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Evidence for a conserved relationship between an acceptor stem and a tRNA for aminoacylation

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ABSTRACT

The anticodon-independent aminoacylation of RNA hairpin helices that reconstruct tRNA acceptor stems has been demonstrated for at least 10 aminoacyl-tRNA synthetases. For *Escherichia coli* cysteine tRNA synthetase, the specificity of aminoacylation of the acceptor stem is determined by the U73 nucleotide adjacent to the amino acid attachment site. Because U73 is present in all known cysteine tRNAs, we investigated the ability of the *E. coli* cysteine enzyme to aminoacylate a heterologous acceptor stem. We show here that a minihelix^{Cys} based on the acceptor-TΨC stem of yeast tRNA^{Cys} is a substrate for the *E. coli* enzyme, and that aminoacylation of this minihelix is dependent on U73. Additionally, we identify two base pairs in the acceptor stem that quantitatively convert the *E. coli* acceptor stem to the yeast acceptor stem. The influence of U73 and these two base pairs is completely retained in the full-length tRNA. This suggests a conserved relationship between the acceptor stem alone and the acceptor stem in the context of a tRNA for aminoacylation with cysteine. However, the primary determinant in the species-specific aminoacylation of the *E. coli* and yeast cysteine tRNAs is a tertiary base pair at position 15:48 outside of the acceptor stem. Although *E. coli* tRNA^{Cys} has an unusual G15:G48 tertiary base pair, yeast tRNA^{Cys} has a more common G15:C48 that prevents efficient aminoacylation of yeast tRNA^{Cys} by the *E. coli* enzyme. Our results support the notion that the conserved determinants in the acceptor stem may have provided a primordial code for cysteine, and that this code evolved in the development of tRNA sequences to include an additional element, such as the 15:48 tertiary base pair.

Keywords: cysteine tRNA synthetase; minihelix; operational RNA code

INTRODUCTION

The rules of the genetic code are determined by the specific aminoacylation of tRNAs by aminoacyl tRNA synthetases. The 20 synthetases are divided into two classes of 10 each based on conserved sequence motifs (Eriani et al., 1990). To a first approximation, synthetases in either class consist of two domains: the conserved class-defining domain for interaction with the acceptor stem of the cognate tRNAs, and a nonconserved domain for interaction with nucleotides outside of the acceptor stem, including the anticodon (Rould et al., 1989, 1991; Krishnaswamy et al., 1991; Biou et al., 1994). Although the majority of synthetases interact with their anticodon and thus provide a logic for relating the anticodon of the genetic code with the amino acid attached to the acceptor end, alanine- and serine-tRNA synthetases do not interact with their respective tRNA anticodons (Giegé et al., 1993, and references therein). These exceptions obscure the determinative role of the anticodon in establishing the genetic code. Recent demonstrations of aminoacylation based on the acceptor-TΨC stems of tRNAs (minihelices) have suggested an operational RNA code for amino acids (Schimmel et al., 1993). In this system, the molecular interaction between the conserved domain of a synthetase and the tRNA acceptor stem alone provides the necessary discrimination in the absence of the anticodon, such that the specificity and efficiency of aminoacylation is entirely determined by nucleotides in the acceptor stem that are in contact with the synthetase. These nucleotides have been found exclusively located within the first three base pairs of the acceptor stem and at the NCCA-OH terminal sequence (Francklyn et al., 1992a; Hamann & Hou, 1995).

The relationship between the operational RNA code and the genetic code has not been explored extensively. The conservation of the genetic code in evolu-

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tion raises the question of whether the operational RNA code is conserved. Previous studies showed that the G3:U70 base pair that is the operational RNA code for alanine is conserved in alanine tRNAs from bacteria to mammals (Francklyn & Schimmel, 1988; Hou & Schimmel, 1989; Steinberg et al., 1993). Because the anticodon sequence for alanine tRNAs is not required for aminoacylation (Hou & Schimmel, 1988; McClain & Foss, 1988), the functional conservation of G3:U70 in alanine tRNAs implies a functional conservation of this base pair in the acceptor stems. The operational RNA code for *Escherichia coli* $tRNA^{Gly}$ consists of U73 and the C2:G71 base pair (Francklyn et al., 1992b; Hamann & Hou, 1995). Although C2:G71 is functionally conserved in evolution, all eucaryotic glycine tRNAs have A73 (Steinberg et al., 1993). The *E. coli* and human enzymes do not cross-charge their respective acceptor stems. However, an interchange of U73 and A73 enables the human enzyme to charge the bacterial substrate, but not the mammalian substrate. Conversely, this interchange confers aminoacylation of the mammalian substrates by the *E. coli* enzyme (Hipps et al., 1995). Thus, despite the differences of nucleotides at position 73, the positional locations of specificity elements in the acceptor stems for aminoacylation with glycine appear conserved from bacteria to human.

