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Switching Recognition of Two tRNA Synthetases with an Amino Acid Swap in a Designed Peptide

Douglas S. Auld and Paul Schimmel

The genetic code is based on specific interactions between transfer RNA (tRNA) synthetases and their cognate tRNAs. The anticodons for methionine and isoleucine tRNAs differ by a single nucleotide, and changing this nucleotide in an isoleucine tRNA is sufficient to change aminoacylation specificity to methionine. Results of combinatorial mutagenesis of an anticodon-binding–helix loop peptide were used to design a hybrid sequence composed of amino acid residues from methionyl- and isoleucyl-tRNA synthetases. When the hybrid sequence was transplanted into isoleucyl-tRNA synthetase, active enzyme was generated in vivo and in vitro. The transplanted peptide did not confer function to methionyl-tRNA synthetase, but the substitution of a single amino acid within the transplanted peptide conferred methionylation and prevented isoleucylation. Thus, the swap of a single amino acid in the transplanted peptide switches specificity between anticodons that differ by one nucleotide.

Isoleucyl- and methionyl-tRNA synthetases are two of the most closely related class I tRNA synthetases (*1*). Each has a characteristic nucleotide binding fold in the NH_2-terminal domain that forms the active site for amino acid activation and for the transfer of the activated amino acid to the bound tRNA. A polypeptide insertion into this domain is believed to provide for interaction with the tRNA acceptor helix (*2*). The COOH-terminal domain is rich in α helices and provides for interaction with the anticodon of the respective tRNAs. Within this second domain, a helix loop peptide provides for interactions with the anticodon. Chemical crosslinking, molecular modeling, and mutagenesis have implicated Trp^{461} (*3*) of the peptide element of *Escherichia coli* methionyl-tRNA synthetase (MetRS) as essential for this anticodon interaction (Fig. 1) (*4–9*). In particular, Trp^{461} is proposed to interact with the first base cytosine of the CAU anticodon of $tRNA^{Met}$ (*10*). Sequence alignments show that in *E. coli* isoleucyl-tRNA synthetase (IleRS), the analogous position is Arg^{734} (Fig. 1).

Muramatsu *et al.* (*11*) further demonstrated the relatedness of these enzymes by the ease with which their respective tRNA specificities could be switched. Although the minor isoacceptor of $tRNA^{Ile}$ has an LAU anticodon, where L is lysidine (lysidine is cytosine with the ε-amino group of lysine covalently linked to C-2 of the pyrimidine ring of cytosine), the unmodified isoacceptor has a CAU anticodon—the same as $tRNA^{Met}$. This unmodified $tRNA^{Ile}$ is aminoacylated efficiently by MetRS but not by IleRS. Modification of C → L yields efficient aminoacylation with isoleucine and eliminates aminoacylation with methionine. We sought to determine whether a complement of the experiment performed by Muramatsu *et al.* could be achieved by manipulation of the two enzymes. Our initial experiments concentrated on gathering more information about the role of 10 amino acids within the helix loop element that includes Arg^{734} of IleRS and Trp^{461} of MetRS (Fig. 1).

Synthetic deoxyoligonucleotide cassettes that encode variants of the helix loop peptide were introduced into plasmids pKSNB (which encodes MetRS) and pDUG03 (which encodes IleRS) through unique restriction sites (*12*). The genes that encode

