RNA Determinants for Translational Editing

MISCHARGING A MINIHELIX SUBSTRATE BY A tRNA SYNTHETASE*

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The fidelity of protein synthesis requires efficient discrimination of amino acid substrates by aminoacyl-tRNA synthetases. Accurate discrimination of the structurally similar amino acids, valine and isoleucine, by isoleucyl-tRNA synthetase (IleRS) results, in part, from a hydrolytic editing reaction, which prevents misacylated valine from being stably joined to tRNA^Ile. The editing reaction is dependent on the presence of tRNA^Ile, which contains discrete D-loop nucleotides that are necessary to promote editing of misacylated valine. RNA minihelices comprised of just the acceptor-TΨC helix of tRNA^Ile are substrates for specific aminoacylation by IleRS. These substrates lack the aforementioned D-loop nucleotides. Because minihelices contain determinants for aminoacylation, we thought that they might also play a role in editing that has not previously been recognized. Here we show that, in contrast to tRNA^Ile, minihelix^Ile is unable to trigger the hydrolysis of misacylated valine and, in fact, is mischarged with valine. In addition, mutations in minihelix^Ile that enhance or suppress charging with isoleucine do the same with valine. Thus, minihelix^Ile contains signals for charging by IleRS that are independent of the amino acid and, by itself, minihelix^Ile provides no determinants for editing. An RNA hairpin that mimics the D-stem/loop of tRNA^Ile is also unable to induce the hydrolysis of misacylated valine, both by itself and in combination with minihelix^Ile. Thus, the native tertiary fold of tRNA^Ile is required to promote efficient editing. Considering that the minihelix is thought to be the more ancestral part of the tRNA structure, these results are consistent with the idea that, during the development of the genetic code, RNA determinants for editing were added after the establishment of an aminoacylation system.

Aminoacyl-tRNA synthetases establish the genetic code by attaching amino acids to their cognate tRNAs (1–3). These reactions are comprised of two steps. Initially the amino acid is condensed with ATP to give an activated aminoacyl adenylate. Subsequently, the aminoacyl moiety of this reactive intermediate is transestfered to the 3’ terminus of the tRNA. The fidelity of the genetic code depends upon the precise molecular recognition of both the amino acid and tRNA substrates by aminoacyl-tRNA synthetases. It has long been recognized that the accurate transduction of molecular information is made increasingly difficult when the molecular structures of two candidate substrates are highly similar (4–7). A prominent example of the need to discriminate between closely related substrates is in the recognition of isoleucine over valine by IleRS. Valine, which differs from isoleucine by a single methylene unit, is activated by Escherichia coli IleRS at a rate approximately 180-fold slower than that of isoleucine (8). IleRS prevents this relatively high error rate from being realized in protein synthesis through two editing reactions that result in the net hydrolysis of misacylated valine (7, 9, 10). These reactions provide a second “sieve” (11) of discrimination against valine and indeed occur at a second, “editing” active site on IleRS (8, 12).

Editing of misacylated valine has a strict requirement for tRNA^Ile (9, 13). In the absence of tRNA^Ile, enzymatically generated Ile-AMP and Val-AMP remain sequestered in the active site. Upon addition of tRNA^Ile to the IleRS-Ile-AMP complex, the aminoacyl group is stably attached to tRNA^Ile. In contrast, addition of tRNA^Val to the IleRS-Val-AMP complex results in immediate hydrolysis of the valyl adenylate. This occurs partly through a pretransfer editing reaction where misacylated Val-AMP is directly hydrolyzed. Alternatively, the valyl moiety of Val-AMP is transferred to tRNA^Ile to make a transient Val-tRNA^Ile intermediate that is rapidly hydrolyzed. The net result of either pathway is an abortive cycle of valine activation followed by tRNA^Ile-dependent hydrolysis that continues until all available ATP is consumed.