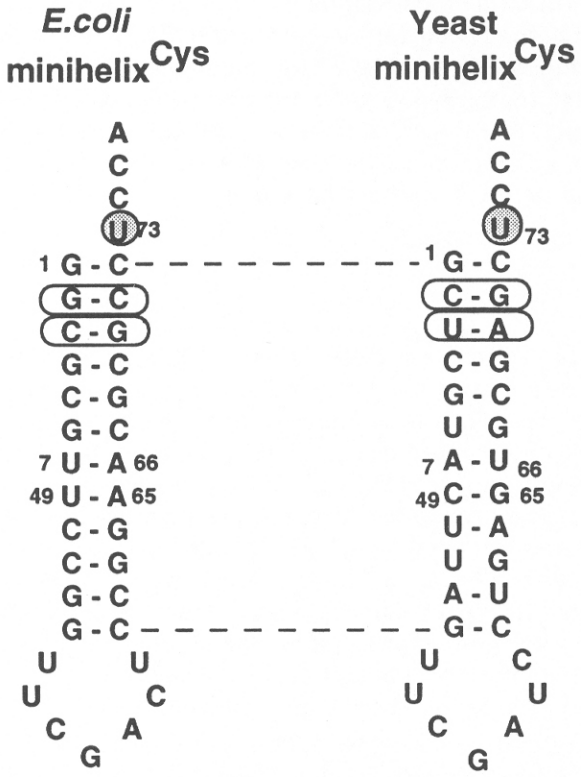

FIGURE 1. *E. coli* and yeast RNA minihelices based on the sequences of acceptor-TΨC stem-loops of respective cysteine tRNAs. Numbers refer to nucleotides in the conventional system for a 76-nt tRNA molecule (Steinberg et al., 1993). Dashed lines connect positions where the two sequences share identical nucleotides. The U73 major determinant for aminoacylation of both minihelices with cysteine is shaded and circled, whereas the nucleotides at the second and third base pairs of the acceptor stems that allow discrimination of Yminihelix^{Cys} from Eminihelix^{Cys} by the *E. coli* cysteine enzyme are circled.

In *E. coli*, the only other acceptor stem that contains U73 is minihelix^{Cys}. We recently showed that U73 alone is sufficient to establish the operational RNA code for cysteine. Substitution of U73 eliminates aminoacylation, whereas transfer of U73 to minihelix^{Ala} confers cysteine acceptance. Even minihelix^{Gly} is a relatively efficient substrate for the cysteine enzyme (Hamann & Hou, 1995). This suggests a dominant role of U73 in aminoacylation with cysteine such that it overrides the major determinants for alanine and glycine. Inspection of available tRNA sequences shows that U73 is present in all cysteine tRNAs (Steinberg et al., 1993). The conservation of U73 raises the question of whether it is functionally conserved as an operational RNA code for cysteine. Because the anticodon of *E. coli* $tRNA^{Cys}$ is important for aminoacylation (Pallanck et al., 1992; Hou et al., 1993; Komatsoulis & Abelson, 1993), a relevant question is how the U73-based operational RNA code might be converted to the genetic code, whereby the synthetase is allowed to interact with the anticodon sequence and the rest of the tRNA. To address these questions, we focused on the *E. coli* cysteine enzyme and investigated its ability to aminoacylate *E. coli* and yeast $tRNA^{Cys}$ and their respective acceptor stems. We also tested the ability of the yeast enzyme to aminoacylate *E. coli* $tRNA^{Cys}$ to gain insight into the structural and functional variations that may exist from the *E. coli* enzyme.

