Enzymatic Aminoacylation of tRNA Acceptor Stem Helices with Cysteine Is Dependent on a Single Nucleotide†

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ABSTRACT: The discriminator base U73 at the acceptor terminus of *Escherichia coli* $tRNA^{Cys}$ is a determinant for the specific aminoacylation of this tRNA by the cognate cysteine tRNA synthetase. Substitution of U73 has a major deleterious effect on the catalytic efficiency of aminoacylation. Here, we show that an RNA hairpin minihelix and an RNA hairpin microhelix that recreate, respectively, the 12-base pair acceptor-TΨC stem and the 7-base pair acceptor helix of *E. coli* $tRNA^{Cys}$ were aminoacylated with cysteine. As in $tRNA^{Cys}$, alteration of U73 to A73, C73, or G73 in the cysteine mini- and microhelices eliminated aminoacylation. This established that the strong influence of U73 on aminoacylation is fully retained from the full-length $tRNA^{Cys}$ to the mini- and microhelix^{Cys}. Transfer of U73 to the noncognate minihelix^{Ala} conferred cysteine acceptance to the latter, despite the presence of the major determinant for alanine tRNA synthetase. Even minihelix^{Gly}, which shares U73 with minihelix^{Cys}, was an efficient substrate for aminoacylation with cysteine. Conversely, as long as U73 was present in minihelix^{Cys}, introduction of the glycine or alanine determinant could not block charging by cysteine tRNA synthetase. Although the catalytic efficiency of aminoacylation of these small RNA helices with cysteine was reduced by orders of magnitude from that of $tRNA^{Cys}$, the single nucleotide U73 determines the ability of these RNA helices to be aminoacylated with cysteine. These results demonstrated a dominant role of U73 for aminoacylation of small RNA helices by cysteine tRNA synthetase.

Aminoacylation of tRNAs is a two-step reaction that involves activation of amino acids by ATP and esterification of the activated amino acids with the 3′-end of the tRNA acceptor stem. This reaction is catalyzed by the aminoacyl tRNA synthetases. Structural and sequence comparison have divided the 20 *Escherichia coli* enzymes into two classes of 10 each based on conserved structural motifs (Eriani *et al.*, 1990; Cusack *et al.*, 1991). Although aminoacyl tRNA synthetases establish the relationship between amino acids and the anticodon trinucleotides of tRNAs, which in turn correspond to the codon triplets in the genetic code, many of the synthetases do not recognize the anticodon trinucleotides as the identity determinant for aminoacylation (Normanly & Abelson, 1989). In fact, members of both classes have been shown to aminoacylate small RNA stem loop mini- and microhelices that are derived from, respectively, the acceptor-TΨC and acceptor domains of the cognate tRNAs (Musier-Forsyth & Schimmel, 1993; Francklyn *et al.*, 1992a). These acceptor stem RNA helices lack the anticodon nucleotides but nonetheless retain the specificity of aminoacylation. The anticodon-independent aminoacylation of small acceptor stem RNA helices thus establishes a relationship between the amino acids and the acceptor stem sequences (Schimmel, 1991; Francklyn *et al.*, 1992a). Conceptually, this relationship is distinct from the relationship between the amino acids and the anticodon trinucleotides of the classical genetic code (Martinis & Schimmel, 1995).

The acceptor stem and the anticodon trinucleotides, separated at opposite ends of the L-shaped tRNA structure (Kim *et al.*, 1974; Robertus *et al.*, 1974), contain the identity determinants for most tRNAs. The major determinants of aminoacylation for $tRNA^{Ala}$, $tRNA^{His}$, and $tRNA^{Ser}$ are located in the acceptor stem and outside the anticodon (Hou & Schimmel, 1988; Himeno *et al.*, 1989; Yan & Francklyn, 1994; Normanly *et al.*, 1992). RNA helices whose sequences are based on the acceptor stems of each of these tRNAs are aminoacylated by the cognate synthetases (Francklyn & Schimmel, 1989; 1990; Sampson & Saks, 1993). The alanine, histidine, and serine enzymes are all class II synthetases. In contrast, the anticodon itself is a major determinant for aminoacylation of $tRNA^{Met}$, $tRNA^{Val}$, and $tRNA^{Ile}$ (Schulman & Pelka, 1988; Muramatsu *et al.*, 1988), yet RNA minihelices of each of these tRNAs lacking the anticodon domain are also aminoacylated by the class I methionine, valine, and isoleucine enzymes, respectively (Martinis & Schimmel, 1992; Frugier *et al.*, 1992; Nureki *et al.*, 1993). In a third class of examples, the major determinants of aminoacylation of $tRNA^{Gly}$, $tRNA^{Gln}$, and $tRNA^{Asp}$ are located both at the anticodon and the N73 nucleotide (also known as the discriminator base) adjacent to the CCA end (Carbon & Roberts, 1974; Rould *et al.*, 1989; Ruff *et al.*, 1991; Pütz *et al.*, 1991). The class II glycine, glutamine, and aspartyl enzymes were able to aminoacylate their cognate acceptor stem helices (Francklyn *et al.*, 1992b; Wright *et al.*, 1993; Frugier *et al.*, 1994). In these examples of anticodon-independent aminoacylation of RNA helices, the specificity of aminoacylation was largely determined by N73 and by base pairs adjacent to this nucleotide.

