Journal of Biochemistry and Molecular Biology, Vol. 31, No. 6, November 1998, pp. 525–535

JOURNAL OF
Biochemistry
and
Molecular Biology

Review

Transfer RNA Acceptor Stem Determinants for Specific Aminoacylation by Class II Aminoacyl-tRNA Synthetases

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Received 22 June 1998, Accepted 20 August 1998

A critical step in the faithful translation of genetic information is specific tRNA recognition by aminoacyl-tRNA synthetases. These enzymes catalyze the covalent attachment of particular amino acids to the terminal adenosine of cognate tRNA substrates. In general, there is one synthetase for each of the twenty amino acids and each enzyme must discriminate against all of the cellular tRNAs that are specific for the nineteen noncognate amino acids. Primary sequence information combined with structural data have resulted in the division of the twenty synthetases into two classes. In recent years, several high-resolution co-crystal structures along with biochemical data have led to an increased understanding of tRNA recognition by synthetases of both classes. The anticodon sequence and the amino acid acceptor stem are the most common locations for critical recognition elements. This review will focus on acceptor stem discrimination by class II synthetases. In particular, the results of *in vitro* aminoacylation assays and site-directed and atomic group mutagenesis studies will be discussed. These studies have revealed that even subtle atomic determinants can provide signals for specific tRNA aminoacylation.

Keywords: Acceptor stem recognition, Atomic group mutagenesis, Minihelices, Positive and negative discrimination, Semi-synthetic tRNAs.

Introduction to Aminoacyl-tRNA Synthetases

Aminoacyl-tRNA synthetases are a family of twenty enzymes that carry out the covalent attachment of specific amino acids to cognate transfer RNA (tRNA) substrates in a two-step reaction known as the aminoacylation reaction. First, the amino acid is activated by ATP to form an enzyme-bound intermediate known as the aminoacyl-adenylate (amino acid-AMP) with release of PP_i. This reaction requires Mg^{2+} and, in the majority of the twenty systems, it can occur in the absence of tRNA. In the second step, the activated amino acid is transferred to either the 2′- or 3′-hydroxyl of the terminal adenosine (A76) of enzyme-bound tRNA with release of AMP. Although a similar reaction is catalyzed by all twenty synthetases, these enzymes are diverse with respect to their sequence, size, and oligomeric structure (Meinnel *et al.*, 1995; Martinis and Schimmel, 1996). It was not until all aminoacyl-tRNA synthetases from a single species (*Escherichia coli*) were cloned and sequenced that limited sequence relationships between all the members of this enzyme family were discovered. This information was used to group the twenty synthetases into two distinct classes of ten members each (Eriani *et al.*, 1990; Burbaum and Schimmel, 1991; Cusack *et al.*, 1991).

The class I synthetases share a common catalytic core domain made up of a series of α-helices and β-sheets that form a Rossmann nucleotide binding fold, which is typical of many nucleotide-binding enzymes (Rould *et al.*, 1989). The ten class II enzymes, on the other hand, share a catalytic core consisting of an antiparallel β-sheet structure and three highly degenerate consensus sequences known as motifs 1, 2, and 3 (Cusack *et al.*, 1990; Ruff *et al.*, 1991). Despite the distinct topology of the catalytic sites of the two classes of synthetases, chemical similarities in the active sites and the proposed mechanisms are observed (for a recent review, see Delarue, 1995). However, there are also some notable functional differences between the two classes (for a recent review, see Arnez and Moras, 1997). While all tRNAs are aminoacylated or "charged" on the 3′-terminal adenosine in the acceptor stem domain, the class I enzymes initially aminoacylate the 2′-hydroxyl of A76, whereas the initial site of amino acid attachment for class II synthetases is the 3′-hydroxyl group. A second

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functional distinction appears to be in the mode of tRNA binding. The class I synthetases approach the minor groove side of the tRNA acceptor stem, whereas the class II synthetases approach the major groove side. Thus, there is a mirror image relationship in the mode of tRNA binding between the two classes of synthetases.

Based mainly on homologous anticodon binding domains, the class II synthetases have been further divided into sub-classes (Cusack *et al.*, 1991; Cusack, 1993; 1998). Five enzymes (GlyRS, HisRS, ProRS, SerRS, and ThrRS)[1] belong to class IIa (the GlyRS here refers to all species except for the *E. coli* enzyme) and four of these (all but SerRS) share a C-terminal anticodon binding domain with a unique α/β fold. SerRS lacks an anticodon binding domain and instead has a novel tRNA recognition feature, namely, a helical arm that forms a 60 Å long antiparallel coiled-coil. This motif is used to recognize the unusually long variable arm of $tRNA^{Ser}$ (Cusack *et al.*, 1996). Class IIb synthetases (AspRS, AsnRS, and LysRS) possess an N-terminal anticodon binding domain that consists of a five-stranded β-barrel motif. While most LysRSs are class II synthetases, it was recently reported that LysRSs from some archaea and bacteria share more similarity to class I synthetases (Ibba *et al.*, 1997a; 1997b). To date, this is the only known example of a class-switch in evolution. AlaRS is an example of another class II enzyme that, like SerRS, does not recognize the anticodon sequence of its cognate tRNA. Unlike class IIa and IIb synthetases, which are all α_2 homodimers, AlaRS, PheRS, and *E. coli* GlyRS are class II tetramers.