RESULTS

We synthesized *E. coli* minihelix^{Cys} (Eminihelix^{Cys}) and yeast minihelix^{Cys} (Yminihelix^{Cys}) based on the available sequences of the acceptor-TΨC stem and loop of the respective tRNAs (Fig. 1). The two hairpins differ at 10 of the 12 base pairs in the stem and two nucleotides in the loop, but share the U73 discriminator base in common. Despite the large sequence differences between the two minihelices, we show in Table 1 that Yminihelix^{Cys} is a substrate for *E. coli* cysteine tRNA synthetase, and that aminoacylation is dependent on U73. As with Eminihelix^{Cys}, substitution of U73 with A73 in Yminihelix^{Cys} reduces aminoacylation to background levels. This confirms the functional significance of U73 for aminoacylation in the context of the yeast sequence. The operational kinetic parameter k_{cat}/K_m for aminoacylation of Eminihelix^{Cys} and Yminihelix^{Cys} by the *E. coli* enzyme are 0.77 and 0.34 $M^{-1}s^{-1}$, respectively. These parameters were obtained from a series of velocities that were measured at substrate concentrations below K_m. We previously established that Eminihelix^{Cys} under the assay conditions has an apparent K_m of 290 μM and k_{cat} of 2.1×10^{-4} s^{-1} (Hamann & Hou, 1995). The high K_m allows for approximation

TABLE 1. Aminoacylation of the wild-type and variants of Eminihelix^{Cys} and Yminihelix^{Cys} by *E. coli* cysteine tRNA synthetase.

RNA	k_{cat}/K_m (M^{-1} s^{-1})[a]	Loss of specificity (x-fold)
Eminihelix^{Cys}	0.77[b]	1.0
A73 Eminihelix^{Cys}	<0.0045	>170
C2:G71/U3:A70 Eminihelix^{Cys}	0.38	2.0
Yminihelix^{Cys}	0.34	2.3
A73 Yminihelix^{Cys}	<0.0023	>330
G2:C71/C3:G70 Yminihelix^{Cys}	0.74	1.0

[a] Catalytic efficiency of aminoacylation (k_{cat}/K_m) is measured by taking the slope of [velocity] versus [substrate] at concentrations of each minihelix substrate below K_m.

[b] The value of k_{cat}/K_m for Eminihelix^{Cys} is taken from Hamann and Hou (1995). The k_{cat}/K_m values for the variants of Eminihelix^{Cys} and those for Yminihelix^{Cys} and variants are the average of three determinations and have standard deviations of 14–18%.

of k_{cat}/K_m based on the slope of the initial rates versus substrate concentrations in the range of 10–100 μM. Table 1 shows that the k_{cat}/K_m of Yminihelix^{Cys} is twofold below that of Eminihelix^{Cys} for the *E. coli* enzyme. The relatively efficient aminoacylation of the yeast substrate compared to the *E. coli* substrates suggests that there are no apparent "negative" elements in the yeast sequence that prevent recognition of U73 by the *E. coli* enzyme.

Because aminoacylation of both Eminihelix^{Cys} and Yminihelix^{Cys} by the *E. coli* enzyme depends on U73, we investigated how this enzyme distinguishes Yminihelix^{Cys} from Eminihelix^{Cys}. We sought to elucidate the nucleotides in Yminihelix^{Cys} that are responsible for the twofold effect on k_{cat}/K_m. Among the first three base pairs of the acceptor stem, Yminihelix^{Cys} shares G1:C72 with Eminihelix^{Cys}, but has C2:G71 and U3:A70 rather than G2:C71 and C3:G70 (Fig. 1). A simple exchange of the second and third base pairs in Yminihelix^{Cys} with those in Eminihelix^{Cys} improved the k_{cat}/K_m of Yminihelix^{Cys} to the level of Eminihelix^{Cys}. Similarly, simultaneous substitution of these two base pairs in Eminihelix^{Cys} with those in Yminihelix^{Cys} reduced k_{cat}/K_m of Eminihelix^{Cys} by twofold to the level of Yminihelix^{Cys}. The reciprocal conversion of k_{cat}/K_m between Yminihelix^{Cys} and Eminihelix^{Cys} achieved by changing the second and third base pairs in the stem suggests that the origins of the species-specific aminoacylation of the *E. coli* enzyme reside at just two base pairs.