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The importance of the discriminator base and the first three base pairs of the acceptor stem has been demonstrated for the alanine, histidine, and glycine microhelix substrates (Francklyn *et al.*, 1992b). A G3:U70 base pair that is unique to $tRNA^{Ala}$ and an extra G-1:C73 base pair unique to $tRNA^{His}$ were required, respectively, for alanine and histidine acceptance. The discriminator base U73 and C2:G71 were required for glycine acceptance. These nucleotides are located at adjacent and overlapping positions in the acceptor stem. However, when any of the three sets of identity determinants were combined in a reference RNA helix, only one of the aminoacyl tRNA synthetases would recognize the substrate. For example, a reference stem loop that contained both the alanine and histidine determinants was aminoacylated with histidine but not alanine, while a variant that contained both the glycine and alanine determinants were aminoacylated only with glycine. In essence, aminoacylation with alanine, histidine, and glycine minihelices is mutually exclusive, whereby the major determinant of each prevents aminoacylation by a different synthetase.

Because the alanine, histidine, and glycine enzymes all belong to class II, the above results raise the question of whether their major determinants can interfere with aminoacylation of a class I substrate by the cognate class I enzyme. We and others have recently shown that the class I cysteine tRNA synthetase depends on U73 and the anticodon nucleotides for aminoacylation (Pallanck *et al.*, 1992; Hou *et al.*, 1993; Komatsoulis & Abelson, 1993). In addition, we have shown that an unusual tertiary interaction at G15:G48 in $tRNA^{Cys}$ is critical for cysteine tRNA synthetase (Hou *et al.*, 1993; Hou, 1994). Here, we investigated aminoacylation of acceptor stem RNA helices with cysteine. We showed that the acceptor stem RNA helices of $tRNA^{Cys}$ were substrates for cysteine tRNA synthetase and that aminoacylation depended on the single nucleotide U73. This provided us with the opportunity to determine if U73 is mutually compatible or exclusive with the major determinant of the clas II alanine or glycine enzyme. In *E. coli*, only $tRNA^{Cys}$ and the three $tRNA^{Gly}$ isoacceptors have U73 (Sprinzl *et al.*, 1991). The sharing of U73 as the major determinant for the cysteine and glycine enzymes also allowed us to investigate cross-charging of acceptor stem substrates that contain U73.

MATERIALS AND METHODS

Materials. The DNA templates with the upstream consensus promoter sequence (5′ TAA TAC GAC TCA CTA TA 3′) for transcription by T7 RNA polymerase were synthesized on an Applied Biosystem 381A DNA synthesizer and purified by reverse-phase C18 column chromatography at the Jefferson Cancer Institute. *E. coli* cysteine tRNA synthetase (Hou *et al.*, 1991) and alanine tRNA synthetase (Hill & Schimmel, 1989) were purified from overproducer strains pYM107/JM109 and pTK875N/W3110, respectively. *E. coli* glycine tRNA synthetase was the gift of Dr. Debbie Hipps (MIT). Phage T7 RNA polymerase was purified from pAR1219/BL-21 according to published procedures (Grodberg & Dunn, 1988). L-[^{35}S]Cysteine (1075 Ci/mmol), L-[3-^{3}H]alanine (76.9 Ci/mmol), and [2-^{3}H]glycine (42 Ci/mmol) were purchased from DuPont NEN.

Synthesis of RNA Stem−Loop Helices. RNA stem−loop minihelices and RNA microhelices were synthesized by *in vitro* transcription of single-stranded synthetic templates that were annealed to a primer sequence for T7 RNA polymerase. Transcription was performed for 4 h at 37 °C in a reaction that contained 40 mM Tris-HCl, pH 8.0, 5 mM DTT (dithiothreitol), 0.05 mg/mL BSA (bovine serum albumin), 80 mg/mL PEG (polyethylene glycol 8000), 0.01% Triton X-100, 50 μM template, 5 mM GMP, 4 mM each ATP, CTP, GTP, and UTP, 1 mM spermidine, 10 mM $MgCl_2$, and 120 μg of T7 RNA polymerase. Full-length transcripts were isolated from a preparative 15% polyacrylamide (29:1, acrylamide:bis)/7 M urea gel, and purified by electroelution and ethanol precipitation. Concentrations of RNA solutions were determined by absorption at 260 nm (1 OD_{260} = 0.04 μg/μL RNA).

