The Catalytic Flexibility of tRNAIle-lysidine Synthetase Can Generate Alternative tRNA Substrates for Isoleucyl-tRNA Synthetase

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Bacteria decode the isoleucine codon AUA using a tRNA species that is posttranscriptionally modified at the wobble position of the anticodon with a lysine-containing cytidine derivative called lysidine. The lysidine modification of tRNAIle2 is an essential identity determinant for proper aminoacylation by isoleucyl tRNA synthetase (IleRS) and codon recognition on the ribosome. The ATP- and lysine-dependent formation of lysidine is catalyzed by tRNAIle-lysidine synthetase. Using the purified recombinant enzyme from Escherichia coli and an in vitro transcribed tRNA substrate, we have confirmed that lysidine modification is both necessary and sufficient to convert tRNAIle2 into a substrate for IleRS. A series of lysine analogs were tested as potential inhibitors during the mechanistic characterization of tRNAIle-lysidine synthetase. Gel electrophoresis revealed that many of these analogs, including some simple alkyl amines, were alternative substrates. Incorporation of these amines into alternative tRNA products was confirmed by mass spectrometry. The availability of tRNAIle2 with differential modifications enabled an exploration of the structural requirements of the anticodon for aminoacylation by methionyl tRNA synthetase and IleRS. All of the modifications were effective at creating negative determinants for methionyl tRNA synthetase and positive determinants for IleRS, although the tolerance of IleRS differed between the enzymes from E. coli and Bacillus subtilis.

The fidelity of protein synthesis requires precise decoding of the genetic code. It is essential that aminoacyl tRNA synthetases match the appropriate amino acid-tRNA pairs and that the anticodons of charged tRNAs interact with the correct mRNA codons. Posttranscriptional modification of the first, or wobble, position of the tRNA anticodon contributes to the accuracy of the latter process (1). One example in eubacteria is the single gene responsible for lysidine formation in both Bacillus subtilis and E. coli, named tilS (for tRNAIle-lysidine synthetase) (5). The tilS gene (formerly yaca) is essential for viability in B. subtilis (6), and homologs are nearly universally present in eubacteria, including human pathogens. Partial inactivation of the tilS gene in E. coli leads to an AUA codon-dependent translational defect (5). Loss or inhibition of TilS protein function is expected to disrupt bacterial protein synthesis as deletion of the pool of isoleucine-chargeable tRNAIle2 would leave ribosomes unable to translate past AUA codons. There is no mammalian counterpart of TilS, as eukaryotes use tRNAIle species with either inosine or a modified uridine at the wobble position of the anticodon to recognize all three isoleucine codons (7). Several high resolution protein structures of bacterial TilS are available (8, 9), and a straightforward reaction mechanism has been proposed (5, 10). Together these attributes contribute to the potential attractiveness of TilS as a novel, albeit unproven target for a broad-spectrum antibacterial agent.

Using a simplified assay format, we have begun mechanistic studies of this enzyme and confirmed that lysidine modification of tRNAIle2 is both necessary and sufficient to convert this tRNA into a substrate for isoleucyl tRNA synthetase (IleRS). During a systematic study of substrate requirements, we found that a number of lysine analogs and smaller primary amine-containing compounds can act as alternative substrates for the TilS-mediated modification of tRNAIle2. The availability of these differentially modified tRNAs has in turn enabled an exploration of the structural requirements of the tRNA anticodon for aminoacylation by IleRS.

EXPERIMENTAL PROCEDURES

Materials—L-[4,5-3H]Lysine monohydrochloride, L-[4,5-3H]isoleucine, L-[methyl-3H]methionine, and wheat germ agglutinin polyethyleneimine-treated type A polyvinyltoluene scintillation proximity assay (SPA) beads were obtained from GE Healthcare. Lysine analogs, αβ-methyleneadenosine-5′-triphosphate, and βγ-methyleneadenosine-5′-triphosphate were obtained from Sigma-Aldrich. Other ATP analogs were obtained from TriLink BioTechnologies (San Diego, CA). TCEP was obtained from Pierce.