We have not tested the effect of individual substitutions at the second or the third base pair. We previously showed that the second and third base pairs of Eminihelix^{Cys} differ from those in Eminihelix^{Gly} and that these differences prevent the *E. coli* glycine enzyme from aminoacylating Eminihelix^{Cys}. The single exchange at the second base pair between the two minihelices does not confer aminoacylation of Eminihelix^{Cys} with glycine. However, substitutions of both base pairs in Eminihelix^{Cys} with those in Eminihelix^{Gly} convert the former into a substrate that has the same k_{cat}/K_m as Eminihelix^{Gly} for the glycine enzyme (Hamann & Hou, 1995). It is likely that the *E. coli* cysteine enzyme behaves in the same way as the glycine enzyme such that a single base pair exchange in the stem will not alter the species specificity.

We tested if the species specificity of aminoacylation established by the second and third base pairs in the acceptor stem is quantitatively conserved in the full-length tRNA for the *E. coli* enzyme. We show in Table 2 that introduction of C2:G71 and U3:A70 of yeast tRNA^{Cys} to replace G2:C71 and C3:G70 of *E. coli* tRNA^{Cys} decreased k_{cat}/K_m of aminoacylation by the *E. coli* enzyme by twofold. This is the same effect as that observed in Eminihelix^{Cys}. Conversely, alteration of C2:G71 and U3:A70 in yeast tRNA^{Cys} to G2:C71 and C3:G70 increased k_{cat}/K_m by near twofold. Thus,

TABLE 2. Aminoacylation of the wild-type and variants of *E. coli* tRNA^{Cys} and yeast tRNA^{Cys} transcripts by *E. coli* cysteine tRNA synthetase.

tRNA transcripts	K_m[a] (μM)	k_{cat}[a] (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	Loss of specificity (x-fold)
E. coli tRNA^{Cys}	2.81[b]	0.458[b]	1.63×10^5	1.00
E. coli A73 tRNA^{Cys}	n.d.[c]	n.d.	<4.67	>34,900
E. coli tRNA^{Cys} C2:G71/U3:A70	18.1	1.40	7.73×10^4	2.11
Yeast tRNA^{Cys}	4.76	9.74×10^{-3}	2.05×10^3	79.5
Yeast A73 tRNA^{Cys}	n.d.	n.d.	<0.826	>197,000
Yeast tRNA^{Cys} G2:C71/C3:G70	13.1	4.50×10^{-2}	3.42×10^3	47.7
E. coli tRNA$^{Cys/CUA}$	n.d.	n.d.	33.2[d]	4,910
E. coli G15:C48 tRNA^{Cys}	13.3	4.00×10^{-2}	3.34×10^3	48.8

[a] Kinetic parameters K_m and k_{cat} were measured by Lineweaver–Burk analysis. Each measurement was an average of three determinations and has standard deviation of 4–8%.

[b] The K_m and k_{cat} determinations for *E. coli* tRNA^{Cys} are from Hamann and Hou (1995).

[c] n.d., not determined.

[d] The kinetic parameter k_{cat}/K_m for this tRNA substrate was an approximation under sub-K_m concentration of substrate.

the discrimination of Yminihelix^{Cys} from Eminihelix^{Cys} by the *E. coli* enzyme based on the second and third base pairs is completely replicated in the acceptor stems of the respective full-length tRNAs. Additionally, as with Eminihelix^{Cys}, Yminihelix^{Cys}, and *E. coli* tRNA^{Cys}, substitution of the discriminator base U73 with A73 in yeast tRNA^{Cys} reduces aminoacylation by the *E. coli* enzyme to background levels. This suggests that U73 is the major determinant for aminoacylation with cysteine of yeast tRNA^{Cys} for the *E. coli* enzyme. The significance of U73, and the quantitatively retained influence of the second and third base pairs of the acceptor stem, therefore establish a conserved relationship between minihelix^{Cys} and the acceptor stem of tRNA^{Cys} in the species specificity of aminoacylation. The implication is that, whether the acceptor stem is a separate entity by itself or incorporated into the full-length tRNA, the structural basis for the interaction between the acceptor stem and the *E. coli* enzyme is the same.

