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RNA binding determinant in some class I tRNA synthetases identified by alignment-guided mutagenesis

(aminoacyl-tRNA synthetase/multiple sequence alignment/RNA recognition)

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ABSTRACT **The N-terminal nucleotide binding folds of all 10 class I tRNA synthetases (RSs) contain characteristic conserved sequence motifs that define this class of synthetases. Sequences of C-terminal domains, which in some cases are known to interact with anticodons, are divergent. In the 676-amino acid *Escherichia coli* methionyl-tRNA synthetase (MetRS), interactions with the methionine tRNA anticodon are sensitive to substitutions at a specific location on the surface of the C-terminal domain of this protein of known three-dimensional structure. Although four class I synthetases of heterogeneous lengths and unknown structures are believed to be historically related to MetRS, pair-wise sequence similarities in the region of this RNA binding determinant are obscure. A multiple alignment of all sequences of three of these synthetases with all MetRS sequences suggested a location for the functional analog of the anticodon-binding site in these enzymes. We chose a member of this set for alignment-guided mutagenesis, combined with a functional analysis of mutant proteins. Substitutions within two amino acids of the site fixed by the multiple sequence alignment severely affected interactions with tRNA but not with ATP or amino acid. Multiple individual replacements at this location do not disrupt enzyme stability, indicating this segment is on the surface, as in the MetRS structure. The results suggest the location of an RNA binding determinant in each of these three synthetases of unknown structure.**

The aminoacyl-tRNA synthetases (RSs) are a diverse family of enzymes in terms of their primary and quaternary structures. Yet based on characteristic sequence motifs, two groups or classes of enzymes have been identified (1–8). A subgroup of the class I enzymes consists of the cysteinyl, isoleucyl-, leucyl-, methionyl-, and valyl-tRNA synthetases (CysRS, IleRS, etc.). Each of these five enzymes has the conserved 11-amino acid signature sequence ending in the HIGH tetrapeptide (1, 3, 9) and the KMSKS pentapeptide (2) that contribute to the structure of the ATP binding site in all 10 class I enzymes. Within the framework of the solved three-dimensional structure of the *Escherichia coli* MetRS (10), the sequences of the N-terminal domains of the other four subgroup I enzymes can be placed. This N-terminal domain forms the catalytic core of the enzyme; it is responsible for ATP and amino acid binding, adenylate formation, and transfer of the aminoacyl-adenylate to the 3′ end of the tRNA. Inserted into this domain are various-sized segments of nonconserved residues designated connective polypeptides (CP1 and CP2), which reflect the size differences in N-terminal domain of these five enzymes. The connective polypeptide sequences are predicted to be involved in recognition of the tRNA acceptor stem (11, 12).

Fused to the N-terminal nucleotide binding fold in these five related synthetases are C-terminal sequences that are substantially less-well conserved. The sequences of these C-terminal domains reflect some degree of similarity in that they are all predominantly α-helical, in contrast to the β-barrel structure seen in the GlnRS (11), another class I enzyme that is not a member of the MetRS-related subgroup. However, sequence similarities in this domain are difficult to detect among the five related synthetases, with only a few semiconserved peptides present in a multiple sequence alignment of the known sequences (13).

In the cocrystal structure of the GlnRS–tRNA^{Gln} complex (11), interactions between the tRNA anticodon and the C-terminal domain are evident. Similarly, despite a different structure, crosslinking and mutagenesis demonstrated that the C-terminal domain of MetRS is also important for anticodon recognition (14–18). In particular, mutagenesis demonstrated that W461 is a specific determinant for recognition of the tRNA^{Met} CAU anticodon (15). This residue is at the N-terminal end of a short α-helix that projects out from the C-terminal domain (10).