Table 1: Relative k_{cat}/K_M for Aminoacylation of Various Acceptor Stem RNA Helices[a]

RNA substrates	relative k_{cat}/K_M of aminoacylation		
	cysteine	alanine	glycine
transcript $tRNA^{Cys}$	2.2×10^5		
transcript A73 $tRNA^{Cys}$	0		
transcript C73 $tRNA^{Cys}$	0		
transcript G73 $tRNA^{Cys}$	0		
$minihelix^{Cys}$	1.00		
$microhelix^{Cys}$	0.84		
$minihelix^{Cys}$	1.00	0	
G3:U70 $minihelix^{Cys}$	1.38	0.058	
A73 $minihelix^{Cys}$	0	0	
$minihelix^{Ala}$	0	1.00	
U73 $minihelix^{Ala}$	0.96	0.034	
$minihelix^{Cys}$	1.00		0
C2:G71 $minihelix^{Cys}$	0.64		0
C2:G71/G3:C70 $minihelix^{Cys}$	0.56		1.53
$minihelix^{Gly}$	0.34		1.00
A73 $minihelix^{Gly}$	0		0
G2:C71 $minihelix^{Gly}$	0.58		0

[a] Relative kinetic parameters for aminoacylation of the wild type and variants of $tRNA^{Cys}$ and RNA minihelix and microhelix substrates derived from $tRNA^{Cys}$, $tRNA^{Ala}$, and $tRNA^{Gly}$. Assays were performed, and substrate concentration ranges were as described in Materials and Methods. Assay time points were selected such that initial rate plots were linear. Each plot contained 4−6 determinants. The k_{cat}/K_M values are reported as relative to the respective wild-type minihelix and have standard deviations of 7−12%.

Aminoacylation of Acceptor Stem RNA Helices with Cysteine. Aminoacylation of acceptor stem RNA helices with cysteine was assayed at 37 °C in a 24-μL reaction containing 20 mM KCl, 10 mM $MgCl_2$, 25 mM DTT, 2 mM ATP, 20 mM Tris-HCl, pH 7.5, 50 μM cysteine, 0.385 μM [^{35}S]cysteine, and 10−400 μM RNA helices or 0.4−25 μM tRNA transcripts that had been previously heated (80 °C, 3 min) and reannealed (37 °C, 20 min). The assay was initiated by adding 4 μL of purified cysteine tRNA synthetase to a final concentration of 4.2 μM for the acceptor helices or 0.8 nM for the tRNA transcripts. At appropriate time points, a 4-μL aliquot of the aminoacylation reaction was transferred to a 20-μL alkylating solution that contained 0.24 M iodoacetic acid/0.1 M sodium acetate (pH 5.0) in formamide at 37 °C for 30 min. A 12-μL aliquot of the alkylating reaction was spotted onto a Whatman 3 MM filter pad, precipitated with trichloroacetic acid (TCA), washed, and counted for radioactivity as described (Schreier & Schimmel, 1972). The concentrations of minihelices for cysteine aminoacylation ranged from 30 to 400 μM for experiments in Figure 2 and from 10 to 100 μM for experiments in Table 1. The concentration of tRNA for cysteine aminoacylation ranged from 0.3 to 25 μM.