Although the *E. coli* enzyme discriminates Yminihelix^{Cys} from Eminihelix^{Cys} with a twofold difference in k_{cat}/K_m, it discriminates yeast tRNA^{Cys} from *E. coli* tRNA^{Cys} by almost two orders of magnitude. The much larger effect of k_{cat}/K_m suggests that elements outside of the acceptor stem play a strong role in discrimination at the tRNA level. We show in Table 2 that, besides U73, the anticodon GCA and a G15:G48 tertiary base pair unique to *E. coli* tRNA^{Cys} are important for aminoacylation of *E. coli* tRNA^{Cys} by the *E. coli* enzyme. Substitution of the GCA anticodon with the amber-reading CUA reduces k_{cat}/K_m of aminoacylation by 5,000-fold, and substitution of G15:G48 with G15:C48 reduces k_{cat}/K_m by 50-fold. Although the overall effect of the G15:G48 tertiary base pair is not the strongest compared to the effect of U73 and the GCA anticodon sequence, it is absent from yeast tRNA^{Cys}. Figure 2 shows that yeast tRNA^{Cys} shares with *E. coli* tRNA^{Cys} the U73 nucleotide and the GCA anticodon sequence, but contains a G15:C48 tertiary base pair. Because the tertiary base pair at position 15:48 connects the dihydrouridine loop with the variable loop to stabilize the overall L-shaped tRNA structure, substitution of this base pair may have an effect on aminoacylation. We previously showed that the G15:G48 tertiary base pair of *E. coli* tRNA^{Cys} is structurally distinct from the G15:C48 tertiary base pair (Hou et al., 1993; Hou, 1994). It is worth noting that aminoacylation of yeast tRNA^{Cys} by the *E. coli* enzyme has a relative k_{cat}/K_m within twofold difference of that of the G15:C48 variant of *E. coli* tRNA^{Cys}. This twofold difference is eliminated as the acceptor stem of yeast tRNA^{Cys} is changed to G2:C71 and C3:G70. Thus, the kinetic defect of the G2:C71/C3:G70 variant of yeast tRNA^{Cys} quantitatively resembles that of the G15:C48 variant of *E. coli* tRNA^{Cys}. This suggests that the lack of the G15:G48 tertiary base pair (or the presence of G15:C48) in yeast tRNA^{Cys} may account for the discrimination of this heterologous tRNA substrate by the *E. coli* enzyme.

The ability of the *E. coli* enzyme to discriminate tRNAs based on the tertiary base pair at 15:48 probably reflects the unusual structure of G15:G48. The *E. coli* alanine enzyme and the yeast phenylalanine enzyme, which have G15:C48 in their cognate tRNAs, do not effectively discriminate at 15:48 (Hou et al., 1995; Sampson et al., 1990). This raises the question of whether the yeast cysteine enzyme discriminates *E. coli* tRNA^{Cys} as a result of their different tertiary base pairs at 15:48. Because the gene for the yeast enzyme has not been cloned and the enzyme has not been highly purified, we used a yeast cell extract that was enriched