To determine whether a conserved structural framework supporting tRNA anticodon recognition exists in the C-terminal domains of the five related class I enzymes, we sought to identify a functional homolog of the MetRS W461 region in *E. coli* IleRS. Although it has not been demonstrated that the tRNA^{Ile} anticodon alone can direct aminoacylation, a lysidine (L, a modified cytidine) to cytidine change in the LAU anticodon of the AUA-reading tRNA^{Ile} minor isoacceptor switched aminoacylation specificity from isoleucine to methionine (19). This observation is consistent with the idea that the anticodon recognition domains in MetRS and IleRS are similar. If the five enzymes in the related class I subgroup possess similar C-terminal structures despite an evolutionary drift in their primary sequences, then a multiple alignment of the known sequences in this subgroup (13) might identify the anticodon recognition region in IleRS that is analogous to the one in MetRS. For this purpose, a putative functional homolog of *E. coli* MetRS W461 was targeted for mutagenesis, and the resulting mutant enzymes were characterized for their ligand binding capability.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* K-12 TG1 (Δ[*lac-pro*], *supE, thi, hsdD5*/F′ *traD36, proA*$^+$*B*$^+$, *lacI*^{q}, *lacZΔM15*; Amersham) was used as a host for site-directed mutagenesis. The *E. coli* K-12 IQ844/pRMS711 (*ΔileS203*::*kan, recA56, araD139*, Δ[*argF-lac*]*U169, rpsL150, relA1, flbB5301, deoC1, ptsF25, rbsR*/F′[*lacI*^{q}*lac*$^+$*pro*$^+$]; ref. 13) was used as the tester strain for IleRS activity. This strain contains a chromosomal

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Abbreviation: RS, tRNA synthetase.

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deletion of the *ileS* gene encoding IleRS and is propagated by expression of IleRS from the maintenance plasmid pRMS711 (R. Starzyk and P.S., unpublished data) harboring the wild-type IleRS gene and the temperature-sensitive pSC101 replicon (20). Hence, viability of IQ844/pRMS711 is maintained at the permissive temperature of 30°C. At the nonpermissive temperature of 42°C, however, replication of the maintenance plasmid is disrupted, yet growth is complemented by IleRS expression from the phagemid pAS205, which encodes wild-type IleRS in pBluescript KS+ (Stratagene). The pAS205 phagemid was used as a parent for the site-directed mutagenesis of codons K732 and R734.

Construction of Amino Acid Substitution Mutants. Single-stranded DNA isolated from the phagemid pAS205 was used as a template for site-directed mutagenesis with the Amersham oligonucleotide-directed mutagenesis system. Oligonucleotides were designed to randomize codons K732 and R734 by replacing the specified codon with NNC/G (where N = A, T, C, or G), such that all amino acids are encoded while limiting the pool of isoacceptors specifying identical amino acids. Progeny phagemids were sequenced and ampicillin-resistant transformants of the tester strain IQ844/pRMS711 were selected.

***In Vivo* Complementation Assays.** Phagemid pAS205 and mutant derivatives were introduced into the tester strain IQ844/pRMS711 by selecting for transformants at the permissive temperature (30°C). Transformants were scored for growth at the nonpermissive temperature (42°C). The elimination of the temperature-sensitive maintenance plasmid pRMS711 was confirmed by testing for chloramphenicol sensitivity resulting from the loss of the drug-resistance marker carried by this plasmid.

Western Blot Analysis. Small-scale cultures of transformed IQ844/pRMS711 cells harboring wild-type and mutant *ileS* genes were propagated at the permissive temperature. The expression of IleRS derivatives from the pAS phagemid series was induced by the addition of 1 mM isopropyl β-D-thiogalactoside during midlogarithmic growth. After 2 h, cells were harvested, resuspended into SDS sample buffer and fractionated by SDS/PAGE. The separated polypeptides were transferred onto Immobilon-P poly(vinylidene difluoride) membranes using a Milliblot semi-dry blotting apparatus (Millipore) and were incubated with a rabbit anti-*E. coli* IleRS antiserum (21) followed by a horseradish peroxidase-linked donkey anti-rabbit antibody (Amersham). The immunoreactive polypeptides were detected with the ECL chemiluminescent system (Amersham).