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Preparation of Recombinant E. coli TilS—The tilS gene in accession number Z50870 was obtained by PCR amplification from E. coli K12 genomic DNA (American Type Culture Collection, Manassas, VA) using the following primers: 5’-GGCATATGACACTCAGCTCAATAAG-3’ (forward primer) and 5’-GGGAAGTTAATTAGGTTTCTGCAAAAGC-3’ (reverse primer). The PCR product was ligated into the expression vector pET26B+ (Novagen, Madison, WI), and the clones were sequenced on an ABI377 DNA sequencer using BigDye Terminator Version 3.1 chemistry. The C-terminal hexahistidine-tagged TilS fusion protein was expressed in E. coli BL21 (DE3) cells with 1 mM isopropyl 1-thio-β-D-galactopyranoside induction and purified using nickel-nitrilotriacetic acid resin (Qiagen, Valencia, CA) following the manufacturer’s instructions. The pooled protein was further purified and buffer-exchanged on a Superdex 200 column (GE Healthcare). The mass of the recombinant TilS was verified by electrospray ionization mass spectrometry performed at NovaTia, LLC (Monmouth Junction, NJ).

Preparation of Recombinant B. subtilis IleRS—The IleRS gene in accession number Q45477 was obtained from B. subtilis strain 168 (obtained from the Bacillus Genetic Stock Center). PCR amplification of the gene was conducted with the following primers: 5’-CACATTAATATGGATTTTTAAGACACGCTCTTAATG-3’ (forward primer) and 5’-GTCGGCTGCGTATTTTGTGATAGTTTTTCCAACGATTTC-3’ (reverse primer). The PCR product was ligated into the expression vector pET3D-FLAG (Novagen) and confirmed by DNA sequencing. The N-terminal FLAG-tagged fusion protein was expressed in E. coli BL21 (DE3) cells with 1 mM isopropyl 1-thio-β-D-galactopyranoside induction and purified using M2-FLAG resin (Sigma-Aldrich) following the manufacturer’s instructions. The pooled protein peak was further purified and buffer-exchanged on a Superdex 200 column. The mass of the recombinant IleRS protein was verified by electrospray ionization mass spectrometry.

Preparation of E. coli S100 Extract—E. coli BL21 (DE3) cells, 3 g, were suspended in 7 ml of 50 mM HEPES (pH 7.5), 100 mM NaCl, 10 mM MgCl2, 10% glycerol and lysed in a French pressure cell at 18,000–20,000 p.s.i. After centrifugation at 100,000 x g for 60 min, the soluble proteins in the supernatant were loaded onto a Superdex 200 column. The peak fractions were pooled, and 0.1 volume of 10% streptomycin sulfate was added. The precipitate was removed by centrifugation at 16,000 x g for 10 min at 4 °C, and solid ammonium sulfate was added to the supernatant to 65% saturation. After collection of the precipitate by centrifugation at 16,000 x g for 10 min at 4 °C, the pellet was resuspended in 0.5 ml of 50 mM HEPES (pH 7.6), 10 mM MgCl2 and dialyzed against the same buffer at 4 °C overnight in a Slide-A-Lyzer-10K (Pierce). The protein preparation was frozen after the addition of glycerol to 10%.

Preparation of tRNA\textsuperscript{ille2}—A template for in vitro transcription was built by putting the tRNA gene behind a T7 promoter (11). Overlapping oligonucleotides were prepared on an ABI 394 DNA synthesizer to construct a cDNA flanking the E. coli tRNA\textsuperscript{ille2} sequence with a 5’ T7 RNA polymerase promoter (5’-AATTCCTCCTTAACTAGACCTCACTATAAGGCCCCTTGAGCTAGTGGTTAGGAGCCAGCGACTCATATAATC-GC-TTGGTCGC-3’) and a 3’ BstNI restriction site (5’-AATTCT-TGGTGCGGCCCTTGCTGAGCTTTAAACCAGGCGACCAAGC-GATTATGAGTCGCCGCTTCAACCAGTGAGC-3’). Vector pLDR24 was generated via mutagenesis of pLDR21 (ATCC #87206) to add EcoRI and PstI restriction sites downstream of the tac promoter. The cDNA was cloned into pLDR24, and the sequence was verified. The resulting pLDR24(Ile2) clone was cut with BstNI to generate the transcription template and used without further purification at 50 µg/ml in the Ribomax Large Scale RNA Production System T7 kit (Promega, Madison, WI) following the manufacturer’s instructions. After incubation, the synthesis reaction was extracted with phenol:chloroform: isoamyl alcohol (25:24:1) and purified on a Superdex 75 column (GE Healthcare). Purity and mass were verified by polyacrylamide gel electrophoresis on Tris borate-EDTA urea gels (Invitrogen) and electrospray ionization mass spectrometry.