In contrast to nucleotide determinants for charging that are located in the anticodon loop and acceptor stem of tRNA^Ile (14), nucleotides in the D-loop of tRNA^Ile trigger the editing reaction (13). For example, replacement of G-16, D-20, and D-21 in the D-loop of tRNA^Ile with their counterparts from tRNA^Val has little effect on aminoacylation. In contrast, these substitutions abolish the editing response. Conversely, transfer of the three D-loop nucleotides from tRNA^Ile into the framework of a specially designed tRNA^Val confers editing activity to the chimerized tRNA. Thus, tRNA determinants for editing and aminoacylation are discrete.

Similarly, a single (G56A) mutation in the active site (for aminoacylation) of IleRS eliminates the discrimination between isoleucine and valine in amino acid activation (8). However, the G56A mutant enzyme still discriminates between isoleucine and valine in the post-transfer editing reaction. These and other mutational analyses, along with chemical cross-linking data (12, 15), showed that the editing and aminoacylation sites are physically distinct and functionally independent. Specifically, the editing site is located within a large insertion known as CP1 (connective polypeptide 1) (16). This 277-amino acid insertion divides the characteristic class I catalytic domain in half (17). A recently determined x-ray structure places the two active sites about 25-Å apart (18).

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1 The abbreviations used are: IleRS, isoleucyl-tRNA synthetase; Ile-AMP, isoleucyl adenylate; Val-AMP, valyl adenylate.

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A present working hypothesis is that specific nucleotides in the D-loop of tRNA^{Ile} trigger the translocation of the valyl group from the aminoacylation to the editing site. Here we tested whether the presumptive translocation and editing response could be recreated by dividing the critical domains of tRNA^{Ile} into two pieces. One piece is an oligonucleotide substrate that recreates the acceptor stem of the tRNA in the form of a minihelix. Previous work showed that IleRS could charge minihelix^{Ile} with isoleucine (19, 20). The other piece is an RNA hairpin ligand designed after the D-loop of tRNA^{Ile}. Thus, we asked whether the two pieces in concert could reproduce the editing reaction or whether continuity of the tRNA structure was required.

If the editing response requires the full tRNA structure and if the D-loop provides critical determinants only within the context of the full tRNA, then we imagined that minihelix^{Ile} might be a good substrate for mischarging with valine. In that event, we wondered whether mischarging depended on the same nucleotide determinants as those required for correct aminoacylation. This part of the analysis was motivated by the prospect of discovering acceptor stem determinants that modulate discrimination against valine.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Wild-type E. coli IleRS was overexpressed in the E. coli strain MV1184 harboring the multicopy plasmid pKS21 (21). Protein purification was essentially as described previously (22).

RNA Substrates—RNA hairpins were chemically synthesized using N'-phenoxyacetyl-protected ribonucleoside phosphoramidites (ChemGenes, Waltham, MA) on an Amersham Pharmacia Biotech Gene Assembler Special (23). RNA concentrations were determined using absorbance at 260 nm at room temperature. Extinction coefficients were determined using absorbance at 260 nm at room temperature. Extinction coefficients were estimated using the Biopolymer Calculator available online. Mature E. coli tRNA^{Ile} (GAU) was isolated from E. coli strain MV1184 containing the plasmid pES300, which allows for the lac-inducible overexpression of tRNA^{Ile} (24, 25).

Editing Assays—The RNA-dependent hydrolysis of valyl adenosylate was assayed by following the consumption of [γ-32P]ATP using a protocol that has been described in detail elsewhere (26). The reaction mixture contained 140 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.5 mM CaCl₂, 2 mM [γ-32P]ATP (25 μCi/μmol), 1 mM valine, 75 mM inorganic pyrophosphatase, 5 μM IleRS, and either 40 μM tRNA^{Ile} or 200 μM minihelix^{Ile} and/or D-loop^{Ile}. Reactions with no RNA were used to control for a small background rate of hydrolysis (typically about 1% of the tRNA^{Ile}-dependent rate).