Enzyme Purification. Transformed IQ844/pRMS711 cells harboring wild-type and mutant *ileS* genes were propagated at the nonpermissive temperature to eliminate wild-type IleRS expressed from the maintenance plasmid, and loss of pRMS711 was confirmed by testing for chloramphenicol sensitivity. Liter cultures grown at 37°C were induced with 1 mM isopropyl β-D-thiogalactoside in late-logarithmic phase for 6 h. Cells were harvested by centrifugation, resuspended in 50 mM potassium phosphate, pH 7.5/0.1 M NaCl/50 mM 2-mercaptoethanol/0.1 mM phenylmethylsulfonyl fluoride. Cells were disrupted by passage through a French press at 16,000 pounds per square inch gauge (1 psi = 6.9 kPa), and the cell lysate was clarified by centrifugation at 10,000 × *g* for 30 min. After a 45–65% (wt/vol) ammonium sulfate fractionation, the extract was dialyzed against 25 mM Tris·HCl, pH 7.5/1 mM 2-mercaptoethanol/0.1 mM phenylmethylsulfonyl fluoride, applied to an HR10/10 MonoQ column (Pharmacia), and eluted with a linear gradient of 0–350 mM NaCl. Column fractions containing IleRS as determined by SDS/PAGE were pooled and concentrated; due to a high level of overexpression the enzyme preparations were estimated to be >95% homogeneous. Enzyme concentration was initially assessed by dye-binding (Bio-Rad) and then by active-site titration as described (22).

Enzyme Assays. The extent of adenylate formation catalyzed by the purified enzymes was assessed by pyrophosphate exchange (23). IleRS (10 nM) was added at 25°C to the reaction mixture (0.1 M Tris·HCl, pH 7.5/5 mM $MgCl_2$/10 mM 2-mercaptoethanol/10 mM KF/2 mM ATP/2 mM [^{32}P]pyrophosphate/1 mM L-isoleucine). Aliquots were removed at various times and quenched in 7% (vol/vol) perchloric acid, followed by the addition of 3% (wt/vol) charcoal suspended in 0.5% HCl. The ATP adsorbed to charcoal was filtered onto glass-fiber pads (Schleicher & Schuell) and quantified by liquid scintillation counting in Hydrofluor (National Diagnostics, Manville, NJ).

The tRNA-dependent aminoacylation reaction catalyzed by purified IleRS was assayed at 37°C in 20 mM Hepes, pH 7.5/0.1 mM EDTA/0.15 M NH_4Cl/bovine serum albumin (100 μg/ml)/2 mM ATP/4 mM $MgCl_2$/20 μM L-[4,5-^{3}H]isoleucine (0.1 μCi/μl; 1 Ci = 37 GBq; Amersham). Purified $tRNA^{Ile}$ (Subriden RNA, Rolling Bay, WA) was added at a concentration of 0.1–100 μM, and 1–100 nM of active sites of IleRS were used. Aliquots were removed at various times and applied to 3MM filter paper disks (Whatman) that had been presoaked with 10% (wt/vol) trichloroacetic acid. Filters were washed for three 30-min periods in 10% trichloroacetic acid and rinsed in 95% ethanol and then in ether, and radioactivity was measured in Betafluor by scintillation counting. Lineweaver–Burk plots of the aminoacylation data were generated by plotting [E]/*v* vs. 1/[tRNA], where [E] is the enzyme concentration and *v* is rate in seconds.

RESULTS AND DISCUSSION

Targeting Mutagenesis by Multiple Sequence Alignment. Based on multiple sequence alignments of the known sequences of enzymes in the class I subgroup containing CysRS, IleRS, LeuRS, MetRS, and ValRS (13, 24), N-terminal secondary structural elements of *E. coli* IleRS can be modeled after those in the crystal structure of *E. coli* MetRS (10) (Fig. 1*A*). The alternating α-helicies and β-sheets in the nucleotide binding fold are shown as rectangles and pentagons, respectively, with the two insertions or connective polypeptides in this structure shown as loops. The major difference between the proposed nucleotide binding fold domains of MetRS and IleRS is the size of the N-terminal insertions.