Transfer RNA Structure, Identity, and Model RNA Substrates for *In Vitro* Aminoacylation

The three-dimensional fold of tRNAs was determined nearly twenty-five years ago when the structure of yeast $tRNA^{Phe}$ was elucidated by X-ray crystallography (Kim *et al.*, 1974; Robertus *et al.*, 1974). Transfer RNAs share a common cloverleaf secondary structure and are all likely to fold into a similar L-shaped structure in solution (Fig. 1). In recent years, a vast amount of structural data on synthetases and synthetase/tRNA complexes has accumulated, especially for class II systems (for recent reviews, see Francklyn *et al.*, 1997; Cusack, 1998; Cusack *et al.*, 1998). This data has led to insights into how synthetases discriminate among similarly folded tRNAs. However, even for systems where there are no known synthetase/tRNA co-crystal structures, detailed recognition features have in some cases been elucidated at the atomic level. This has been accomplished, in part, due to the development of *in vitro* RNA transcription systems in the late 1980s, and the demonstration that modified nucleotides found in natural tRNAs are not essential for synthetase recognition in most systems (Sampson and Uhlenbeck, 1988; Milligan and Uhlenbeck, 1989). In addition, advances in automated chemical RNA synthesis in recent years (Scaringe *et al.*, 1990; Sproat *et al.*, 1995) contributed significantly to tRNA recognition studies. While *in vivo* genetic approaches have also been extraordinarily useful in elucidating tRNA "identity sets", which are the positive and negative elements used for synthetase discrimination, *in vivo* assays provide an indirect measure of aminoacylation (Schulman, 1991; Pallanck *et al.*, 1995). *In vitro* approaches, on the other hand, allow the aminoacylation step in translation to be measured directly. The contribution to aminoacylation of specific tRNA domains, nucleotides, and even single atomic groups can, therefore, only be determined *in vitro*.

***In vitro* aminoacylation of model RNA substrates** The technical advances in methods of *in vitro* RNA transcription contributed to the discovery that many synthetases can aminoacylate RNAs derived only from the top half of the L-shaped tRNA structure (Fig. 1) (Francklyn and Schimmel, 1989). The so-called minihelices and microhelices contain the first 12 or 7 base-pairs of the acceptor-TΨC stem-loop or acceptor stem, respectively. RNA duplexes and four base-pair tetraloop-containing RNAs are also substrates for some synthetases (Fig. 1, right). All of these truncated RNA constructs lack the domain that contains the trinucleotide anticodon. The acceptor stem typically ends in four single-stranded nucleotides ($tRNA^{His}$ is an exception), including the universally conserved CCA-3′ end and the "discriminator" base at position 73 (Fig. 1, circled). The latter is important for tRNA discrimination by a majority of synthetases (Shimizu *et al.*, 1992; Hou, 1997). A second major site for specific synthetase interaction in many tRNA systems is the anticodon (Saks *et al.*, 1994), which is located approximately 75 Å from the amino acid attachment site in the full-length L-shaped tRNA. However, the fact that model RNAs that lack the anticodon are substrates for at least eleven of the synthetase systems (Frugier *et al.*, 1994; Hamann and Hou, 1995; Martinis and Schimmel, 1995) demonstrates that the anticodon is dispensable for specific *in vitro* aminoacylation (for a recent review on minihelices, see Musier-Forsyth and Schimmel, 1998). Moreover, many of the recognition features identified in the full-length tRNAs are recapitulated in the small RNA stem-loops. Thus, these model substrates, which are also readily prepared via automated chemical RNA synthesis, can be used to investigate the details of acceptor stem recognition using atomic group "mutagenesis". In this approach, modified nucleotides are incorporated into synthetic RNAs to probe the functional effect of subtle atomic group deletions on aminoacylation efficiency.

[1] Throughout the text, individual aminoacyl-tRNA synthetases are abbreviated by the three-letter code of the amino acid for which they are specific, followed by the "RS" suffix. Unless otherwise noted, the results described refer to experiments with the *E. coli* system.

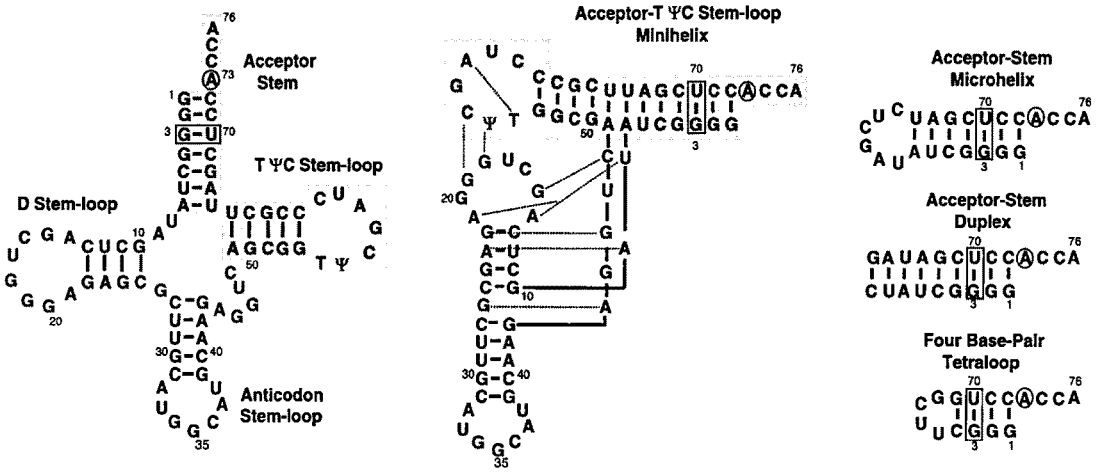

Fig. 1. Sequence of *E. coli* $tRNA^{Ala}$ and model RNA substrates for aminoacylation with Ala. For the tRNA, both the secondary structural representation (left) and the folded L-shaped structure (middle) with tertiary interactions (dotted lines) are shown. The shaded portion of the tRNA indicates the acceptor-TΨC stem-loop minihelix. The microhelix (right, top) is based on just the acceptor stem domain with a loop derived from nucleotides 8–13. The duplex (right, middle) is derived from the first 9 base pairs of the acceptor-TΨC stem, and the tetraloop (right, bottom) is based on only the first 3 base pairs of the acceptor stem with a nonspecific fourth base pair and loop. In all of the RNAs, the G3:U70 base pair and A73 discriminator base are boxed and circled, respectively.