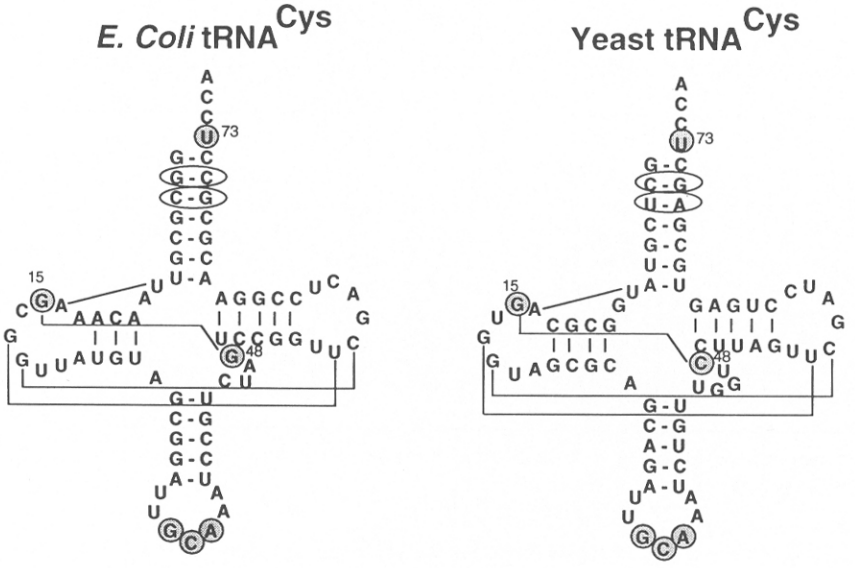

FIGURE 2. The sequence and cloverleaf structures of *E. coli* and yeast cysteine tRNAs. Both have the anticodon sequence GCA. The major determinants for aminoacylation of *E. coli* tRNA^{Cys} by *E. coli* cysteine tRNA synthetase are located at U73, the GCA anticodon, and the G15:G48 tertiary base pair. The corresponding positions in yeast tRNA^{Cys} are shaded for comparison. The tertiary interactions at p8:14, p15:48, p18:55, and p19:56 are indicated by solid lines.

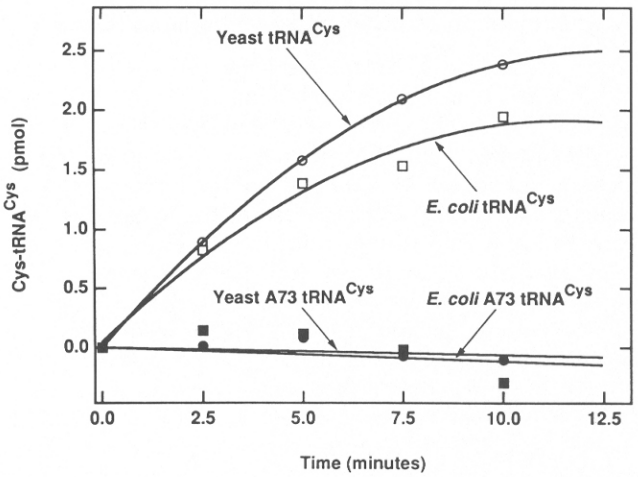

FIGURE 3. Aminoacylation of the wild-type and A73 variants of yeast and *E. coli* tRNA^{Cys} by the crude extract that contained yeast cysteine tRNA synthetase. The ordinates represent the total amount of aminoacylation from 2 μM of each substrate, and are the averages of three determinations with standard deviations of 2–3%. The curvature of the time course indicates the presence of inhibitory contaminants in the extract. The plateau charging of the *E. coli* and yeast tRNA^{Cys} substrates is estimated as 7%.

for cysteine tRNA synthetase as a source of the yeast enzyme. Although this extract contained other aminoacyl tRNA synthetases, no aminoacylation with cysteine was detected without a tRNA substrate or with a noncognate tRNA such as *E. coli* tRNA^{Ala}. This indicates the specificity for cysteine under the assay conditions that contained ^{35}S-cysteine. With this extract, we showed that the yeast enzyme aminoacylates *E. coli* tRNA^{Cys}, and that aminoacylation of both the homologous and heterologous substrates is dependent on U73. Replacement of U73 with A73 decreases aminoacylation to background levels. However, unlike the *E. coli* enzyme, the yeast enzyme does not discriminate *E. coli* tRNA^{Cys}. Although the enzymatic aminoacylation in the crude extract decreased with time (thus indicating inhibitory activities in the extract), the initial rate of aminoacylation of *E. coli* tRNA^{Cys} by the yeast enzyme is within 80% of that of yeast tRNA^{Cys} at 2 μM substrate concentration (Fig. 3). This is a small difference compared to the difference of two orders of magnitude between the two substrates for the *E. coli* enzyme. Thus, although the yeast enzyme shares with the *E. coli* enzyme the ability to use U73 as the major determinant for aminoacylation, it is capable of accommodating the G15:G48 tertiary base pair that is unique to *E. coli* tRNA^{Cys}.