The C-terminal domains of these enzymes exhibit little sequence homology. Although *E. coli* MetRS is the only dimer of this class I subgroup, limited proteolysis of the region shown as a wavy line in Fig. 1 results in a fully active truncated monomer (27), suggesting that the removed sequences are responsible for oligomerization. This is consistent with the observation that yeast MetRS is monomeric and has a smaller C-terminal extension (28). In the absence of this oligomerization domain, the C-terminal sequences of the four known sequences of MetRS can be loosely aligned with those of IleRS, by using small blocks of semiconserved sequences (13). The five MetRS sequences are derived from *E. coli* (M-Ec; ref. 29), thermophilic eubacteria *Thermus thermophilus* (M-Tmt; ref. 30), yeast *Saccharomyces cerevisiae* mitochondria (M-Scm; ref. 31), yeast cytoplasm (M-Sc; ref. 28), and *Bacillus stearothermophilus* (Bst-M; ref. 26); the four IleRS sequences are derived from *E. coli* (I-Ec; ref. 1), yeast (I-Sc; refs. 32 and 33), a thermophilic archaebacteria *Methanobacterium thermoautotrophicum* (I-Mt; ref. 34), and the protozoa *Tetrahymena thermophila* (I-Tt; ref. 25). Segments of this multiple sequence alignment are shown in Fig. 1*B*. The sequence similarities are high in the N-terminal regions of the enzymes, exemplified by the sequences span-

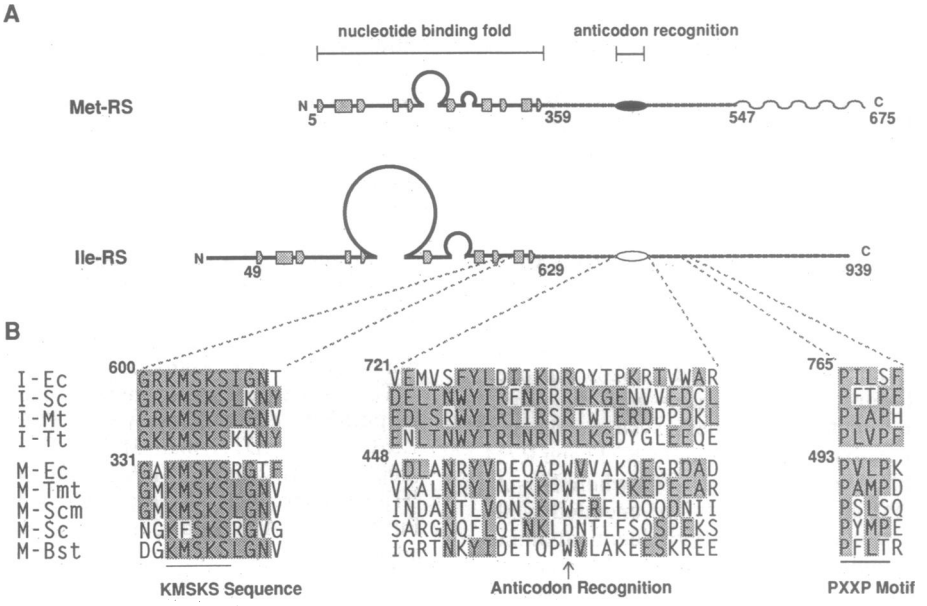

FIG. 1. Primary and secondary sequence similarities between MetRS and IleRS. (*A*) Modeling of the secondary structural elements of IleRS based on the crystal structure of *E. coli* MetRS (10). The N-terminal nucleotide binding fold and domain important for tRNA anticodon recognition are indicated. Rectangles, α-helices; pentagons, β-sheets. (*B*) Three segments of an alignment (13) containing the nine available sequences for MetRS and IleRS. The *Tetrahymena thermophila* IleRS sequence (I-Tt; ref. 25) and *Bacillus stearothermophilus* MetRS sequence (Bst-M; ref. 26) have been added to the previously reported alignment. Shaded residues represent semiconservative substitutions within the IleRS sequences that are shared by individual MetRS enzymes. The three regions shown correspond to the highly conserved KMSKS sequence, the less-well conserved C-terminal region spanning residues important in tRNA anticodon recognition in MetRS (15), followed by the conserved PXXP motif. Ec, *E. coli*; Sc, *S. cerevisiae*; Mt, *M. thermoautotrophicum*; Tt, *Tetrahymena thermophila*; Tmt, *Thermus thermophilus*; Scm, mitochondrial *S. cerevisiae*; Bst, *B. stearothermophilus*.

ning the KMSKS motif. The shaded residues in Fig. 1*B* are semiconservative residues shared among the four IleRS enzymes that may or may not be present in the MetRS sequences. The degree of sequence conservation is much lower in the C-terminal regions of the two enzyme families, specifically around W461 of *E. coli* MetRS that is important for anticodon recognition (highlighted by an arrow). W461 is conserved in four of the five MetRS sequences and is postulated to interact with C34, the first base of the $tRNA^{Met}$ CAU anticodon (15). This poorly conserved region is flanked on the C-terminal side by a block of more conserved amino acids, the PXXP motif (13), that forms a loop between two α-helicies in *E. coli* MetRS (10).