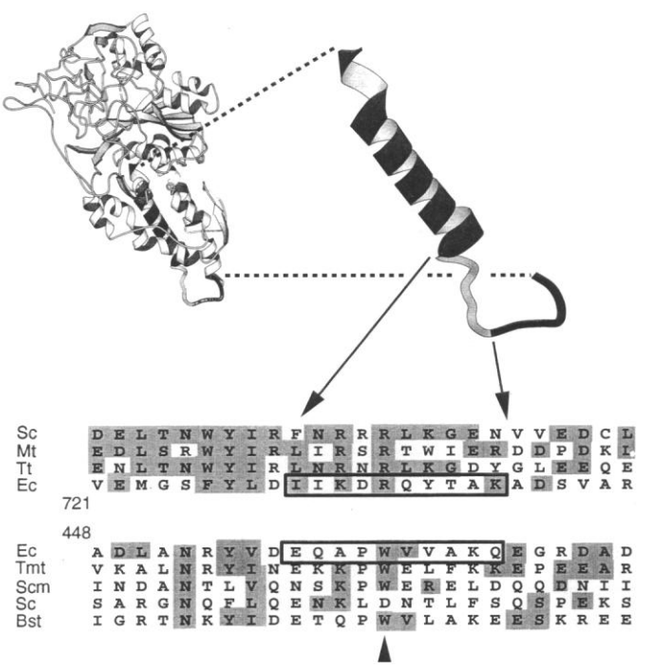

Fig. 1. (Top) The helix loop structure derived from the crystal structure of the 547–amino acid monomeric fragment of MetRS (*5*); the fragment was drawn with the program Molscript (*29*). (Bottom) A portion of the alignment used to define the anticodon-binding region in IleRS (upper four sequences) and MetRS (*3, 13, 20, 30*). Also shown is the 10–amino acid region that was combined (boxed residues) by binary codon mutagenesis (*18*). Shaded regions represent semiconserved residues in IleRS sequences that also are semiconserved in MetRS sequences. The arrowhead at the bottom points to the anticodon recognition region. Ec, *E. coli*; Sc, *Saccharomyces cerevisiae*; Mt, *Methanobacterium thermoautotrophicum*; Tt, *Tetrahymena thermophila*; Tmt, *Thermus thermophilus*; Scm, mitochondrial *Saccharomyces cerevisiae*; and Bst, *Bacillus stearothermophilus*.

Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

the recombined enzymes in these and subsequent experiments were introduced into Δ*metG*(MN9261/pRMS615) and into Δ*ileS*(IQ844/pRMS711) tester strains that contain null alleles (complete ablations) of chromosomal *metG* and *ileS*, respectively (*8*, *13*). These null strains are maintained by plasmids pRMS615 and pRMS711, respectively, which encode the missing enzyme activity on a vector that has a temperature-sensitive replicon. Because of the temperature-sensitive replicon, these strains do not grow at 42°C, unless the introduced gene contained on plasmid pDUG03 or on plasmid pKSNB provides a variant enzyme with sufficient activity to rescue the temperature-sensitive growth phenotype. Thus, the activities of the recombined enzymes were tested initially by the criteria of whether they could serve as the sole source of enzyme activity in vivo.

Neither recombined enzyme, with the transplanted 10–amino acid peptide of the other, could complement its respective null allele. The recombined MetRS could not be isolated, presumably because of reduced stability. In the case of the IleRS that contains the peptide from MetRS, the recombined enzyme accumulated in vivo and therefore could be characterized in vitro (*14*). Although amino acid activation (*15*) was not affected, the ratio of the catalytic rate constant to the Michaelis constant (k_{cat}/K_m) for aminoacylation of $tRNA^{Ile}$ was reduced by a factor of at least 10^4, compared with the wild-type enzyme (*16*, *17*). This result demonstrates that the ability to aminoacylate tRNA depends on the particular peptide sequence that is introduced.

Our objective was to design a peptide that could be accommodated into the structure of each protein and to direct specificity toward a tRNA through a simple amino acid substitution within the context of the cognate enzyme. For this purpose, a library of 2^{10} (1024) combinatorial variants of IleRS was constructed. This library was designed to contain binary combinations of the 10 residues found in each enzyme (*18*), as defined by the alignment (Fig. 1). The plasmid-encoded library was transformed into strain IQ844/pRMS711, and the transformed cells were checked for growth at 30°C and 42°C. In this way, a set of complementing and noncomplementing hybrid enzymes was obtained (*19*).

The sequences of a randomly chosen representative set of active and inactive mutants were determined by DNA sequence analysis. This analysis showed that the combined library of active and inactive mutants had no biases for mutations at particular locations. However, the pattern of allowed substitutions in the active mutants was not random. For example, of 26 sequenced active variants, all retained Lys^{732} and Tyr^{736} and 24 of 26 retained Arg^{734} (*17*). The retention of Lys^{732} in the 26 active variants was consistent with earlier observations of Shepard *et al.* (*20*) that point substitutions of Lys^{732} severely diminish the activity for the tRNA-dependent step of aminoacylation. Because representatives of the same 26 active variants were obtained from independent isolates of additional clones, we believe that this set of 26 variants represents most of the active enzymes in the library.