Preparation of C34G-tRNA\textsuperscript{ille2}—Mutagenesis on clone pLDR24(Ile2) was performed via PCR amplification with the Vent and Vent exo- polymerases (New England Biolabs, Ipswich, MA) using the following oligonucleotides 5’-GTGGTTAGGACGCAGCGACTGATACTGCTTGCTGCT-3’ (forward primer) and 5’-CCACGGACCAACGGTATTACGCGCTGCTCCTAACCCAG-3’ (reverse primer). The sequence of the pLDR24(G34Ile2) clone was verified, and the vector was digested with BstNI for the preparation of tRNA as above.

Assay of TilS Enzymatic Activity—The standard TilS enzymatic assay was performed in a 96-well white polystyrene microwell plate with 30 µl of a reaction mix comprised of 50 mM TAPS buffer (pH 8.5), 3 mM MgCl\textsubscript{2}, 0.01% Tween 20, 0.5 mM TCEP, 0.1 mg/ml bovine serum albumin, 20 nM E. coli tRNA\textsuperscript{ille2}, 5 µM ATP, 110 nM [3H]lysine (91 Ci/mmol), and 10 nM E. coli TilS enzyme. Samples were incubated for 2 h at room temperature before terminating the reaction by the addition of 30 µl of 2.7 mg/ml SPA beads suspended in 175 mM sodium citrate buffer (pH 2.0) with 100 mM NaCl. After 20 min at room temperature to allow tRNA capture, the plate was centrifuged at 400 x g for 5 min. Radioactivity was measured in a Packard Topcount NXT microplate scintillation counter. Inhibition curves were plotted, and IC50 values were calculated with a 2 parameter fit using KaleidaGraph (Synergy Software).

Preparation of tRNA with Lysine Analogues—Reactions were performed in a 75-µl reaction mix consisting of 50 mM TAPS buffer (pH 8.5), 3 mM MgCl\textsubscript{2}, 0.5 mM TCEP, 0.1 mg/ml bovine serum albumin, 20 µM E. coli tRNA\textsuperscript{ille2}, 100 µM ATP, 500 nM E. coli TilS enzyme, and 100 µM lysine or specified analog concentration for 4 h at 37 °C and then frozen without further workup until analysis. The amount of unmodified tRNA was assayed by diluting an aliquot of the reaction mixture 1000-fold into a 30-µl reaction mix comprised of 50 mM TAPS buffer (pH 8.5), 3 mM MgCl\textsubscript{2}, 0.01% Tween 20, 0.5 mM TCEP, 0.1 mg/ml bovine serum albumin, 50 µM ATP, 110 nM [3H]lysine (91 Ci/mmol), and 20 nM E. coli TilS enzyme. Samples were incubated for 2 h at room temperature before terminating the reaction by the addition of SPA beads as in the standard assay above. The reactions with lysine analogs were also analyzed by gel electrophoresis using a 15% polyacrylamide Tris borate-EDTA, 8 M urea gel run at 120 V for 3 h and stained with ethidium bromide.
Catalytic Flexibility of Lysidine Synthetase

Assay of tRNA Synthetases—Assays for IleRS and MetRS activities were performed in 96-well white polystyrene microplates with 30 μl of a reaction mix comprised of 50 mM HEPES buffer (pH 7.3), 10 mM Mg(OAc)₂, 100 mM KCl, 0.5 mM TCEP, 0.1 mg/ml bovine serum albumin, 100 nM TilS-modified tRNAIle2, 100 μM ATP, 100 nM [3H]amino acid (93 Ci/mmol for isoleucine; 76 Ci/mmol for methionine), and 40 μg/ml partially purified S-100 fraction of E. coli extract or 0.1 nM recombinant B. subtilis IleRS. The enzyme amounts chosen were within the linear response range of the assay to ensure that the measured rates did not reach a plateau. Samples were incubated for 2 h at room temperature before terminating the reaction by the addition of 30 μl of 8.3 mg/ml SPA beads suspended in 175 mM sodium citrate buffer (pH 2.0) with 100 mM NaCl. After 10 min at room temperature to allow tRNA capture, the plate was centrifuged at 400 x g for 5 min. Radioactivity was measured in a Packard Topcount NXT microplate scintillation counter.