Semi-synthetic tRNAs Atomic group mutagenesis can also be performed in the context of full-length tRNAs if a semi-synthetic approach is used. Semi-synthetic tRNA constructs that contain a chemically synthesized oligonucleotide, typically derived from the 3′- or 5′-fifteen to twenty nucleotides of the tRNA, can be annealed to a corresponding 3/4-length tRNA fragment prepared enzymatically by *in vitro* transcription (Fig. 2). This strategy allows modified nucleotides to be incorporated into the chemically synthesized strand. In some systems, it has been shown that heat-annealing is sufficient to produce a functional tRNA from the two fragments and that enzymatic ligation of the phosphodiester bond is not needed for efficient aminoacylation (Yap *et al.*, 1995). Alternatively, RNA ligase or DNA-splinted ligation with DNA ligase can be used to prepare an intact full-length tRNA molecule (Moore and Query, 1998).

Overlapping Acceptor Stem Determinants Specify Recognition by Class II Synthetases

Using the *in vitro* techniques described above, researchers have shown that in addition to the anticodon and the discriminator base, the majority of class II synthetases from *E. coli* recognize one or more additional features within the acceptor stem domain of their respective tRNA substrates. This review will focus on recognition in class II Ala, Gly, His, Pro, Ser, and Thr systems for which elements within the first four base pairs following the single-stranded 3′-end have been shown to be important for synthetase discrimination. The acceptor stem domain of each of these six *E. coli* tRNAs is shown in Fig. 3. To date, for the remaining four class II systems (Phe, Asp, Asn, and Lys), critical acceptor stem nucleotides outside of the discriminator base have not been identified, although in the case of *E. coli* $tRNA^{Asp}$, the second base pair is a very minor recognition element (Nameki *et al.*, 1992).

The recognition elements for the systems shown in Fig. 3 were determined by *in vitro* aminoacylation of $microhelix^{Ala}$, $duplex^{Ala}$ (not shown), $microhelix^{Gly}$, $microhelix^{His}$, $minihelix^{Ser}$, and full-length tRNAs. In this figure, nucleotides that have been classified as major and minor recognition elements are shown in the shaded and open boxes, respectively. In the Ala system, positions

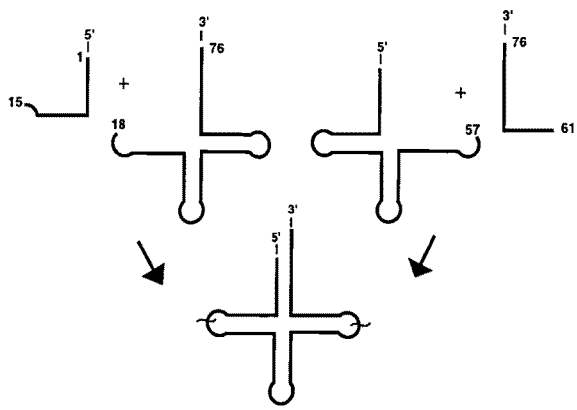

Fig. 2. Two schemes for the preparation of semi-synthetic tRNAs. The shorter oligonucleotides are chemically synthesized and annealed to longer 3/4-length fragments prepared by *in vitro* transcription. The wavy lines represent a break in the phosphodiester backbone in either the D or TΨC loops.