These results allowed us to design a peptide that incorporated the main elements needed for an active IleRS and that required the least alteration to confer activity on MetRS. Recognizing that Trp^{461} is critical for MetRS and that the corresponding Arg^{734} in IleRS is conserved in the library of active variants, we hypothesized that an interchange between Trp^{461} and Arg^{734} would be part of any scheme to interchange the functionality of these peptides. Pro^{460} and Val^{462} flank Trp^{461} in MetRS. The importance of Pro^{460} in MetRS is well established (*7*, *8*, *10*), and it was encouraging that Asp^{733} could be replaced by Pro in six of the characterized active variants of IleRS (*17*). The valine (Val^{462}) on the COOH-terminal side of Trp^{461} of MetRS was the most common substitution (16 occurrences of Q735V) (*3*) in the library of active IleRS variants. The Q735V replacements were coupled in 50% of the cases with either a D733P or a T737A substitution (*17*). After a review of these features of the library, an IleRS variant was constructed with the double D733P and T737A substitution, combined with Q735V, to give the peptide designated HLP^{27}: I^{730}IKPRVYAAK (HLP^{27} is helix loop peptide number 27; substituted residues from MetRS are shown in boldface). This sequence retains the critical residues (for IleRS) of K732, R734, and Y736.

The variant IleRS containing HLP^{27} complemented the IQ844 null allele of *ileS*. When the 10–amino acid peptide was transplanted into MetRS, there was no complementation of the *metG* null strain MN9261 (Fig. 2), although the enzyme accumulated in vivo and could be isolated. A single R734W replacement in the HLP^{27} variant of IleRS was then constructed to give peptide $HLP^{27,\ R\rightarrow W}$:$I^{730}$IKPWVYAAK. This enzyme, however, failed to complement the strain IQ844 *ileS* null allele (Fig. 2), even though the enzyme accumulated in vivo and could

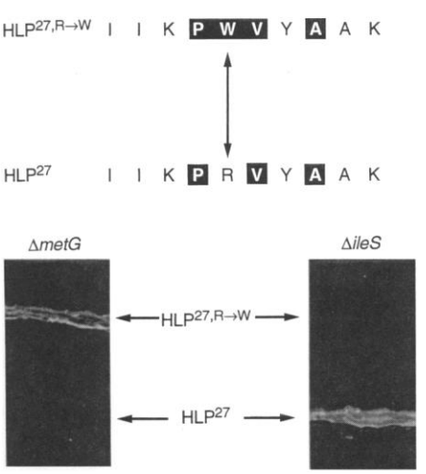

Fig. 2. Activity in vivo for HLP variants. (Top) Sequences of the helix loop peptides that were transplanted into IleRS and MetRS (*3*). MetRS-derived residues are shown in blocked font. (Bottom) The in vivo complementation results are shown for the MetRS null strain (Δ*metG*) and the IleRS null strain (Δ*ileS*). Cells were streaked in horizontal lines on agar plates that contained rich media and ampicillin. Photographs of these plates were processed into bitmap images with an Apple Scanner (model A9M0337) and with Applescan software from Apple Computer. Bitmaps were contrast-equalized with the graphical scanning tool GScan and presented with Showcase (version 3.2) on an IRIS 4D Silicon Graphics workstation. Conditions for complementation were as described (*19*).

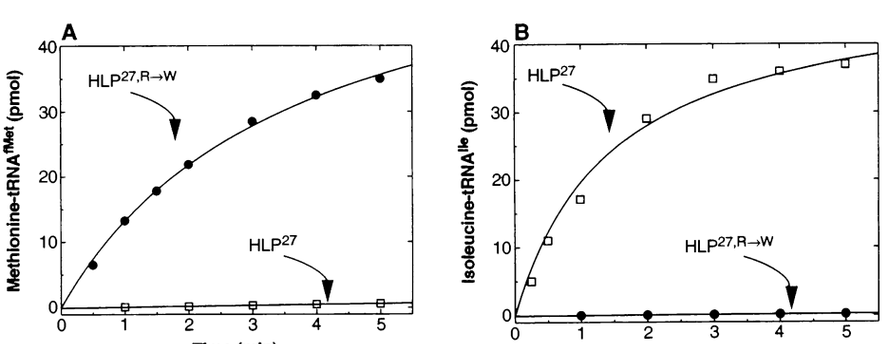

Fig. 3. Activity in vitro for the HLP variants. Examples of methionine acceptance (**A**) and isoleucine acceptance (**B**) for the MetRS and IleRS variants, respectively, are shown. Conditions for the aminoacylation assay were as described (*16*), with 100 nM MetRS and 4 μM $tRNA^{fMet}$ (A) or 20 nM IleRS and 4 μM $tRNA^{Ile}$ (B).