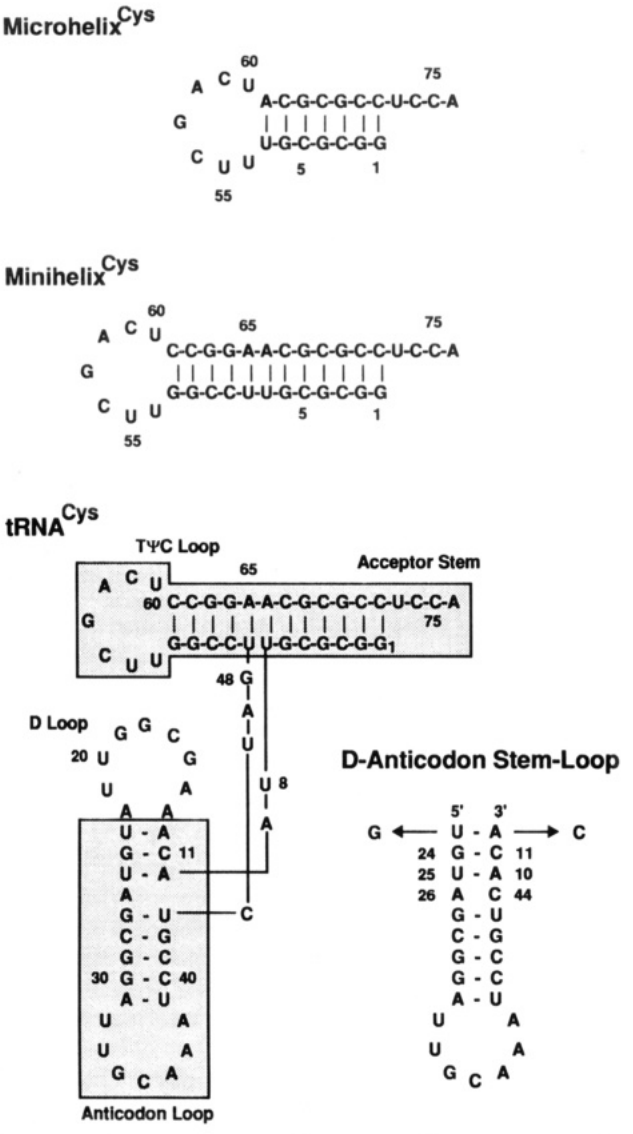

FIGURE 1: Structures of tRNA^{Cys}, minihelix^{Cys}, and microhelix^{Cys} stem loops and D-anticodon stem loop. The shaded regions of the full-length tRNA^{Cys} molecule indicate the portions of this molecule that are recapitulated in the smaller stem loop molecules. To facilitate transcription of the D-anticodon stem loop by the T7 RNA polymerase, the A12:U23 base pair was changed to C12:G23.

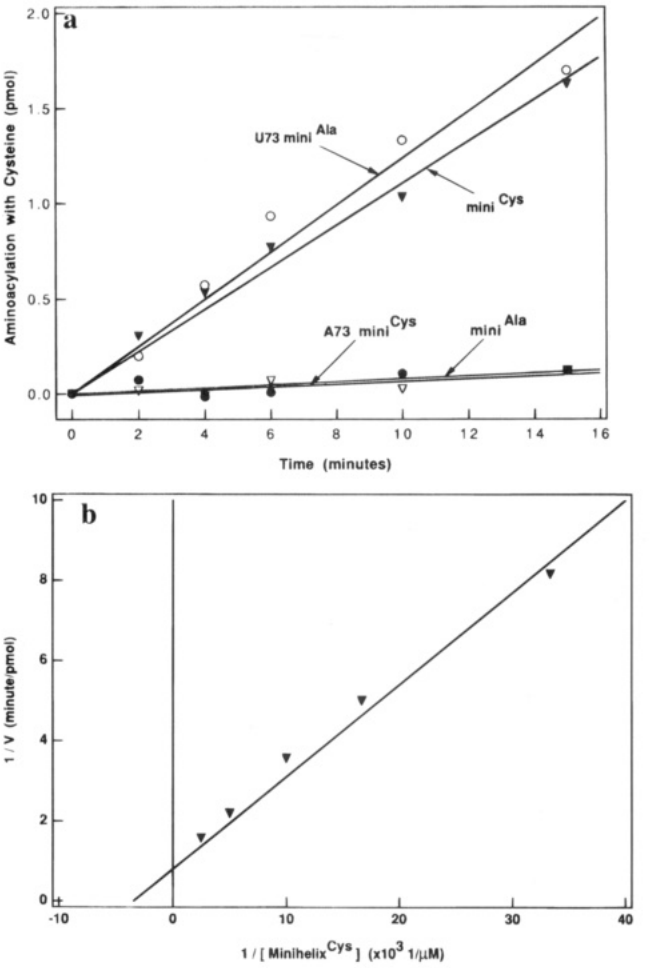

FIGURE 2: (a): Time-dependent aminoacylation with cysteine by cysteine tRNA synthetase of the wild-type minihelix^{Cys} (closed triagles), the wild-type minihelix^{Ala} (closed circles), the A73 variant of minihelix^{Cys} (open triagles, A73 mini^{Cys}), and the U73 variant of minihelix^{Ala} (open circles, U73 mini^{Ala}). Each point that is presented in the figure has been corrected from a parallel no-RNA control. The concentration of each RNA substrate was 50 μM, while the concentration of cysteine tRNA synthetase was 4.2 μM (b): Lineweaver−Burk plot for aminoacylation of minihelix^{Cys} as a function of substrate concentation. Data were collected at 30, 60, 100, 200, and 400 μM substrate. Total enzyme used in the reaction was 100 pmol. The correlation coefficient was 0.997.

Aminoacylation with Glycine and Alanine. The conditions for aminoacylation with glycine were 50 mM Tris-HCl, pH 7.5, 4 mM DTT, 0.2 mg/mL BSA, 10 mM $MgCl_2$, 2 mM ATP, 100 μM glycine (7.5 Ci/mmol), and 1 μM glycine tRNA synthetase. The assay reactions were stopped by pipetting aliquots onto filter pads and precipitating the pads with TCA as described (Schreier & Schimmel, 1972). The conditions for aminoacylation with alanine were similar to those of aminoacylation with glycine, except 21.2 μM alanine (2.83 Ci/mmol) and 0.12 μM alanine tRNA synthetase were used. The concentrations of minihelices for glycine aminoacylation ranged from 20 to 160 μM, while those for alanine aminoacylation ranged from 1 to 10 μM for the cognate substrate and from 10 to 100 μM for the noncognate substrates.