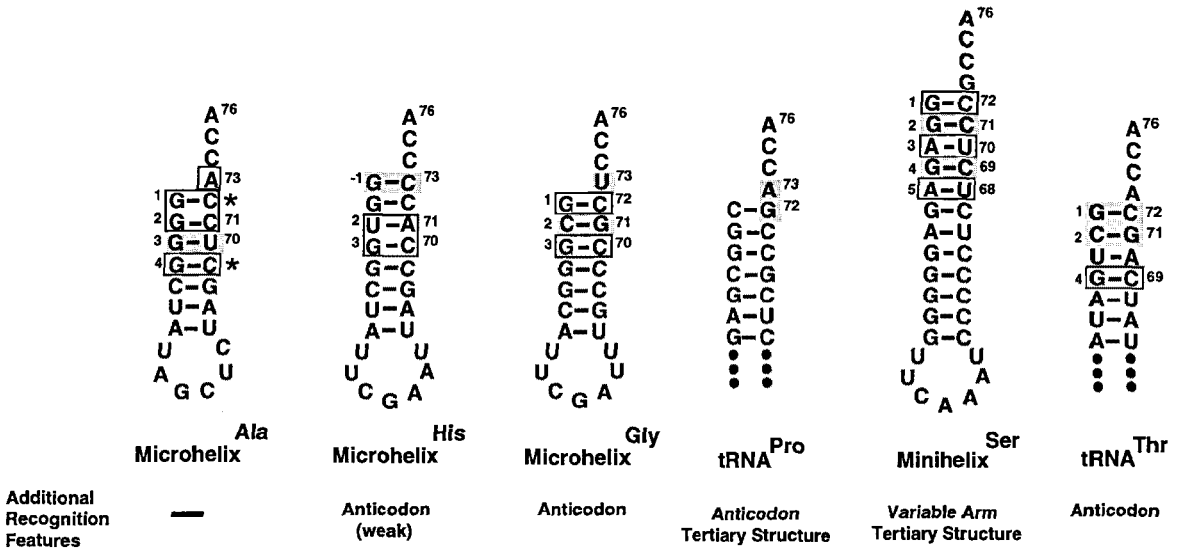

Fig. 3. Sequences of microhelices derived from *E. coli* $tRNA^{Ala}$, $tRNA^{His}$, and $tRNA^{Gly}$, a minihelix derived from *E. coli* $tRNA^{Ser}$, and the acceptor stem domain of *E. coli* $tRNA^{Pro}$ and $tRNA^{Thr}$. Numbering in all cases is according to full-length tRNA. The boxes indicate major (shaded) and minor or modulating (open) recognition elements as determined by *in vitro* aminoacylation assays of full-length tRNA transcripts, or mini-substrates prepared either by *in vitro* transcription or chemical synthesis. The relative importance of major recognition elements varies widely between systems. The asterisks indicate positions in the Ala system where negative discrimination are known to occur. Additional recognition features located outside of the acceptor stem domain in each system are indicated below each RNA.

where base substitutions result in negative discrimination by the cognate synthetase have also been identified and are indicated by an asterisk. In general, mutagenesis of minor or modulating elements has a less severe effect on aminoacylation relative to changes at major elements. Minor positions can often be substituted with one or more nucleotides without affecting aminoacylation efficiency significantly, whereas this is generally not the case for a major element. The term negative element refers to positions where the wild-type base is not necessarily important for positive recognition by the synthetase, but where a specific substitution introduces a blocking feature. The location of positive and negative elements may overlap. In most cases, it is known that a minor element modulates activity, but whether the effect is primarily due to positive recognition, negative discrimination, or a combination of both has not been clearly established.

Recognition of G3:U70 by AlaRS AlaRS is one of the most thoroughly studied class II enzymes with respect to acceptor stem recognition. A detailed picture of the determinants used for specific aminoacylation by AlaRS has emerged, in part, through atomic group mutagenesis studies. In particular, the importance of the unique G3:U70 wobble base pair (Fig. 3, shaded), initially established by *in vivo* assays (Hou and Schimmel, 1988; McClain and Foss, 1988), has been extensively analyzed in numerous contexts *in vitro*, including full-length tRNAs (Park *et al.*, 1989; Shi *et al.*, 1990; Beuning *et al.*, 1997), minihelices (Francklyn and Schimmel, 1989; Shi *et al.*, 1990), microhelices (Francklyn and Schimmel, 1989; Francklyn *et al.*, 1992), duplexes (Musier-Forsyth *et al.*, 1991a; 1991b; 1995; Beuning *et al.*, 1997; Musier-Forsyth and Schimmel, 1998), and four base pair tetraloop-containing RNAs (Shi *et al.*, 1992) (Fig. 1). In all these contexts, aminoacylation with Ala is G3:U70-dependent.

Using $duplex^{Ala}$ substrates, the contribution of specific functional groups presented by the G:U wobble pair to aminoacylation efficiency has been investigated. By incorporating a series of standard bases and base analogs at position 3:70 of $duplex^{Ala}$ variants, it was established that the presence of a *free* minor groove exocylic amino group at position 3 is critical for recognition (Musier-Forsyth *et al.*, 1991b). In particular, a G3 to inosine (I) substitution eliminates aminoacylation of duplex substrates. Inosine is a guanine analog that lacks the minor groove exocylic amino group of G, but maintains all of the same major groove functional groups. From these studies, it was estimated that the 2-amino group contributed at least 3.3 kcal/mol to aminoacylation catalytic efficiency (Fig. 4). The wobble configuration of the third base pair and the overall helix geometry in an I3:U70 variant is expected to be similar to that of the wild-type duplex (Limmer *et al.*, 1996). Moreover, alternate base analogs that recreate the presence of a free amino group in a similar position of the acceptor stem helix also confer Ala acceptance on RNA

duplexes (Musier-Forsyth *et al.*, 1995). These studies highlight the importance of a specific minor groove atomic group rather than a particular base pair. In addition to the G3 amino group, backbone 2′-hydroxyls of G4, U70, and C71 that line the minor groove and are all located within ~5 Å of the critical amino group have been shown to play a significant role in aminoacylation (Musier-Forsyth *et al.*, 1991a; 1991b) (Fig. 4).

Both low and high resolution NMR structures of microhelix^{Ala} show that the acceptor stem has an overall helix geometry similar to the A-form, with some deviations occurring near the G:U pair (Limmer *et al.*, 1996; Ramos and Varani, 1997). Recent *in vitro* experiments showed that the presence of a helical distortion, however, is not the major factor for efficient tRNA^{Ala} aminoacylation (Beuning *et al.*, 1997). This was established by introducing