RESULTS

The reaction catalyzed by TilS has been assayed with a radio-labeled lysine substrate by following the incorporation of acid precipitable radioactivity into tRNA (5, 10). To further simplify the procedure and make the TilS reaction amenable to high throughput screening, we applied SPA technology. This approach has been successfully used for aminocyl tRNA synthetases, which also perform the ATP-dependent coupling of an amino acid to a tRNA (12). We found polyethyleneimine-coated wheat germ agglutinin type I polyvinyltoluene beads to perform best for the nonspecific capture of total tRNA. The tRNA substrate was prepared by run-off transcription; previous work indicates that the synthetic polynucleotide is nearly equivalent to the native tRNA substrate (10). The use of high specific activity tritiated lysine enabled good detection sensitivity using only nM levels of enzyme and tRNA.

TilS was found to have an alkaline pH optimum in the range of 8.5–9.5; pH 8.5 was adopted for the standard assay. Kinetic analysis established that the substrate levels were not saturating for the standard assay described under “Experimental Procedures.” Our examination of the enzymatic reactivity of TilS for the standard assay described under “Experimental Procedures.” Our examination of the enzymatic reactivity of TilS for the standard assay described under “Experimental Procedures.” Our examination of the enzymatic reactivity of TilS for the standard assay described under “Experimental Procedures.” Our examination of the enzymatic reactivity of TilS for the standard assay described under “Experimental Procedures.” Our examination of the enzymatic reactivity of TilS for the standard assay described under “Experimental Procedures.”

A series of commercially available lysine analogs were examined for inhibition in the standard TilS assay. The structures of lysine and its analogs studied in this report are shown in Fig. 1 and Table 3. Lysine, itself appears as an inhibitor of the TilS reaction (Table 2), although its catalytic efficiencies, as determined by Vmax/Km, were at least 10-fold lower than ATP. Four other analogs (N⁰-methyladenosine-5′-triphosphate, 2-amino-6-chloropurineriboside-5′-triphosphate, 2-amino-6-chloropurineriboside-5′-triphosphate, and 6-carboxyadenosine-5′-triphosphate) were neither substrates nor inhibitors.

A series of commercially available lysine analogs were examined for inhibition in the standard TilS assay. The structures of these compounds and their inhibition constants are listed in Fig. 1 and Table 3. Lysine, 1, itself appears as an inhibitor because of the isotopic dilution of the tritiated lysine in the assay; the IC₅₀ in this case approximates Kᵥ, and provides a useful reference point for the binding affinity of the other compounds. Alteration of the amino or carboxyl functionalities on the α carbon of lysine resulted in only very weak inhibitors (compounds 5–8). Likewise, shortening the side chain, deleting the ε amino group, or substituting a guanidino moiety were also detrimental (compounds 9, 11, and 12). Modifications within the lysine side chain, however, were much better tolerated. Although the hydroxylysine 4 had reduced potency, dehydrolysine 10 and aminoethylserine 3 were very similar to lysine itself. Surprisingly, aminoethylcysteine 2 appeared to have sig-

### TABLE 1
Inhibition constants for ATP analogs in the TilS reaction

| ATP analog                                      | IC₅₀ (μM) |
|-----------------------------------------------|----------|
| Benzimidazoleriboside-5′-triphosphate         | 7.6 ± 3.3 |
| 8-Azidoadenosine-5′-triphosphate              | 2.7 ± 0.2 |
| Adenosine-5′-O-(1-thiotriphosphate)           | 2.4 ± 0.2 |
| α,β-Methyladenosine-5′-triphosphate           | 1.8 ± 0.5 |
| β,γ-Methyladenosine-5′-triphosphate           | 1.2 ± 0.1 |

### TABLE 2
Kinetic constants for ATP and analogs in the TilS reaction

| Nucleotide substrate            | Kᵥ (μM) | Vmax/Kᵥ (100) |
|---------------------------------|---------|---------------|
| ATP                             | 1.6 ± 0.5 | 100           |
| 8-Azidoadenosine-5′-triphosphate| 8.0 ± 2.4 | 2.3           |
| 7-Deazaadenosine-5′-triphosphate| 1.8 ± 0.5 | 8.6           |
| N⁰-Methyladenosine-5′-triphosphate| 2.1 ± 0.7 | 4.7           |

FIGURE 1. Structures of lysine and its analogs studied in this report.
versus the enzyme contained a primary carbon.