RESULTS

Minihelix^{Cys} and microhelix^{Cys} are based on the L-structure of *E. coli* tRNA^{Cys} (Figure 1). In this structure, the acceptor and TΨC stems are coaxially stacked while the dihydrouridine (D) and anticodon stems are coaxially stacked. The minihelix substrates were produced by transcription of DNA templates that encode the wild type or variants of the 12-base pair acceptor-TΨC stem loop. The microhelix substrates were prepared similarly but with DNA templates that encode only the 7-base pair acceptor stem and the seven nucleotides of the TΨC loop. Transcription was performed with 5 mM GMP over 4 mM GTP to facilitate synthesis of minihelix^{Cys} and microhelix^{Cys} that initiate with a monophosphate at the 5′-end (Francklyn & Schimmel, 1990). With purified *E. coli* cysteine tRNA synthetase at catalytic concentration (4.2 μM) relative to substrates (50 μM), aminoacylation showed that both minihelix^{Cys} (Figure 2a) and microhelix^{Cys} were charged with cysteine. While the rate of aminoacylation of mini- and microhelix^{Cys} was significantly reduced from that of the full-length tRNA^{Cys} transcript, under the same reaction conditions, minihelix^{Ala} (Figure 2a) and microhelix^{Ala} were not aminoacylated with cysteine above background levels. Conversely, mini- and microhelix^{Cys} were not substrates for alanine tRNA synthetase. This establishes that acceptor stem RNA helices of

tRNA^{Cys} are specifically recognized by cysteine tRNA synthetases. However, aminoacylation of mini- and microhelix^{Cys} was not quantitative. With low concentration of cysteine tRNA synthetase (4.2 μM), mini- and microhelix^{Cys} were charged to 0.07%. Even with substrate levels of cysteine tRNA synthetase (40 μM) after 45 min of a typical aminoacylation reaction, mini- and microhelix^{Cys} were only charged to 0.3%. This indicates that there is a synthetase-dependent, substrate-independent deacylation reaction by cysteine tRNA synthetase toward the charged mini- and microhelices (Dietrich *et al.*, 1976). Longer incubation time was impractical due to further deacylation of the aminoacyl group from the RNA substrates. Under similar reaction conditions, the total level of aminoacylation of tRNA^{Cys} transcript is 30%. Thus, the low plateau-level charging of mini- and microhelix^{Cys} and the incomplete aminoacylation of tRNA^{Cys} transcript *in vitro* reflect a slow forward rate of aminoacylation that is offset by the chemical and enzymatic deacylation reactions (Dietrich *et al.*, 1976; Hou & Schimmel, 1988).

Although the overall charging of minihelix^{Cys} is low, this RNA substrate showed characteristics of Michaelis−Menten kinetics. We define the operational kinetic parameters K_M and k_{cat} as those for the observed rate of the forward aminoacylation reaction (Dietrich *et al.*, 1976). Determination of initial rates of aminoacylation over a 13-fold range of substrate concentrations showed that the K_M for this substrate is 290 μM and the k_{cat} is 2.1×10^{-4} s^{-1} (Figure 2b). The catalytic efficiency of aminoacylation of minihelix^{Cys} is thus reduced approximately 2.5×10^5-fold relative to the full-length transcript, which has a K_M of 2.8 μM and a k_{cat} of 0.45 s^{-1}. The more than 100-fold increase in K_M for minihelix^{Cys} suggests a loss of binding free energy of 2.9 kcal mol^{-1} from the full-length tRNA [$\Delta\Delta G = -RT \ln (K_M^{Mini}/K_M^{tRNA})$, where $R = 1.987$ cal K^{-1} mol^{-1}, and $T = 310$ K]. However, the primary loss of efficiency of aminoacylation for the minihelix substrate compared to tRNA^{Cys} is in k_{cat}, which is reduced 2×10^3-fold from that of the full-length tRNA. Using initial rates of aminoacylation at substrate concentrations below K_M, we estimate that k_{cat}/K_M for microhelix^{Cys} is approximately the same as that of minihelix^{Cys} (0.84 vs 1.0, Table 1). Thus, while removing the dihydrouridine and the anticodon stem loops from the full-length tRNA reduced the catalytic efficiency of aminoacylation by 5 orders of magnitude, there is almost no effect in further removing the TΨC stem from the minihelix substrate. The large difference in k_{cat}/K_M for mini- and microhelix^{Cys} from tRNA^{Cys} demonstrates the importance of the nucleotide sequence outside of the acceptor stem for efficient aminoacylation. Because the anticodon has a role in aminoacylation, a second RNA hairpin helix that reconstructed the D-anticodon stem loop (Figure 1) of tRNA^{Cys} was added to test if this helix stimulated aminoacylation of minihelix^{Cys}. However, addition of the D-anticodon stem loop at a variety of concentrations did not enhance aminoacylation of the acceptor stem helix (data not shown). This suggests that transduction of the anticodon signal for efficient aminoacylation may require covalent continuity of the tRNA polynucleotide chain.