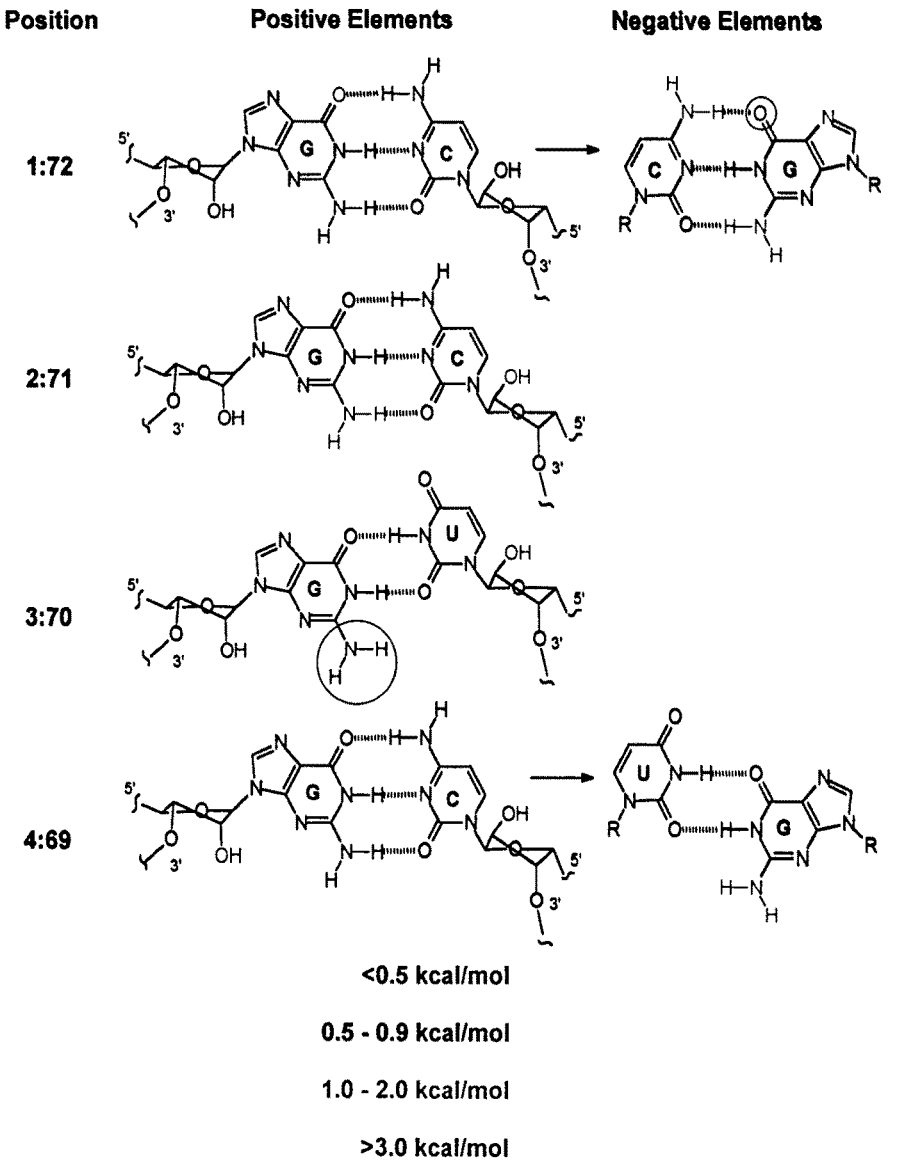

Fig. 4. Summary of acceptor stem atomic groups that specify *in vitro* aminoacylation by *E. coli* AlaRS. Structures of the first four base pairs of *E. coli* tRNA^{Ala} are shown in the middle, with the corresponding helix position indicated on the left. Base pair substitutions at 1:72 and 4:69 that have negative effects on aminoacylation are shown on the right. Functional groups that have been tested by atomic group mutagenesis are highlighted in color. The contribution to aminoacylation catalytic efficiency is indicated in terms of $\Delta\Delta G^{\circ}$, which was calculated as described (Musier-Forsyth and Schimmel, 1992; Beuning *et al.*, 1997). Functional groups whose deletion had little or no effect are shown in green ($\Delta\Delta G^{\circ} < 0.5$ kcal/mol) whereas the strongest positive and negative elements are circled and shown in red ($\Delta\Delta G^{\circ} > 3$ kcal/mol). Intermediate effects were observed at positions shown in blue ($\Delta\Delta G^{\circ} = 0.5$–$0.9$ kcal/mol) and pink ($\Delta\Delta G^{\circ} = 1$–$2$ kcal/mol).

alternative wobble pairs including C3:A70 into full-length $tRNA^{Ala}$ and $duplex^{Ala}$ substrates (Beuning *et al.*, 1997). Earlier, alternative mismatched variants were reported to confer Ala aminoacylation *in vivo* (Gabriel *et al.*, 1996). Additionally, a recent NMR study demonstrated that C3:A70 induces helix 'destacking' in the vicinity of the mismatch, albeit in the opposite direction than in the G3:U70 wild-type duplex (Vogtherr *et al.*, 1998). The *in vitro* aminoacylation studies showed that the presence of alternate mismatched wobble base pairs significantly reduces or eliminates aminoacylation catalytic efficiency in both full-length and mini-substrates. For example, the k_{cat}/K_M of full-length C3:A70-$tRNA^{Ala}$ is reduced by >5000-fold relative to the wild-type G3:U70-tRNA. Based on these studies, it was estimated that a helical distortion may contribute at most up to 1.5 kcal/mol to lowering the free energy of activation ($\Delta G°$) for aminoacylation.

Taken together, the large body of *in vivo* (McClain *et al.*, 1988; 1991; 1996; McClain and Foss, 1988; Gabriel *et al.*, 1996) and *in vitro* data on the role of G:U in the Ala system suggest that an array of minor groove functional groups in and around the third base pair together with conformational features induced by the wobble pair all contribute to AlaRS recognition. Based on *in vitro* experiments that directly measure the aminoacylation reaction, however, specific atomic groups appear to contribute more to catalytic efficiency than a helical irregularity. In the RNA-synthetase complex, whether the critical functional groups interact with AlaRS directly or whether they mediate their effects through an indirect mechanism remains to be established.