The initial assumption was that the compounds in Table 3, other than lysine, were dead-end inhibitors of TilS, i.e. they formed completely nonproductive complexes with the enzyme. It was noted, however, that every compound with measurable inhibitory potency versus the enzyme contained a primary amine and, thus, was potentially a competitive alternate substrate. Because the assay relies on incorporation of radioactive lysine into tRNA, alternative substrates that replace lysine will appear to be inhibitors and require other methods to discern their true character. This question was addressed in several ways. Analogs were incubated at \( \pm 10 \)-fold their \( IC_{50} \) (see Table 4) with high concentrations of ATP, tRNA\(^{15N2}\), and TilS to maximize the modification of the tRNA. An aliquot of each analog reaction was then diluted into a standard assay containing tritiated lysine to assess whether any unmodified tRNA\(^{15N2}\) remained. As recorded in Table 4, complete conversion with most analogs could be inferred from the complete disappearance (\( \geq 99\% \)) of the initial substrate; in reactions K and L about a quarter of the residual tRNA\(^{15N2}\) substrate remained. Formation of fully modified tRNA products in reactions B–J could be directly detected by gel electrophoresis. As shown in Fig. 2, reactions B–E and G–J clearly produced tRNA species with slower migration than the unmodified substrate. The migration shift was minimal for cadaverine (reaction F), species with slower migration than the unmodified substrate. The migration shift was minimal for cadaverine (reaction F), possibly because this reactant uniquely added some positive charge to the polyanionic tRNA in the electrophoresis at pH 8.7, whereas the other reactants made either zwitterionic (reactions B, C, D, E, G) or neutral (reactions H, I, J) additions.

Finally, the product tRNAs were also analyzed by electrospray ionization mass spectrometry; each reaction with lysine significantly improved binding affinity relative to lysine. The relatively relaxed requirements of the lysine side chain prompted examination of some primary alkyl amines as potential side chain mimics. Although the simple amino acids 16 and 17 were not inhibitors of the TilS reaction, three other amines containing neutral functional groups instead of carboxylates (13–15) retained detectable binding affinity. The potency of N-acetylatedenylamine (13) is surprisingly good considering it completely lacks the binding determinants of the lysine \( \alpha \) carbon.

![FIGURE 2. Analysis of TilS reactions with tRNA\(^{15N2}\) and lysine analogs by gel electrophoresis.](image-url)

Samples of the reaction products were run as described under “Experimental Procedures.” Lane headings refer to the reactions listed in Table 4; A, control; B, lysine; C, aminoethylcysteine; D, aminohexamidoamine, E, (5R)-5-hydroxlysine; F, cadaverine; G, 4,5-trans-dehydrolysine; H, ethanolamine; I, N-acetylatedenylamine; J, histamine.

\( IC_{50} \) values for lysine and the analogs in Fig. 1 in the TilS reaction

| No. | Compound              | \( IC_{50} \)     | R1 | R2      | X   | R3  | R4  |
|-----|-----------------------|-------------------|----|---------|-----|-----|-----|
| 1   | Lysine\( ^a \)        | 0.68 ± 0.04       | NH\( ^+ \) | CO\( ^- \) | CH\( \_ \) | H | NH\( ^+ \) |
| 2   | Aminomethylcysteine   | 0.045 ± 0.003     | NH\( ^+ \) | CO\( ^- \) | S  | H  | NH\( ^+ \) |
| 3   | Aminohexamidoamine    | 0.40 ± 0.02       | NH\( ^+ \) | CO\( ^- \) | O  | H  | NH\( ^+ \) |
| 4   | (5R)-5-Hydroxlysine   | 17.6 ± 0.8        | NH\( ^+ \) | CO\( ^- \) | CH\( \_ \) | OH | NH\( ^+ \) |
| 5   | Lysinamide            | 620 ± 70          | NH\( ^+ \) | CONH\( _2 \) | CH\( \_ \) | H  | NH\( ^+ \) |
| 6   | Cadaverine            | 910 ± 40          | NH\( ^+ \) | CO\( ^- \) | CH\( \_ \) | H  | NH\( ^+ \) |
| 7   | N-α-methyl-lysine\( ^a \) | 1300 ± 120       | NH\( ^+ \) | CO\( ^- \) | CH\( \_ \) | H  | NH\( ^+ \) |
| 8   | 6-Aminohexanoic acid  | >1000             | NH\( ^+ \) | CO\( ^- \) | CH\( \_ \) | H  | NH\( ^+ \) |
| 9   | Norleucine             | >1000             | NH\( ^+ \) | CO\( ^- \) | CH\( \_ \) | H  | NH\( ^+ \) |
| 10  | 4,5-trans-Dehydrolysine | 1300 ± 120      | NH\( ^+ \) | CO\( ^- \) | CH\( \_ \) | H  | NH\( ^+ \) |
| 11  | Ornithine\( ^a \)     | 700 ± 150         | NH\( ^+ \) | CO\( ^- \) | CH\( \_ \) | H  | NH\( ^+ \) |
| 12  | Arginine              | >1000             | NH\( ^+ \) | CO\( ^- \) | CH\( \_ \) | H  | NH\( ^+ \) |
| 13  | N-Acetylatedenylamine  | 6.8 ± 0.9         | NH\( ^+ \) | CO\( ^- \) | CH\( \_ \) | H  | NH\( ^+ \) |
| 14  | Histamine             | 330 ± 20          | NH\( ^+ \) | CO\( ^- \) | CH\( \_ \) | H  | NH\( ^+ \) |
| 15  | Ethanolamine          | 1770 ± 150        | NH\( ^+ \) | CO\( ^- \) | CH\( \_ \) | H  | NH\( ^+ \) |
| 16  | Glycine               | >1000             | NH\( ^+ \) | CO\( ^- \) | CH\( \_ \) | H  | NH\( ^+ \) |
| 17  | β-Alanine             | >1000             | NH\( ^+ \) | CO\( ^- \) | CH\( \_ \) | H  | NH\( ^+ \) |