DISCUSSION

Cysteine tRNA synthetase is a member of the class I synthetases that have a conserved Rossmann fold of alternating β-strands and α-helices in the N-terminal domain for interaction with the acceptor stem of their cognate tRNAs (Rossmann et al., 1967; Eriani et al., 1990; Hou et al., 1991). We show here that the acceptor stems of *E. coli* and yeast tRNA^{Cys} are aminoacylated by the *E. coli* cysteine enzyme, and that aminoacylation is solely dependent on U73. Although the catalytic efficiencies of aminoacylation of the acceptor stems are reduced from those of the respective tRNAs, the dependence on U73 for aminoacylation is completely recapitulated in the acceptor stems. This suggests that the operational RNA code for cysteine consists of U73, and that this code has been preserved for the *E. coli* enzyme with both the bacterial and yeast substrates. Further, we show that the *E. coli* enzyme discriminates between the *E. coli* and yeast sequences by nucleotides at the second and third base pairs of the acceptor stem. The kinetic basis for the interaction and discrimination at the acceptor stems is quantitatively conserved whether the acceptor stem is separated from or contained within the framework of the full-length tRNA. This suggests that the specific interaction between the N-terminal domain of the cysteine enzyme and the acceptor stem of tRNA^{Cys} is independent of the anticodon-binding domain. Although the GCA anticodon of *E. coli* tRNA^{Cys} is important for the overall aminoacylation of this tRNA, the interaction between GCA and the C-terminal domain of the cysteine enzyme does not contribute directly to the recognition of the acceptor stem.

That the acceptor stem-binding domain of synthetases behaves as if it is operationally independent of the anticodon-binding domain has been observed with the class I methionine enzyme and the class II alanine enzyme. The methionine enzyme is closely related to the cysteine enzyme in structure, and interacts with both the acceptor stem and the anticodon of tRNA^{Met} for aminoacylation (Schulman & Pelka, 1988; Hou et al., 1991; Meinnel et al., 1991; Martinis & Schimmel, 1992). In the methionine enzyme, a looped-out peptide between two helices of the C-terminal domain interacts with the anticodon (Ghosh et al., 1990; Kim et al., 1993). Although deletion of this peptide reduces the rate of aminoacylation, this deletion has no effect on the catalytic rate of aminoacylation of a microhelix based on the acceptor stem (Kim & Schimmel, 1992). The alanine enzyme shares with other class II synthetases a conserved motif of a seven-stranded antiparallel β-sheet (Eriani et al., 1990). The nonconserved region of the alanine enzyme at the C-terminus can be truncated without perturbing the ability of the conserved domain to interact with the acceptor stem of tRNA^{Ala} (Buechter & Schimmel, 1993). The results for the methionine, alanine, and cysteine enzymes together support the notion that the conserved domains may have been derived from primordial synthetases that aminoacylated acceptor stems, whereas the nonconserved domains may have been added to the conserved structures for interaction with the distal parts

of the tRNA molecule that include the D-anticodon biloop (Schimmel et al., 1993).

Because of the possible significance of the acceptor stem for the development of the full-length tRNA, we investigated if the operational RNA code can be expanded to establish the genetic code and how this expansion is manifested in species specificity. We show here that U73 is the major determinant for aminoacylation of *E. coli* and yeast $tRNA^{Cys}$ by the respective homologous and heterologous cysteine tRNA synthetases. However, the species specificity of aminoacylation of *E. coli* $tRNA^{Cys}$ against yeast $tRNA^{Cys}$ by the *E. coli* enzyme is provided by the G15:G48 tertiary base pair. This tertiary base pair can achieve its effect by directly binding to the synthetase or by indirectly altering the structure of *E. coli* $tRNA^{Cys}$ so that it is distinct from that of yeast $tRNA^{Cys}$. Although the molecular basis of discrimination based on G15:G48 is unknown, the kinetic defect of yeast $tRNA^{Cys}$ is reminiscent of that of the G15:C48 variant of *E. coli* $tRNA^{Cys}$. This reinforces the notion that the G15:G48 tertiary base pair provides the primary determinant for species specificity of the *E. coli* enzyme. Interestingly, although the yeast enzyme retains the ability of the *E. coli* enzyme to recognize U73 as the major determinant, it does not effectively discriminate against *E. coli* $tRNA^{Cys}$. This lack of discrimination suggests an origin of diversity based on a tRNA tertiary feature that is necessary to extend the operational RNA code to the genetic code for aminoacylation with cysteine.