Despite poor primary sequence conservation between IleRS and MetRS in the region of W461, we wondered whether these enzymes shared a similar tertiary structure in this region and, if so, whether the anticodon binding domain of IleRS could be identified using MetRS as a model. A conserved arginine (R734) in the IleRS sequences is at the position analogous to W461 in the alignment. Also, the semiconserved K732 is proximal to the site fixed by the alignment.

To test this alignment and to determine whether an RNA binding site can be predicted for a synthetase of unknown structure, R734 and K732 in *E. coli* IleRS were randomized and the resulting substitution mutants were assessed for their ability to aminoacylate $tRNA^{Ile}$. Mutagenesis of IleRS codons R734 and K732 were individually accomplished by site-directed mutagenesis with oligonucleotides that randomized the original codons. The mutagenic oligonucleotides encoded an equal mixture of each base in the first two positions of the original codon, and a 50% mixture of guanosine and cytidine at the wobble position. These oligomers collectively encode all 20 amino acids but limit the number of possible clones and eliminate the two stop codons that end in adenosine. Clones from the mutagenic library were sequenced, and mutant alleles were introduced into the IleRS *E. coli* null strain IQ844 (13) to assess their activities. This strain is propagated by a temperature-sensitive maintenance plasmid that complements the deficiency resulting from the chromosomal deletion. At the nonpermissive temperature of 42°C, the maintenance plasmid is eliminated and the ability of the mutant alleles to complement the chromosomal deletion was determined.

Characterization of Substitution Mutants. Nine R734 and 16 K732 substitutions were generated and are listed in Table 1, along with their relative abilities to complement the IleRS null strain IQ844. The substitutions did not appear to affect the stability of these expressed alleles as judged by Western blot analysis of crude cell extracts with anti-IleRS polyclonal antibodies. All mutant proteins were detected as full-length proteins with molecular masses on SDS gels identical to the wild-type enzyme (≈105 kDa; data not shown). Of the 9 substitutions of R734, all of the mutants, with the exception of the truncated version, complemented the null strain. These *in vivo* results suggest that the conserved R734 does not play an important role in tRNA recognition and is not likely to serve as the functional homolog of W461 in MetRS. This conclusion was confirmed by an *in vitro* kinetic analysis of the K734A mutant (see below).

Mutagenesis of K732 yielded different results. Despite their overproduction as full-length proteins, the majority of the K732 substitution mutants were unable to support the growth of the IQ844 null strain at 42°C. Only the conservative K732R mutant supported growth as well as the wild-type enzyme. A few of the mutants, namely, those substituted with small hydrophobic and hydroxyl side chains, complemented the null allele yet growth of the complemented strain was very poor. A plus/minus sign in the complementation column (Table 1) represents poor growth after 2 days; a plus/double minus sign represents very poor growth only noticeable after 3 days. Thus, the basic amino group presented by the wild-type lysine or semiconservative arginine is important for enzyme activity. Substitution of a small side chain for lysine or arginine poorly compensates, whereas other side chains are completely inactive. A possible expla-

Table 1. *In vivo* activity of IleRS mutants

Enzyme	Complementation
R734R	+
R734A	+
R734C	+
R734F	+
R734K	+
R734M	+
R734S	+
R734V	+
R734Y	+
R734*	−
K732K	+
K732A	+/−−
K732C	+/−
K732D	−
K732F	−
K732G	+/−−
K732I	−
K732L	−
K732M	−
K732N	−
K732P	−
K732R	+
K732S	+/−−
K732T	+/−
K732V	+/−−
K732W	−
K732Y	−