\( ^a \) The apparent inhibition by lysine is the consequence of isotopic dilution of the radiolabeled substrate.

\( ^a \) As described in the “Results,” these samples apparently contained contaminating lysine.

### TABLE 4

| Reaction | Amine substrate | Concentration | % Residual unmodified tRNA | Predicted tRNA mass | Observed tRNA mass | Mass error |
|----------|-----------------|---------------|---------------------------|---------------------|-------------------|------------|
| A        | None (control)  | (100)         | 24,665.5                  | 24,669.4            | 3.9               |
| B        | Lysine          | 0.1           | 24,793.7                  | 24,793.3            | 1.6               |
| C        | Aminohexamidoamine | 0.1    | 24,811.7                  | 24,813.2            | 1.5               |
| D        | Aminohexamidoamine | 0.1    | 24,795.6                  | 24,796.2            | 0.6               |
| E        | (5R)-5-Hydroxlysine | 0.2    | 24,809.7                  | 24,811.5            | 1.8               |
| F        | Cadaverine      | 10            | 24,791.7                  | 24,752.2            | 2.5               |
| G        | 4,5-trans-Dehydrolysine | 1     | 24,791.7                  | 24,793.0            | 1.3               |
| H        | Ethanolamine    | 20            | 24,708.6                  | 24,710.0            | 1.4               |
| I        | N-Acetylatedenylamine | 0.1    | 24,748.6                  | 24,750.1            | 1.5               |
| J        | Histamine       | 5             | 24,758.6                  | 24,759.7            | 1.1               |
| K        | N-α-Methyl-lysine | 20    | 24,807.7                  | 24,794.9            | -12.8             |
| L        | Ornithine       | 10            | 24,779.6                  | 24,794.3            | 14.7              |
Catalytic Flexibility of Lysidine Synthetase

TABLE 5
Evaluation of alternatively TilS-modified tRNAs from Table 4 as substrates for tRNA synthetases

| tRNA substrate (amine modifier) | Relative aminoacylation rate* | E. coli MetRS | E. coli IleRS | B. subtilis IleRS |
|---------------------------------|-------------------------------|--------------|--------------|------------------|
| tRNA\textsuperscript{Met}       | (100)                         | 7            | 0            | 32               |
| Reaction A (control)            |                               | 23           | 2            | 0                |
| Reaction B (lysine)             | (100)                         | 0            | (100)        | (100)            |
| Reaction C (aminoethylcysteine) |                               | 1            | 104          | 127              |
| Reaction D (aminoethylysine)    |                               | 0            | 44           | 95               |
| Reaction E ([SIR]-5-hydroxylsine) |                             | 4           | 23           | 130              |
| Reaction F (cadaverine)         |                               | 1            | 356          | 133              |
| Reaction G (4,5-trans-dehydrolysine) |                           | 2           | 19           | 105              |
| Reaction H (ethanolamine)       |                               | 0            | 123          | 99               |
| Reaction I (N-acetylatediethylamine) |                         | 3            | 31           | 92               |
| Reaction J (histamine)          |                               | 0            | 205          | 116              |