Table 1 shows the relative operational k_{cat}/K_M for the wild type and variants of minihelix^{Cys}. To test the role of U73, the A73 variant was constructed. In tRNA^{Cys}, substitution of U73 with A73, C73, or G73 eliminated aminoacylation by cysteine tRNA synthetase (Table 1). We show here that

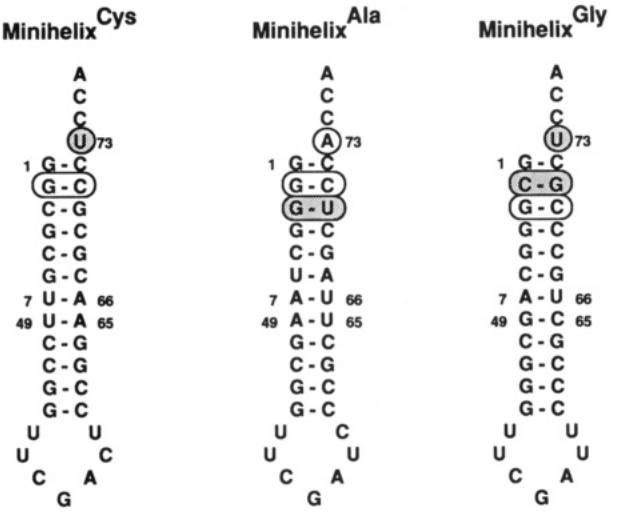

FIGURE 3: Sequence and structure of the minihelix^{Cys}, minihelix^{Ala}, and minihelix^{Gly} stem loops that can be aminoacylated. Shaded, circled nucleotides indicate major identity determinants. Unshaded, circled nucleotides indicate minor identity determinants. The effect of substitution on aminoacylation of these nucleotides is discussed in the text.

the U73 to A73 change in minihelix^{Cys} also completely abolished aminoacylation with cysteine (Figure 2a). The A73 minihelix^{Cys} variant gave the same background signal as the noncognate minihelix^{Ala} for aminoacylation with the cysteine enzyme (Figure 2a). Likewise, the G73 and C73 variants of minihelix^{Cys} and the A73, G73, and C73 variants of microhelix^{Cys} were all defective for aminoacylation (data not shown). These results collectively demonstrate that the single nucleotide U73 is a major determinant in establishing the amino acid identity of the acceptor stem helices of tRNA^{Cys} and that its role in the acceptor stem is as important as in the intact tRNA^{Cys}.

We next tested the possibility of whether U73 alone can confer cysteine acceptance in a different acceptor stem sequence framework. We constructed U73 minihelix^{Ala} in which the wild-type A73 of minihelix^{Ala} was replaced by U73. For alanine acceptance, A73 modulates the efficiency of aminoacylation with alanine, but by itself does not confer charging by alanine tRNA synthetase (Shi *et al.*, 1990). This is supported by the lack of alanine acceptance of A73 minihelix^{Cys} (Table 1). The U73 variant of minihelix^{Ala} was weakly charged by the cognate alanine tRNA synthetase (30-fold down from the wild-type minihelix^{Ala}, Table 1). However, U73 minihelix^{Ala} retains the primary and secondary determinants for aminoacylation with alanine, which are the G3:U70 and G2:C71 base pairs, respectively (Figure 3). Even in the presence of the major and minor determinants for alanine acceptance, U73 minihelix^{Ala} recruited interaction with cysteine tRNA synthetase and was efficiently aminoacylated with cysteine. The efficiency of cysteine charging was indistinguishable from that of the wild-type minihelix^{Cys} (Table 1, Figure 2). This suggests that U73 is a positive element for cysteine acceptance and that the presence of alanine determinants (G3:U70 and G2:C71) does not interfere with charging by cysteine tRNA synthetase. Also, because U73 minihelix^{Ala} is still recognized by alanine tRNA synthetase, the positive element for cysteine acceptance does not block alanine acceptance.

Conversely, introduction of the alanine determinant G3:U70 to minihelix^{Cys} created a minihelix that contained U73 for cysteine acceptance as well as the major and minor

determinants for alanine acceptance (G3:U70 and G2:C71). Aminoacylation showed that G3:U70 minihelix^{Cys} is a substrate for both the cysteine and the alanine enzymes and that its efficiencies for cysteine and alanine acceptance are nearly the same as those for U73 minihelix^{Ala}. This implies that G3:U70 minihelix^{Cys} behaves the same as U73 minihelix^{Ala} for both the cysteine and the alanine enzymes. Thus, the major determinant for cysteine identity and those for alanine identity are mutually compatible within the sequence framework of each substrate, where they act independently to confer charging by their cognate enzymes.