A73, G1:C72, and G2:C71 modulate AlaRS recognition The discriminator base, A73, and the first two base pairs modulate activity in the Ala system (Francklyn *et al.*, 1992; Liu *et al.*, 1996; Beuning *et al.*, 1997) (Fig. 3). Both positive recognition and negative discrimination have been shown to occur at these positions. In the case of the second base pair, inosine substitution showed that the 2-amino group of G2 contributes significantly to positive recognition (Musier-Forsyth and Schimmel, 1992) (Fig. 4). Specific atomic groups of A73 have not yet been probed, however, it is known that the discriminator base is needed for transition state stabilization (Shi and Schimmel, 1991). The high resolution NMR structure of $microhelix^{Ala}$ indicates that A73 is stacked over G1 rather than C72 (Ramos and Varani, 1997). Although the significance of this stacking interaction is unclear, based on an NMR study of the *Thermus thermophilus* Ala system there is likely to be a functional enzyme interaction at G1:C72 (Lechler *et al.*, 1997). In this work, a pronounced broadening of the imino and amino resonances of the first base pair is observed upon enzyme addition, indicating the likelihood of a direct interaction at this site. Biochemical data also support the importance of the first base pair in the Ala system. Despite the fact that 75% of all *E. coli* tRNAs contain a G1:C72 base pair, substitutions at this semi-conserved position have surprisingly large effects on aminoacylation efficiency. In fact, the single base pair transversion of G1:C72 to C1:G72 results in complete loss (>1000-fold decrease) of $duplex^{Ala}$ aminoacylation and a $>10^4$–fold reduction in k_{cat}/K_M for aminoacylation of full-length $tRNA^{Ala}$ (Liu *et al.*, 1995).

To further investigate this dramatic effect, atomic group mutagenesis studies were carried out, wherein over 30 standard and modified base pairs were incorporated into $duplex^{Ala}$ at 1:72. These studies demonstrated that G1:C72 is used as both a minor positive recognition element and as a site for negative discrimination by AlaRS (Beuning *et al.*, 1997). Individual major groove functional groups of G1 and C72 were determined to contribute up to ~1 kcal/mol toward transition state stabilization. However, these studies also showed that the complete loss of aminoacylation activity in the C1:G72-$duplex^{Ala}$ variant can be largely attributed to the negative influence of G72. In particular, 2-aminopurine (2AP) substitution at position 72, which results in deletion of the major groove 6-keto oxygen and N1 proton of guanine, restored aminoacylation activity to within 18-fold of the wild-type level (Beuning *et al.*, 1997). Moreover, a 2AP72 substitution restored activity independent of the nucleotide present at position 1. These data, together with a series of pyrimidine base analog substitutions at position 72, support the conclusion that a major groove carbonyl oxygen at this site functions to block aminoacylation by AlaRS (Fig. 4). Whether the negative effect is caused by a direct steric block or whether it is due to an indirect mechanism such as altering critical stacking interactions with A73 remains to be determined.

Importance of a nonessential element at 4:69 in aminoacylation by AlaRS Placement of a specific functional group in the minor groove of the fourth base pair that is adjacent to the critical G3:U70 pair, also has a negative effect on AlaRS recognition. Whereas all of the standard Watson-Crick base pairs and a G4:U69 wobble pair are well tolerated at this position, a U4:G69 substitution results in a 20-fold reduction in aminoacylation efficiency (Henderson *et al.*, 1998). Base analog substitutions showed that this decrease was largely due to the negative effect of the exocyclic 2-amino group of G69 (Fig. 4). In particular, a U4:I69 substitution restored aminoacylation efficiency to near wild-type levels. It has been proposed that the negative effect of U4:G69 may be due to the recruitment of a tightly bound water molecule that bridges the amino group and the 2′-hydroxyl of U4 (Henderson *et al.*, 1998). Such water-mediated hydrogen bonding interactions have been observed in several crystal structures of G:U-containing RNA helices (Westhof *et al.*, 1988; Holbrook *et al.*, 1991; Betzel *et al.*,

1994; Cruse *et al.*, 1994). The presence of a new water-mediated hydrogen-bonding network at U4:G69 may result in the disruption of critical functional interactions at G3:U70. Additional structural work is needed to test this hypothesis.

Overall picture of acceptor stem recognition by AlaRS Figure 4 summarizes the atomic determinants that specify aminoacylation with Ala. These elements may function either via a direct contact with the enzyme, or indirectly via interactions through water molecules or conformational effects. It is important to note that these mechanistic possibilities are not mutually exclusive. The overall picture that is emerging is that AlaRS achieves an extraordinary degree of discrimination of both cognate and noncognate acceptor stems by combining minor groove recognition in and around the critical 3:70 base pair with major groove discrimination at 1:72. The latter conclusion is in accordance with co-crystal structures of class II synthetases complexed to tRNAs, which show that these enzymes approach the top of the acceptor stem from the major groove side (Ruff *et al.*, 1991; Cusack *et al.*, 1996). Using truncated AlaRS variants and RNA microhelices, experiments to identify the enzyme domain that is likely to be involved in both the major and minor groove functional interactions have been carried out (Buechter and Schimmel, 1995). In this work, the N-terminal 368 amino acids of AlaRS that contain the three class II consensus motifs were shown to possess determinants for recognition of A73 and G2:C71. A 76 amino acid polypeptide appended to this catalytic center was shown to play a role in minor groove recognition around G3:U70. Additional experiments are needed to identify the specific amino acids responsible for the key acceptor stem interactions in the Ala system.