Aminoacylation Assays—The IleRS-catalyzed isoleucylate or valylation of RNA substrates was followed using the trichloroactic acid precipitation method of Shepard and co-workers (22). Reactions were carried out at room temperature in a solution containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl₂, 100 μM EDTA, 2 mM ATP, 10 mM inorganic pyrophosphatase, 5 μM IleRS, and 5 μM IleRS. RNA minihelices were used at a concentration of 50–500 μM and tRNA^{Ile} at a concentration of 10–40 μM. No RNA controls were used to correct for background rates.

RESULTS

RNA-dependent Hydrolysis of Misactivated Valine—The L-shaped tRNA^{Ile} structure (Fig. 1, top right) consists of two helical domains arranged in an L-shape. The dotted lines denote tertiary interactions (29). Arrows indicate nucleotides (G-16, D-20, D-21) of tRNA^{Ile} that are important for the RNA-dependent editing of misactivated valine. D-loop^{Ile} (top left) is an RNA hairpin based on the D-stem/loop of tRNA^{Ile}. Minihelix^{Ile} (bottom) mimics the acceptor-TΨC helical domain of tRNA^{Ile}. Two variants of the minihelix are shown: A1 minihelix^{Ile} is missing the 5′-terminal adenosine; A73G minihelix^{Ile} has an A to G mutation at the discriminator position 73.

One domain to the other. D-loop nucleotides previously identified as essential for editing are marked with arrows in Fig. 1. These nucleotides are not among those needed for the universal connection between the two domains.

At least half of the tRNA synthetases charge minihelices based on the acceptor-TΨC stem of their cognate tRNAs (30–33). Although certain synthetases do not make any contacts with the anticodon (e.g. alanyl-tRNA synthetase (34) and seryl-tRNA synthetase (35)), many others interact with both the acceptor stem and anticodon. In the latter cases, the enzymes can still aminoacylate their associated minihelices, although the efficiency is generally severely reduced relative to the full tRNA. (In the E. coli isoleucine system studied here, the minihelix is ~10⁶-fold less active than tRNA^{Ile}.) Nevertheless, aminoacylation of minihelices generally retains the same sequence specificity for acceptor stem nucleotides as seen in the charging of tRNAs. For this reason, minihelices are thought to interact with the active site in a way that closely parallels the full tRNA.

Ile-Ile^{Ile} (Fig. 1, bottom) contains determinants for aminoacylation by IleRS (19, 20). Previously, this domain of tRNA^{Ile} had not been investigated for its ability to stimulate editing of misactivated valine. As can be seen in Fig. 2, the addition of tRNA^{Ile} to a mixture of IleRS, ATP, and valine rapidly leads to the complete consumption of available ATP. In contrast, minihelix^{Ile} is unable to stimulate the hydrolytic editing of misactivated valine, even at the high concentration of 200 μM.
Next we tested whether the addition of an isolated D-stem/loop domain of tRNA\textsubscript{Ile} could induce editing. For this purpose, we constructed a 9-base pair RNA hairpin that extends the D-stem by pairing nucleotide G-26 and including 4 base pairs of the anticodon stem (D-loop\textsubscript{Ile}, Fig. 1, top left). This D-stem/loop RNA hairpin did not induce editing (see Fig. 2). Finally, we obtained no evidence for an editing response when minihelix\textsubscript{Ile} and D-loop\textsubscript{Ile} were used in combination (data not shown for clarity). These results suggest that continuity of the tRNA\textsubscript{Ile} structure is required for the editing response.