The observation that G3:U70 minihelix^{Cys} and U73 minihelix^{Ala} have close to wild-type efficiencies of cysteine aminoacylation suggested a dominant effect of U73 on recognition by cysteine tRNA synthetase. We then tested if this effect would be manifested in the context of a minihelix where N73 plays an equally important role. The discriminator base U73 of *E. coli* microhelix^{Gly} is a major determinant for aminoacylation with glycine. This nucleotide together with the C2:G71 base pair of tRNA^{Gly} contribute to the specificity of microhelix^{Gly} (Francklyn *et al.*, 1992b). We constructed minihelix^{Gly} and showed that aminoacylation of this substrate also depended on U73 and the C2:G71 base pair. Substitution of U73 with A73 (the A73 minihelix^{Gly} mutant) or alteration of C2:G71 to G2:C71 (the G2:C71 minihelix^{Gly} mutant) eliminated aminoacylation of minihelix^{Gly} with glycine (Table 1). The dependence on U73 for glycine aminoacylation is in common with that of minihelix^{Cys} for cysteine aminoacylation and suggests that minihelix^{Gly} would provide the framework for examining cross-charging by cysteine tRNA synthetase. Aminoacylation showed that minihelix^{Gly} is a substrate for the cysteine enzyme and that it has a catalytic efficiency only 3-fold down from that of minihelix^{Cys} (Table 1). Thus, the U73 determinant for cysteine identity in the context of minihelix^{Gly} not only conferred aminoacylation with glycine but also aminoacylation with cysteine. In contrast, minihelix^{Cys} is not a substrate for the glycine enzyme (Table 1). This result is expected because of the lack of the major glycine determinant C2:G71 in minihelix^{Cys}.

Transfer of the C2:G71 glycine determinant to minihelix^{Cys} slightly reduced but did not prevent aminoacylation with cysteine (Table 1). Interestingly, C2:G71 alone was not sufficient to confer aminoacylation with glycine. Additional substitution of C3:G70 in minihelix^{Cys} with G3:C70 from minihelix^{Gly} was necessary to confer glycine charging. While the C2:G71/G3:C70 mutant of minihelix^{Cys} did not differ much from the C2:G71 mutant in its capability of cysteine charging (k_{cat}/K_M 0.56 and 0.64, respectively, Table 1), this mutant was aminoacylated with glycine even more efficiently than the wild-type minihelix^{Gly} (k_{cat}/K_M 1.53 vs 1.00, Table 1). Thus, the C3:G70 base pair of minihelix^{Cys} is a strong negative element that interferes with glycine tRNA synthetase. Once this element is removed, introduction of the C2:G71 glycine determinant conferred glycine acceptance to minihelix^{Cys}. However, even in the presence of the glycine determinant, the C2:G71 and C2:G71/G3:C70 mutants of minihelix^{Cys} were efficiently charged with cysteine. Their catalytic efficiencies are only slightly reduced from that of the wild type. It is possible that the C2:G71 glycine determinant may exert a minor negative effect on cysteine tRNA synthetase. Indeed, elimination of C2:G71 from minihelix^{Gly} enhanced aminoacylation with cysteine (G2:C71 minihelix^{Gly}). In contrast, substitution of U73 in minihelix^{Gly} (the A73 minihelix^{Gly} mutant) completely inactivated cysteine acceptance. Thus, the glycine determinant in minihelix^{Cys} did not significantly compromise the ability of this substrate to be aminoacylated by cysteine tRNA synthetase. Even if the glycine determinant has a negative effect, this effect is extremely minor compared to the effect of U73.

DISCUSSION

Our results show that the discriminator base U73 is a major determinant for aminoacylation of acceptor stem helices with cysteine. Substitution of U73 with any other nucleotide prevents charging by cysteine tRNA synthetase. Because no other *E. coli* tRNAs have U73 (Sprinzl *et al.*, 1991), our results suggest that no other minihelices derived from *E. coli* tRNAs will be recognized by cysteine tRNA synthetase. Thus, the single nucleotide U73 is sufficient to enable cysteine tRNA synthetase to discriminate against 18 of the 20 acceptor stems. The dependence on a single nucleotide at position 73 for aminoacylation of acceptor stems with cysteine is analogous to that of aminoacylation with aspartate by the class II yeast aspartyl tRNA synthetase (Frugier *et al.*, 1994). In the latter, substitution of the discriminator base G73 has a severe effect on the catalytic efficiency of aminoacylation. Specifically, the U73, A73, and C73 variants of minihelix^{Asp} have k_{cat}/K_M values that are reduced by the same order and magnitude as are observed with the U73, A73, and C73 variants of yeast tRNA^{Asp}. This implies that the role of G73 in aminoacylation with aspartate is quantitatively retained from the acceptor stem to tRNA^{Asp}. As with minihelix^{Cys}, addition of a second hairpin helix that reconstructed the anticodon domain of tRNA^{Asp} did not enhance aminoacylation of minihelix^{Asp}. Thus, the single nucleotide-dependent aminoacylation of the acceptor stem has a parallel between the class I cysteine and the class II aspartate enzymes.