Recognition in the Pro system *E. coli* $tRNA^{Pro}$ is another class II system where critical acceptor stem determinants have been identified. In particular, using full-length mutant tRNAs, the unique G72 and the discriminator base, A73, were found to be major recognition elements both *in vivo* (McClain *et al.*, 1994) and *in vitro* (Liu *et al.*, 1995) (Fig. 3). ProRS also recognizes the anticodon and requires a specific tertiary structure for efficient aminoacylation (Liu and Musier-Forsyth, 1994; Liu *et al.*, 1995; Yap and Musier-Forsyth, 1995; Yap *et al.*, 1995). Aminoacylation of acceptor stem minihelices is not detected in this system (Liu and Musier-Forsyth, 1994). Nevertheless, a semi-synthetic strategy was used to examine the atomic determinants for G72 recognition. Using the semi-synthetic tRNA construct shown in Fig. 2 (right), the guanine base analogs I and 2AP were substituted at position 72. These substitutions tested functional groups in both the minor (I) and major (2AP) grooves (Fig. 5). Deletion of the 2-amino group of G72 has no effect on aminoacylation by ProRS, whereas the 2AP substitution results in a 50-fold decrease in aminoacylation efficiency (Liu *et al.*, 1996). Thus, the major groove 6-keto oxygen and/or the N1-proton of G72 are positive recognition elements for ProRS. As mentioned above, the 6-keto oxygen of G72 is a negative or blocking element for AlaRS (Fig. 4). In accordance with these results, AlaRS is unable to charge a G3:U70-$duplex^{Pro}$ variant unless the first base pair is changed from C1:G72 to G1:C72 (Liu *et al.*, 1995). Taken together, these experiments show that in the class II Ala and Pro systems, a single atomic group, namely a carbonyl oxygen in the major groove of position 72, is used for different purposes by ProRS and AlaRS. In

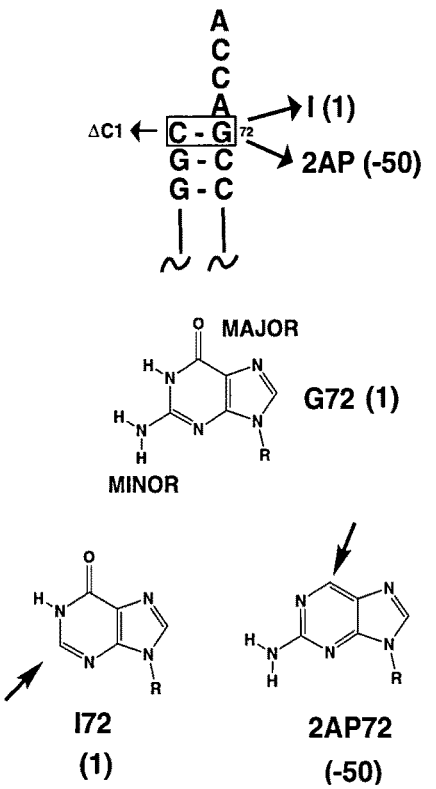

Fig. 5. Base analog substitutions at the 72 position of semi-synthetic $tRNA^{Pro}$ molecules and their effect on aminoacylation by *E. coli* ProRS. A portion of the acceptor stem domain of *E. coli* $tRNA^{Pro}$ is shown on the top with the 1:72 base pair boxed. The small arrow indicates that the first nucleotide was deleted in these constructs (ΔC1) to facilitate *in vitro* transcription of the 5'-3/4-length fragment beginning with G2 (Liu *et al.*, 1995; 1996). Numbers indicate fold-decrease in k_{cat}/K_M relative to the (ΔC1:G72) wild-type tRNA. The structure of the wild-type base, G72, with the location of the major and minor grooves indicated, is shown in the middle. The base analogs are shown at the bottom, with arrows pointing to the minor and major groove positions tested in each case. I is inosine and 2AP is 2-aminopurine. Deoxynucleotide analogs, which are well tolerated at this position (Yap *et al.*, 1995), were used in this study.

particular, they illustrate how synthetases utilize overlapping acceptor stem sites for positive recognition of cognate tRNAs and negative recognition of noncognate tRNAs.

The identification of a key major groove atomic group in the Pro system is consistent with the major groove acceptor stem approach of this class of synthetases. The high resolution co-crystal structures of AspRS/tRNA^{Asp} (Ruff *et al.*, 1991) and SerRS/tRNA^{Ser} (Cusack *et al.*, 1996) (see below) show that major groove acceptor stem contacts are made by the variable loop that lies between the two β-strands of motif 2. By analogy to these other class II systems, cysteine-scanning mutagenesis experiments have recently been carried out to identify the amino acid(s) in the motif 2 loop of *E. coli* ProRS that may interact with the critical 6-keto oxygen of G72. Each of the five motif 2 loop residues (Val143, Arg144, Pro145, Arg146, Phe147) was individually substituted with Cys and it was discovered that only the mutation at Arg144 results in complete elimination of aminoacylation activity (F. Yang and K. Musier-Forsyth, manuscript in preparation). This mutant protein, however, was fully active in aminoacyl-adenylate formation, indicating that the entire defect is in tRNA binding and/or amino acid transfer to the acceptor stem. Cross-linking experiments have also verified that this Arg residue is proximal to the 1:72 base pair of tRNA^{Pro} (B. Burke, B. Chan, and K. Musier-Forsyth, unpublished results). Thus, it is possible that a specific Arg144-G72 interaction is a major determinant of specific acceptor stem recognition in the Pro system. Similar Arg-rich RNA-protein interaction motifs have been identified in other systems such as the Tat-TAR interaction of HIV (Tao and Frankel, 1992).

Recognition in the His and Gly systems A comparison of microhelix aminoacylation in the His, Gly, and Ala systems provided another striking example of overlapping acceptor stem recognition (Francklyn *et al.*, 1992). Based on *in vitro* studies, major determinants in these systems are located at the unique −1:73 base pair for His (Himeno *et al.*, 1989; Francklyn and Schimmel, 1990), 2:71 and 73 for Gly, and 3:70 for Ala (Fig. 3). Analysis of a series of 13 microhelix mutants showed that substitutions that resulted in positive recognition by one enzyme, blocked aminoacylation by the other two. Thus, aminoacylation with His, Gly, and Ala was found to be mutually exclusive. Specific atomic groups that are likely to be responsible for the modulating effects of substitutions at 2:71 and 3:70 for His and 1:72 and 3:70 for Gly (Fig. 3) have not yet been identified. Interestingly, as for the Ala system, the specificity of aminoacylation by GlyRS and HisRS is maintained in RNA tetraloop substrates containing less than one turn of an RNA helix (Fig. 1) (Shi *et al.*, 1992). These RNAs contain only the first three base pairs of the corresponding tRNA appended to a nonspecific fourth base pair and closing loop. These studies highlight the importance of the first three base pairs in specifying recognition in these class II systems.