In vivo activity of stable IleRS mutants. Amino acid substitutions of positions R734 and K732 were assessed for their ability to support cell growth on the IleRS null strain IQ844/pRMS711 (13) at the nonpermissive temperature (42°C) (complementation). +, Wild-type growth after 1 day; +/−, growth after 2 days; +/−−, minimal growth after 3 days; −, no detectable growth after 3 days. Polypeptide stability was determined by Western blot analysis of crude cell extracts containing overexpressed mutant proteins from transformed strain IQ844/pRMS711 at the permissive temperature (30°C). Only proteins of the size expected for full-length enzyme were detected by this analysis, suggesting the mutants had folded into stable structures that were resistant to cellular proteases. R734R and K732K are wild-type IleRSs. R734* contains a stop codon at position 734.

nation for complementation of null strains harboring mutant alleles that contain substitutions of smaller hydrophobic or hydroxyl side chains may be that a small residue provides a pocket for introduction of a new basic residue, such as R734. The poor growth of these complemented strains may be due to the decreased activity of the mutant enzyme or, alternatively, due to a negative effect on cell growth, such as from tRNA mischarging.

To determine the steps of the aminoacylation reaction at which K732-substituted enzymes are defective, representative mutants were purified and characterized *in vitro*. Chosen mutants were purified from the null strain that had been propagated at the nonpermissive temperature to ensure the absence of the wild-type allele. Ammonium sulfate fractionation followed by MonoQ chromatography yielded nearly homogeneous enzyme preparations. The concentrations of these enzyme preparations were determined by active site titrations. Adenylate synthesis and the overall aminoacylation reaction were then assayed.

The characterization of the representative K732T substitution mutant is shown in Fig. 2. The adenylate formation step (Fig. 2*A*) is unaffected by the K732T substitution. The rate of adenylate synthesis is indirectly determined from the rate of isoleucine-dependent exchange of labeled pyrophosphate for ATP catalyzed by the synthetase. The lack of an effect of the K732T substitution on adenylate formation is consistent with the hypothesis that IleRS and MetRS share a

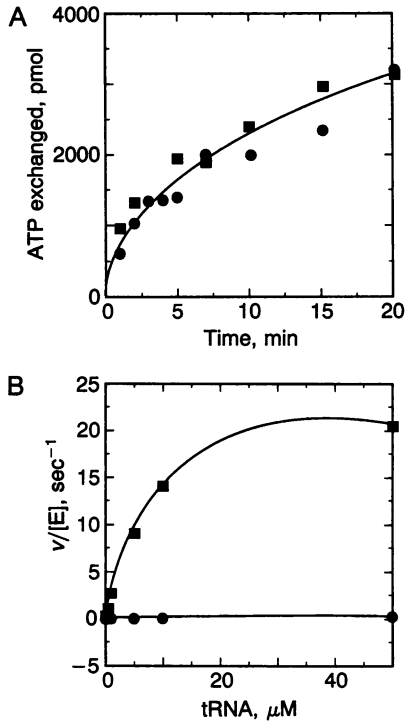

FIG. 2. (*A*) Pyrophosphate exchange catalyzed by wild-type (■) and mutant (●) IleRS. Pyrophosphate exchange was conducted using an enzyme concentration of 10 nM. The rate of ATP–PPi exchange for the K732T mutant is compared to the wild-type enzyme. (*B*) Aminoacylation of tRNA catalyzed by wild-type (■) and mutant (●, K732T) IleRSs. The aminoacylation of $tRNA^{Ile}$ was carried out with synthetases at 3 nM, tRNA at 1 μM, isoleucine at 20 μM, and ATP at 2 mM.

similar structural organization and that the effects of the K732T substitution are localized to the C-terminal tRNA recognition domain. In contrast, the tRNA charging activity of the K732T mutant was severely depressed compared to wild-type IleRS (Fig. 2*B*). Thus, K732 is important for the tRNA-dependent step of aminoacylation.