Figure 3 summarizes the major identity determinants for the cysteine, alanine, and glycine minihelices. For each of these minihelices, the identity of aminoacylation is determined by nucleotides at position 73, 2:71, or 3:70 of the acceptor stem. Although previous results showed that aminoacylation with the class II alanine and glycine enzymes is mutually exclusive (Francklyn *et al.*, 1992b), we show here that aminoacylation with the class I cysteine enzyme is compatible with either alanine or glycine. In essence, introduction of the U73 determinant for cysteine acceptance to minihelix^{Ala} or minihelix^{Gly} conferred aminoacylation with cysteine without blocking aminoacylation with alanine or glycine. Conversely, introduction of the major determinant for alanine or glycine acceptance to minihelix^{Cys} elicited aminoacylation by the alanine or glycine enzyme, respectively, while maintaining aminoacylation by the cysteine enzyme. These results suggest that the cysteine enzyme is able to overcome the major determinants of the class II alanine and glycine enzymes. In contrast, the class I methionine, valine, and glutamine enzymes all recognize the discriminator N73 for aminoacylation of their cognate minihelices (Martinis & Schimmel, 1992; Frugier *et al.*, 1992; Wright *et al.*, 1993). The lack of U73 in these minihelices in principle should prevent them from aminoacylation with cysteine. It is possible that the ability of the cysteine enzyme to accommodate the major determinant of the class II alanine or glycine synthetase, but not those of the other class I synthetases, reflects an origin of specificity

that was developed during the evolution of the two classes of aminoacyl tRNA synthetases.

Because cysteine tRNA synthetase recognizes both minihelix^{Cys} and minihelix^{Gly} as substrates, this raises the question of whether and how this enzyme discriminates tRNA^{Gly} from tRNA^{Cys}. The other synthetase that has significant cross-charging activity with acceptor stem substrates is the *E. coli* glutamine enzyme, which aminoacylates the cognate microhelix^{Gln} as well as the noncognate microhelix^{Met} and microhelix^{Ala} (Wright *et al.*, 1993). The molecular basis of this cross-charging is not clear, because microhelix^{Met} and microhelix^{Ala} do not share obvious sequence homology with microhelix^{Gln} in their acceptor stems. Further, the glutamine enzyme makes specific contact with the anticodon nucleotide U35 for aminoacylation (Jahn *et al.*, 1991; Rould *et al.*, 1991; Perona *et al.*, 1993) and, for this reason, is known to mischarge some amber suppressor tRNAs that have U35 (Normanly & Abelson, 1989). However, the cysteine enzyme has not been reported to mischarge any other tRNA substrates.

Under saturating tRNA concentration and substrate levels of cysteine tRNA synthetase over a period of 25 min, we detected no charging of tRNA^{Gly} (data not shown). The discrimination at the tRNA level, but not at the minihelix level, suggests a role of the nucleotide sequence such as the anticodon in the manifestation of the identity of tRNA^{Cys}. However, introduction of the cysteine anticodon GCA to tRNA^{Gly} (to create tRNA$^{Gly/GCA}$) only weakly conferred cysteine aminoacylation under saturating substrate and enzyme concentrations (data not shown). We estimated that the k_{cat}/K_M of charging tRNA$^{Gly/GCA}$ with cysteine is 2 orders of magnitude down from that of tRNA^{Cys}. Clearly, the anticodon sequence alone does not account for the discrimination against tRNA^{Gly}. We have previously shown that aminoacylation of tRNA^{Cys} depends not only on U73 and the GCA anticodon sequence but also on a novel G15:G48 tertiary interaction that stabilizes the L-shaped tRNA structure (Hou *et al.*, 1993). Substitution of G15:G48 has a major deleterious effect on cysteine tRNA synthetase. All other *E. coli* tRNAs have a purine 15 and a complementary pyrimidine 48 (Sprinzl *et al.*, 1991). For example, *E. coli* tRNA$^{Gly/UCC}$ has an A15:U48 tertiary interaction. Although the structural effect of G15:G58 on cysteine acceptance is not well understood, the lack of G15:G48 in tRNA^{Gly} provides a plausible basis for discrimination at the tRNA level by cysteine tRNA synthetase.

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