutation in this subunit affects adenylate synthesis but has little effect on tRNA binding.[98] Conversely, mutagenesis and chemical modification of residues in the β-subunit decrease activity for aminoacylation but not for amino acid activation.[32,71,98] GlyRS catalyzes the aminoacylation of $tRNA^{Gly}$-based acceptor stem microhelices[26], and mutations in a region of the β-subunit involved in tRNA binding have a selective effect on the aminoacylation of full-length tRNA vs. a glycine microhelix.[32] These observations suggest that the α- and β-subunits represent functional domains. The α-subunit corresponds to the core catalytic domain and contains the active site for adenylate synthesis and acceptor stem aminoacylation, whereas the β-subunit is largely responsible for RNA interactions removed from the acceptor stem. The β-subunit alone can bind tRNA, possibly via β-subunit-anticodon interactions.[62,98]

The two noncovalently associated subunits of *E. coli* GlyRS may correspond to the two covalently linked domains of most other synthetases. These two subunits are encoded on the genome in tandem with only nine nucleotides separating the carboxy terminus of the α-subunit from the amino terminus of the β-subunit.[42] This arrangement is suggestive of a single polypeptide, and fusion of the carboxy terminus of the α-subunit to the amino terminus of the β-subunit through short peptide linkers gives active GlyRS homo-oligomers.[96,97] Thus, it is not obligatory for catalytic activity that the two subunits of GlyRS be separate polypeptide chains. Yeast GlyRS is an α_2 dimer in which presumably, the corresponding α- and β-subunits are covalently linked.[43]

The functional equivalent of the anticodon-binding domain found in some synthetases may be encoded as a separate polypeptide (the β-subunit) in *E. coli* GlyRS. This subunit could have been recruited into a complex with the active site-acceptor helix-binding domain to provide additional RNA discrimination based on anticodon recognition. A similar explanation may hold for the polypeptide insertions into the active site domains of class I and class II synthetases: these insertions were recruited initially as separate polypeptides for specific interactions with acceptor stem nucleotides.

The possibility of constructing an active synthetase from fragments in this manner has been explored with MetRS (see earlier discussion) and with *E. coli* IleRS. The active site domain in *E. coli* IleRS (a monomer of 939 amino acids) is located in the N-terminal region,[15,94] and the anticodon recognition site is in the C-terminal domain.[87] Approximately 145 of the 370 amino acids of the CP1 insertion in IleRS are dispensable for catalytic activity.[94] Thus, the insertions may occur at natural breaks in the secondary structure that makes up the nucleotide-binding fold. Shiba and Schimmel[91] placed breaks in IleRS at 18 locations, including within CP1 and CP2. No fragments were active *in vivo* when expressed alone, but 11 of 18 fragment pairs were active when coexpressed and active bipartite complexes were demonstrated *in vitro*. In an extension of this work, the tripartite assembly of IleRS from specific fragments was demonstrated.[92] One tripartite structure was comprised of an N-terminal piece extending to Gly330 (within CP1), a middle piece from Gly330 to Lys583 in the second half of the nucleotide fold, and a C-terminal fragment extending from Lys583 to the carboxy terminus. These results demonstrate the feasibility of generating multichain, noncovalently associated complexes with aminoacylation activity and raise the possibility that these were intermediates in the evolution of contemporary single-chain synthetases.

Related to these considerations are the locations of the multiple introns in the gene for IleRS from *Tetrahymena thermophila*.[16] Most of these introns occur between exons that encode for discrete secondary structural elements.[84] In particular, there are introns on each side of CP1 and at the boundary of the nucleotide-binding fold and the C-terminal anticodon-binding domain. Possibly, polypeptide insertions (such as CP1) were encoded by distinct exons that were recruited into the nucleotide-binding fold to provide a framework for recognition of RNA oligonucleotides, which subsequently became the acceptor stems of modern RNAs.[84] In a similar manner, recruitment of the exon encoding the anticodon-binding domain to the C-terminal side of the nucleotide-binding fold may have enabled RNA interactions distal to the acceptor stem.

Determinants for the aminoacylation efficiency of tRNA by the synthetases are found in the acceptor stems of tRNAs and have been proposed to constitute a second genetic code.[20,59,83] Specific recognition of acceptor stem nucleotides is mediated both by residues in the class-defining active site domain and by residues located in polypeptide insertions into this domain. Although there are examples where the catalytic domain for aminoacylation of RNA acceptor stem helices is functionally independent of the integrity of the remainder of the protein, an acceptor stem-binding subdomain independent of the amino acid activation domain has not yet been isolated. Determinants for amino acid activation and acceptor stem aminoacylation are closely integrated within the catalytic domain and may be difficult to separate. This core synthetase may represent the primordial enzyme in which RNA interactions that did not extend further than 10 to 20 Å beyond the amino acid attachment site at the 3′-end of a bound RNA substrate provided all of the determinants for aminoacylation efficiency. These nucleotide-specific interactions defined a second genetic code.

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