MATERIALS AND METHODS

Preparation of tRNA and minihelix substrates

The tRNA substrates were prepared by the run-off transcription using T7 RNA polymerase and DNA templates that encode the tRNA genes. The DNA templates were constructed in plasmid pTFMa and variants of the tRNA genes were obtained by site-directed mutagenesis (Hou et al., 1993). The minihelix substrates were prepared using T7 RNA polymerase and single-stranded DNA templates that encode the acceptor-TΨC stem of tRNAs. These synthetic templates were annealed to an oligonucleotide complementary to the T7 RNA polymerase promoter sequence (Hamann & Hou, 1995). Transcription of the tRNA and minihelix substrates was performed using the previously described conditions (Hamann & Hou, 1995). Full-length products were isolated from a preparative 12% polyacrylamide/7 M urea denaturing gel, and purified by electroelution and ethanol precipitation. RNA concentrations were estimated by absorption at 260 nm, where 1 OD unit is equivalent to 0.04 μg/μL. The wild-type *E. coli* $tRNA^{Cys}$ transcript is aminoacylated to 30% plateau charging, whereas $Eminihelix^{Cys}$ is charged to 0.07% by the *E. coli* cysteine tRNA synthetase. The incomplete plateau charging of tRNAs and minihelices is attributed to an enzymatic deacylation of the cysteine enzyme that offsets the forward aminoacylation reaction (Hamann & Hou, 1995).

Aminoacylation of tRNA and minihelix substrates

Aminoacylation of tRNAs and minihelices was assayed at 37 °C in 24 μL of a buffer 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 (New England Nuclear, greater than 600 Ci/mmol), and the purified tRNA or minihelix substrates. Prior to assays, the RNA substrates were heated at 70 °C, 2 min in 10 mM Tris-HCl, pH 8.0, and reannealed at 37 °C for 30 min. Aminoacylation was initiated by adding 4 μL of an appropriately diluted purified *E. coli* cysteine tRNA synthetase (e.g., 4 nM for the wild-type *E. coli* $tRNA^{Cys}$ transcript, and 25 μM for the $Eminihelix^{Cys}$ substrate). At various time points, aliquots of 4 μL of the assay reaction were removed to a 20-μL alkylation solution of 0.24 M iodoacetic acid, 0.1 M sodium acetate, pH 5.0, in formamide for 30 min at 37 °C. A portion of the alkylation reaction (12 μL) was spotted onto a Whatman 3-mm filter pad, precipitated with trichloroacetic acid, and washed as previously described (Hou et al., 1993; Hamann & Hou, 1995). The concentrations of tRNAs for k_{cat} and K_m determinations by Lineweaver–Burk analysis ranged from 0.5 to 16 μM, whereas those for k_{cat}/K_m approximation ranged from 0.3 to 2.0 μM. The concentrations of minihelices for k_{cat}/K_m approximation ranged from 10 to 100 μM.

Enrichment of cysteine tRNA synthetase from yeast cell extract

Yeast protease deficient strain BJ3501 was grown to 1 OD_{600} unit in YPD medium. The cells were harvested and washed with a buffer containing protease inhibitors (20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 1 mM DTT, 0.3 M $(NH_4)_2SO_4$, 1 μg/mL pepstatin A, 0.5 μg/mL leupeptin, 0.1 mM benzamidine, 1 mM each PMSF, NaF, and sodium metabisulfite). The washed cells were resuspended in 40 mL of the same buffer and were vortexed with 40 mL of glass beads at 4 °C for 20 min and sonicated for 5 min. The cell extract was separated from unbroken cells and glass beads by centrifugation, dialyzed in column buffer (10 mM potassium phosphate, pH 7.3, 20 mM β-mercaptoethanol, 1 mM EDTA, 4 mM $MgCl_2$, and 15% glycerol), and applied to 10 mL DEAE-sepharose CL-6B. After the column was washed with four volumes of column buffer, a linear gradient of NaCl from 0 to 0.5 M in column buffer was applied to elute cysteine tRNA synthetase. The fractions that contained cysteine tRNA synthetase were eluted between 0.25 and 0.35 M NaCl. These fractions were pooled, concentrated by dialysis in column buffer containing 50% glycerol overnight at 4 °C, and stored at −20 °C.

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