be isolated. In contrast, transplantation of the $HLP^{27,\ R\rightarrow W}$ peptide into MetRS yielded complementation of the *metG* null strain (Fig. 2).

The results obtained in vivo were recapitulated in vitro (Fig. 3). The HLP^{27}-containing MetRS variant was purified and, although the rate of amino acid activation was unaffected, k_{cat}/K_m for methionylation of $tRNA^{Met}$ was lowered by a factor of 5000 relative to the wild-type enzyme (an increase of 5.2 ± 0.3 kcal mol^{-1} in the free energy of activation) (*16*). In contrast, the k_{cat}/K_m for isoleucylation of $tRNA^{Ile}$ by the HLP^{27}-containing IleRS variant was reduced by a factor of about 5 relative to that of the wild-type enzyme. This reduction corresponds to an increase of approximately 1.0 ± 0.3 kcal mol^{-1} in the apparent free energy of activation for aminoacylation relative to that of the wild-type enzyme.

Similarly, purified $HLP^{27,\ R\rightarrow W}$-containing IleRS has a k_{cat}/K_m value for the isoleucylation of $tRNA^{Ile}$ that was lowered by a factor of 200 relative to the wild-type enzyme (an increase of approximately 3.3 ± 0.3 kcal mol^{-1} in the free energy of activation), whereas the rate of amino acid activation was unaffected. In contrast, the $HLP^{27,\ R\rightarrow W}$-containing MetRS enzyme has a k_{cat}/K_m value for methionylation of $tRNA^{Met}$ that is reduced by a factor of about 30 relative to the wild type (an increase of 2.1 ± 0.3 kcal mol^{-1} in the free energy of activation). These in vitro results indicate that the effect of the W ↔ R swap is similar in each enzyme (an increase in the free energy of activation of 3.1 ± 0.4 kcal mol^{-1} in MetRS and 2.3 ± 0.4 kcal mol^{-1} in IleRS) (Fig. 3). Thus, a single amino acid swap in a transplanted peptide is sufficient to confer activity on either IleRS or MetRS.

The success of these experiments likely is based on the close relation between the anticodon-binding motifs of the two enzymes (*13*, *21*). Although the specificity of this peptide can be changed markedly by a single amino acid replacement in both enzymes, other interactions in the synthetase-tRNA complex no doubt are also needed for specificity. Regardless of these details, the result reported here is operationally the converse of the experiment conducted by Muramatsu *et al.*, in which the change of a single anticodon base led to a switch in tRNA specificity (*11*). Our results strengthen the concept that although many interactions are important for these synthetase-tRNA complexes, much of their specificity depends on a single amino acid in an appropriate sequence context. The discrimination in these instances distinguishes the CAU anticodon of $tRNA^{Met}$ from the GAU and LAU anticodons of isoleucine tRNAs. Given the previous proposal that Trp^{461} of MetRS interacts directly with the C of the CAU anticodon of $tRNA^{Met}$, it is possible that the R ↔ W interchange mentioned here partially reflects an exchange of side chains that are interacting with G (or L in the modified tRNA) or C.