Recognition in the Ser and Thr systems Unlike the majority of synthetases, the discriminator base is not a recognition element for *E. coli* SerRS and ThrRS (Fig. 3). In the Thr system, relatively large decreases (80–4000-fold) in aminoacylation efficiency have been observed when mutations are made at either of the first two base pairs of tRNA transcripts (Hasegawa *et al.*, 1992). Although, based on these data, it is likely that the second base pair is a major positive recognition element, it is unclear whether the observed effects at the semi-conserved 1:72 pair are due to positive or negative discrimination by ThrRS. Weak recognition of the fourth base pair also occurs in this system. The anticodon is critical for aminoacylation by ThrRS and no studies of minihelix^{Thr} aminoacylation have been reported.

The major positive recognition feature in the Ser system is the long variable arm of tRNA^{Ser}, which interacts with the unusual helical arm of SerRS (Biou *et al.*, 1994) and which gives the tRNA a characteristic tertiary structure (Himeno *et al.*, 1990; Asahara *et al.*, 1993; 1994). Structure-specific rather than base-specific recognition seems to be important in this system. Nevertheless, minihelices that lack the variable arm domain are substrates for *E. coli* SerRS (Sampson and Saks, 1993). In the co-crystal structure of the *T. thermophilus* tRNA^{Ser}–SerRS complex (Cusack *et al.*, 1996), the long variable loop of motif 2 interacts with the major groove of the acceptor stem. *In vitro* aminoacylation studies of minihelix substrates have been carried out to understand the determinants for acceptor stem discrimination by *E. coli* SerRS (Saks and Sampson, 1996). In these experiments, base analogs were not employed. Nevertheless, the large number of canonical base pair substitutions that were tested together with the crystal structure data allowed major groove functional interactions to be revealed at the atomic level.

Studies using mutant minihelices established that the first five base pairs all contributed to recognition by SerRS, although the measured decreases in aminoacylation efficiency were relatively minor (≤ 80-fold) (Saks and Sampson, 1996). The "major" acceptor stem recognition nucleotides are located at 2:71 and 4:69 of minihelix^{Ser} (Fig. 3). At G2:C71, major groove recognition of the carbonyl oxygen of G2 and the amino group of C71 is predicted by the X-ray structure, and the mutagenesis data are consistent with this expectation. The fourth base pair in the acceptor stem of all five *E. coli* tRNA^{Ser} isoacceptors is not strictly conserved, but is always a purine:pyrimidine pair. Substitution with pyrimidine:purine at this site results in decreased aminoacylation efficiency. The mutagenesis and X-ray data are consistent with a functional

hydrophobic interaction between the synthetase and the C5-proton of pyrimidine 69. A similar, albeit less important, hydrophobic interaction was proposed to occur between the synthetase and the fifth base pair. The first and third base pairs were also shown to be minor recognition elements in the Ser system. Based on minihelix^{Ser} mutagenesis studies, major groove functional interactions at 1:72 were proposed to involve primarily the carbonyl oxygen of G1. Interactions at 3:70 are less clear from this analysis and effects of substitutions at this position may be indirect, as no contacts with this site are observed in the X-ray structure. It has been proposed that the relatively weak base-specific acceptor stem recognition that is observed in minihelix^{Ser} may reflect the need for negative discrimination against noncognate tRNAs, such as tRNA^{Thr}, which share many of the same acceptor stem elements (Cusack *et al.*, 1996). To fully resolve these issues, additional experiments including those with base analogs would be useful.

Concluding Remarks

Acceptor stem elements that are unique to a particular tRNA system have, in many cases, been found to be important for synthetase recognition. For instance, the G3:U70 wobble pair in tRNA^{Ala}, the unique G-1:C73 base pair in tRNA^{His}, and the G72 that is unique to tRNA^{Pro}, are all key determinants for recognition by the corresponding synthetases. However, the 'code' that specifies aminoacylation depends strongly on minor or modulating elements and context effects (Frugier *et al.*, 1998). Additionally, in many systems, the acceptor stem cannot specify aminoacylation in the absence of the anticodon-containing domain. Nevertheless, based on the *in vitro* studies reviewed here for class II systems, even subtle atomic determinants, such as a minor groove amino group at 3:70 that signals charging with Ala, can specify aminoacylation when presented in the correct context. Single atomic groups can also provide a strong block to recognition, even in the presence of major positive recognition elements. An example is the placement of a carbonyl oxygen in the major groove at position 72, which blocks aminoacylation of G3:U70-containing substrates by AlaRS. Moreover, determinants for both positive interactions with cognate synthetases and negative interactions with noncognate enzymes are very often located in overlapping acceptor stem positions. Thus, the class II aminoacyl-tRNA synthetases appear to achieve specific recognition and discrimination of similarly folded tRNAs, at least in part, by making maximal use of the information content encoded in the major and minor grooves of the tRNA acceptor stem.

Acknowledgments I am grateful to Ms. Penny Beuning for preparation of Fig. 4 and for critical reading of the manuscript. Work on class II aminoacyl-tRNA synthetases is supported by a grant from the National Institutes of Health (GM49928). The donors of The Petroleum Research Fund, administered by the American Chemical Society, are also acknowledged for partial support of this research.

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