**Mischarging of a Minihelix Substrate**—Because minihelix\textsubscript{Ile} is capable of being aminoacylated with isoleucine and yet is unable to induce editing, we tested its ability to be aminoacylated with valine. As shown in Fig. 3, minihelix\textsubscript{Ile} is a relatively robust substrate for mischarging with valine. This level of mischarging was not observed with mature tRNA\textsubscript{Ile}, as the editing reaction prevents the stable attachment of valine to tRNA\textsubscript{Ile}. We tried to force misacylation of tRNA\textsubscript{Ile} by using concentrations (40 mM) well above the $K_m$ ($5$ mM, Ref. 14). Still, no misacylation of the full tRNA could be detected. Thus, although minihelix\textsubscript{Ile} is significantly less active than tRNA\textsubscript{Ile} for charging with isoleucine, it is far more active in mischarging with valine.

**Mischarging of Minihelix Sequence Variants**—Despite the reduced activity of minihelix\textsubscript{Ile} for charging with isoleucine, this aminoacylation is specific. For example, substitution of the A-73 discriminator with G results in a minihelix that is 8.4-fold less active in charging with isoleucine. A qualitatively similar effect is seen when the same substitution is made in the full tRNA (14). Here we found that mischarging A73G minihelix\textsubscript{Ile} with valine showed the same rate reduction that was observed for charging with isoleucine (Fig. 4). In addition, we investigated a variant of minihelix\textsubscript{Ile} designated $\Delta 1$ minihelix\textsubscript{Ile}, in which the 5'-terminal A-1 nucleotide has been deleted. Recently, we discovered that this deletion enhances charging with isoleucine (20). Presumably, this disruption of the first base pair increases the flexibility of the single-stranded 3' terminus. The 3'-end of bound tRNA\textsubscript{Ile} in the co-crystal with glutaminyl-tRNA synthetase is folded back into the active site (36). Because glutaminyl-tRNA synthetase and IleRS are structurally related, the enhanced flexibility of the $\Delta 1$ substrate is thought to ease passage of the minihelix acceptor terminus into the transition state for catalysis.) The enhancement observed in charging $\Delta 1$ minihelix\textsubscript{Ile} with isoleucine is exactly paralleled in misacylation with valine (Fig. 4). Thus, the determinants for charging of minihelix\textsubscript{Ile} are independent of which amino acid is used as the substrate. These results rule out the possibility that the acceptor stem plays a role in amino acid discrimination.

A direct comparison of the initial rates of aminoacylation with both isoleucine and valine reveals that isoleucine is only a 3-fold better substrate under these conditions. This level of discrimination was observed over a large concentration range (50–500 $\mu$M) of minihelix substrate (data not shown), showing that the nature of the amino acid does not modulate minihelix binding. Because the rates of Ile-AMP and Val-AMP synthesis are greater than the rate of aminoacylation of minihelix substrates, it is likely that the aminoacyl adenylates (of both isoleucine and valine) accumulate in the active site to similar levels. Under these circumstances, the rate of aminoacyl-RNA formation should be most correlated to the transfer rate of the aminoacyl group from the adenylate to the minihelix. The
small difference between the isoleucylation and valylation rate of minihelixIle may represent a lack of discrimination toward the amino acid side chain in the transfer step.

**DISCUSSION**

The ability of isoleucyl-tRNA synthetase to discriminate against valine is enhanced in the presence of tRNAIle. In charging of minihelix substrates, this discrimination is reduced to a mere 3-fold preference for isoleucine. This outcome is expected for substrates that have significantly reduced editing activity and yet have retained some charging activity. The mischarging of a minihelix reinforces the evidence that the D-loop of tRNAIle is indispensable for proper discrimination against valine (13).

For example, mutations in the anticodon of minihelixIle are within 20% of each other. For example, none of the D-loop nucleotides critical for editing are protected by bound IleRS in phosphate ethylation experiments (14).

Considering the available evidence, we propose that the effect of the D-loop is derived from its presumed role in transducing conformational information between the two domains of the aminoacylation system where one or more of the discriminatory "sieves" has been attenuated. It should be noted that the notion of aminocyclation systems became more robust, specificity could have become a greater selective advantage. At this point, determinants for editing may have been appended to the tRNA structure.

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