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3. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. Amino acid mutations are also indicated with the single-letter code. Thus, $Gln^{735} \rightarrow$ Val is Q735V.
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12. Xba I and Rsr II restriction sites were introduced into a cloned *ileS* gene. For mutagenesis of MetRS, a previously constructed and cloned *metG* gene containing Bss HII and Nar I restriction sites was used (*8*). Introduction of these restriction sites led to one mutation of S742R in IleRS. In MetRS, introduction of these restriction sites led to the mutations K439A and G478A (*8*). These substitutions are phenotypically silent. The respective enzyme variants show wild-type behavior in vitro and in vivo and therefore will be referred to as wild-type enzymes.
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14. The purification of active enzymes was performed by the transformation of strain IQ844/pRMS711 with plasmid pDUG03 (which encodes IleRS) or strain MN9261/pRMS615 with plasmid pKSNB (which encodes the 547–amino acid monomeric fragment of MetRS). The transformed strains that harbor the wild-type and mutant *ileS* or *metG* alleles were grown at 42°C and were tested initially for chloramphenicol sensitivity to confirm loss of the maintenance plasmid pRMS711 or pRMS615, because these maintenance plasmids harbor the chloramphenicol resistance gene (*8*, *13*). For isolation of inactive enzymes, *E. coli* strain MI1 (for IleRS) (*22*) or MJR (for MetRS) (*23*) was used. The *ileS* and *metG* alleles of these strains confer amino acid auxotrophy because they encode for enzymes defective in amino acid binding (with values of K_m raised 350 to 1000 times) (*24*, *25*). Therefore, inactive enzymes can be overexpressed and purified from these strains grown in rich media, and the isolated enzyme's activity can be assayed in vitro at a concentration of isoleucine or methionine at which there is no concern for activity caused by any contaminating, chromosomally encoded enzyme (*24*, *25*). The procedure for enzyme purification was followed as described (*20*, *24*). Purity was estimated to be approximately 95% by SDS–polyacrylamide gel electrophoresis.
15. The amount of adenylate formed by the enzymes was assessed by amino acid–dependent pyrophosphate–adenosine triphosphate exchange monitored at 25°C (*26*). Conditions for pyrophosphate exchange were as described (*9*, *20*); 10 nM purified enzyme was used.
16. The tRNA-dependent aminoacylation reaction was assayed at 37°C under conditions as described (*9*, *20*). For the determination of k_{cat} and K_m values, purified enzyme was added at concentrations of 1 to 150 nM and the tRNA concentration was varied from 0.5 to 60 μM. Values for k_{cat}/K_m were derived from the slopes of Lineweaver-Burke plots generated from the data. The increase in the free energy of activation for the variants was calculated from the difference in the apparent Gibbs free energy of activation for the designated variant versus that of the wild-type enzyme. Errors were calculated from two to three separate determinations of k_{cat}/K_m. The $tRNA^{fMet}$ was purchased from Sigma, and $tRNA^{Ile}$ was purified from *E. coli* JM109 by the use of an overexpressing plasmid and the extraction method as described (*27*) with Nucleobond–AX columns (Macherey–Nagel).
17. D. S. Auld and P. Schimmel, data not shown.
18. This library was constructed in plasmid pDUG03 with the use of synthetic oligonucleotide cassettes that were made with a codon mutagenesis technique (*28*). DNA synthesis was performed on a Pharmacia Gene Assembler Plus DNA synthesizer model 530. Each oligonucleotide was synthesized on controlled pore glass columns (Pharmacia; 1.3 μmol). The procedure involves two solid-phase oligonucleotide syntheses performed in parallel. One column is programmed to synthesize the IleRS sequence (on a column containing 50% of the total resin), and the other is programmed to synthesize the MetRS sequence (on a column containing the remaining 50% of the total resin). The synthesis is interrupted after the production of each codon, and the resins that contain the incomplete oligonucleotides are mixed and then split in half again to give a 50:50 mix of the MetRS- and IleRS-derived codons at each of the 10 positions.
19. Identification of active mutants was performed by transformation of the library into strain IQ844/pRMS711, followed by selection for 3 days at 42°C on plates that contained rich media and ampicillin. For active mutants, loss of the maintenance plasmid was confirmed by the lack of growth on plates that contained rich media and chloramphenicol. All inactive mutants were grown in *E. coli* strain MV1184, and after DNA sequence analysis each was transformed separately into strain IQ844/pRMS711 to confirm an inactive phenotype. The production of stable enzymes in vivo was confirmed by protein immunoblots with the use of rabbit antibody to *E. coli* IleRS and horseradish peroxidase–linked donkey antibody to rabbit immunoglobulin G (Amersham). The immunoreactive proteins were visualized by chemiluminescence (Amersham).
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