These data, as well as those for purified K732S, K732R, R734A, and R734K proteins were computed as Lineweaver–Burk plots and used to determine the kinetic parameters shown in Table 2. Consistent with the *in vivo* results, purified R734K and R734A mutants exhibited only minor differences in kinetic parameters relative to wild-type IleRS, confirming that this aligned residue is not a functional homolog of W461 of MetRS. It is unclear why R734 is conserved in all four sequences of IleRS when substitutions yield minimal effects. This result is in contrast to the severe decrease in relative catalytic efficiency (k_{cat}/K_m) observed for the K732T mutant, which approaches three orders of magnitude relative to k_{cat}/K_m for the wild-type enzyme. The major contribution to this defect is the 225-fold increase in the K_m for $tRNA^{Ile}$ as

Table 2. Kinetic parameters for wild-type and mutant IleRSs

Substitution mutant	Relative K_m ($tRNA^{Ile}$)	Relative k_{cat}	Relative k_{cat}/K_m
R734K	1.1	0.78	7.1×10^{-1}
R734A	3.1	0.70	2.3×10^{-1}
K732R	10	0.29	2.5×10^{-2}
K732S	30	0.25	9.0×10^{-3}
K732T	225	0.25	1.7×10^{-3}

Kinetic parameters K_m for $tRNA^{Ile}$, k_{cat}, and k_{cat}/K_m were determined relative to those for wild-type IleRS from Lineweaver–Burk plots. For wild-type IleRS, K_m ($tRNA^{Ile}$) = 4.4 μM, k_{cat} = 21.9 sec^{-1}, and k_{cat}/K_m = 4.6 $sec^{-1} \cdot \mu M^{-1}$.

opposed to a 4-fold decrease in k_{cat}, suggesting that the defect stems from weaker tRNA binding. A similar effect of lower magnitude was observed with the K732S mutant. Were it possible to propagate them in the absence of the wild-type maintenance protein, even greater defects might be detected with other mutants. Surprisingly, even the K732R mutant, which complemented the null strain in a manner indistinguishable from that of wild-type IleRS, yielded an ≈35-fold decrease in k_{cat}/K_m. This observation is of interest because the other three IleRS sequences (Fig. 1) contain arginine rather than lysine at this position.

Concluding Remarks. It is unclear whether K732 is important for the specific recognition of $tRNA^{Ile}$ anticodon bases, because a lysine is also present at the analogous position in three of the five known MetRS sequences. However, despite poor primary sequence conservation in the C-terminal regions of MetRS and IleRS, the use of a sequence alignment to target a residue critical for tRNA recognition in an enzyme of unknown structure suggests that similarities exist between the C-terminal domains of these enzymes. If, as proposed, W461 in *E. coli* MetRS interacts with the first base of the $tRNA^{Met}$ CAU anticodon (15), then the putative K732 homolog in *E. coli* IleRS, which is within a different sequence context, may also serve this role by interaction with the first base of the LAU and GAU anticodons (where L is lysidine) of $tRNA^{Ile}$ isoacceptors.

A multiple sequence alignment of the known sequences of the five enzymes in the class I subgroup may also identify the analogous anticodon binding domains in the LeuRS, ValRS, and CysRS. For ValRS, a lysine at this position in the alignment is conserved in all five published ValRS sequences, whereas a conserved asparagine is present in five of the six published LeuRS sequences (ref. 13 and unpublished data), suggesting that these residues may be important for tRNA recognition. Interestingly, the single CysRS sequence is missing this region and the conserved PXXP motif (where X is any amino acid) found in all other sequences of the four enzymes in the class I subgroup. This observation suggests that CysRS may not interact with the $tRNA^{Cys}$ anticodon or, if is does, the contacts are different than with the other four enzymes. Alteration of the $tRNA^{Cys}$ ACA or GCA anticodons to the amber-reading CUA triplet does not prevent aminoacylation with cysteine, suggesting a lesser role for the anticodon in tRNA recognition of CysRS (35, 36). In any event, to predict residues in the C-terminal domain that are important for tRNA recognition in CysRS, sequences from other organisms are needed.

For MetRS, IleRS, ValRS, and LeuRS, the sequence alignment and mutagenesis results suggest that the C-terminal domains organize into a similar structural framework for tRNA recognition, in which different individual residues confer RNA ligand contacts and context-dependent discrimination. Whereas the position of the putative homologs of IleRS K732 and MetRS W461 are slightly shifted in the alignment, their spatial positions may be similar in the two enzymes; alternately, residues at different spatial positions in the same motif may be responsible for mediating binding contacts.

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