*The 100% reference corresponds to 3240, 2210, or 4600 net cpm for E. coli MetRS, E. coli IleRS, or B. subtilis IleRS, respectively, measured after a 2-h assay as described under “Experimental Procedures.”

or an analog was predicted to generate a modified tRNA with a unique molecular weight. As seen in Table 4, tRNA products with the expected masses were indeed found in reactions A–J. In reactions K and L, however, the molecular weights of the products did not match the predictions and instead matched the product of reaction B which used lysine as substrate. This indicates that low level lysine contamination in the N-α-methyl lysine and ornithine (on the order of 0.1%) accounted for the modified tRNAs formed in reactions K and L, respectively. Trace lysine at such a level likely accounts for all of the potency of these two compounds as (apparent) inhibitors in Table 3 as well. For the eight other lysine analogs, however, the evidence is unambiguous that TilS is capable of accepting alternative substrates for modification of tRNA\textsuperscript{Ile2}.

Nucleotides in the anticodon loop are among the common identity elements that define specific recognition of tRNAs by aminoacyl tRNA synthetases. It has previously been reported that the lysidine modification of cytidine 34 in the anticodon of tRNA\textsuperscript{Ile2} is an absolute determinant for switching recognition from MetRS to IleRS (3). We sought to verify and extend these observations using both partially purified native E. coli and purified recombinant B. subtilis tRNA synthetase enzymes. The availability of differentially TilS-modified tRNA\textsuperscript{Ile2} molecules enabled us to explore in greater detail the specificity determinants of the anticodon for aminoacylation. After appropriate assay conditions were established for coupling of methionine and isoleucine to their respective tRNAs, the tRNAs from TilS reaction mixtures A–J in Table 4 were each tested as a substrate for aminoacylation by MetRS or IleRS. The results are presented in Table 5. Consistent with literature precedent, the unmodified tRNA\textsuperscript{Ile2} (reaction A) showed measurable activity as a substrate for the native E. coli MetRS, although its reaction rate was slower than authentic tRNA\textsuperscript{Met}. Also as expected, aminoacylation with methionine was undetectable after TilS modification of tRNA\textsuperscript{Ile2} with lysine (reaction B). Table 5 newly reveals that all of the alternative modifications made to tRNA\textsuperscript{Ile2} in TilS reactions C–J were equally effective at creating negative determinants for MetRS recognition.

The pattern of substrate utilization were reversed with respect to IleRS. Whereas the rate of aminoacylation of unmodified tRNA\textsuperscript{Ile2} (reaction A) with isoleucine was essentially unde-
imine with a primary amine; this can be fulfilled by amines other than lysine, which is consistent with our observation that alternative TilS-mediated modifications of tRNA \(^{\text{Lys}}\) are able to create recognizable substrates for IleRS. There are clearly species differences among IleRS enzymes in this respect, with our data indicating greater discrimination for the modified cytidine by \(E.\) coli enzyme than by \(B.\) subtilis enzyme. Further experimentation will be required to assess the relevance of the relaxed specificity of TilS and IleRS to bacteria in their native environment.

Analysis of bacterial genomes indicates that tRNA \(^{\text{Lys}}\)’s with a CAU anticodon sequence are widely distributed (18, 19). The cytidines are presumptively modified \textit{in vivo} to introduce the proper determinants for AUA codon recognition and aminoacylation by IleRS. The catalytic flexibility of both TilS and IleRS observed in this work indicates that lysine, at least with regard to its chemical properties, is not uniquely qualified for the modification of the wobble position of tRNA \(^{\text{Lys}}\). The choice of this amino acid may instead be part of a more complex mechanism of regulation of protein biosynthesis. It should be noted, however, that the presence of lysidine has been confirmed in only \(E.\) coli, \(B.\) subtilis, and \(Mycoplasma capricolum\) (2, 20, 21). The tRNA recognizing the isoleucine AUA codon in at least one archaeobacterial species contains a different (non-lysidine) modification (22, 23). This raises the interesting possibility that even some eubacteria may have evolved their TilS and IleRS enzymes to recognize alternatives to lysidine, respectively.

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