Different Adaptations of the Same Peptide Motif for tRNA Functional Contacts by Closely Homologous tRNA Synthetases†

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ABSTRACT: The N73 nucleotide at the end of the tRNA acceptor stem is commonly used by tRNA synthetases for discrimination. Because only a few synthetase−tRNA cocrystal structures have been determined, understanding of the molecular basis for N73 discrimination is limited. Here we investigated the possibility that, for at least some synthetases, the capacity to recognize different N73 nucleotides resides in the variable sequence of the loop of motif 2, a motif found in all class II enzymes. In the cocrystal of the class II yeast aspartyl-tRNA synthetase, atomic groups of the G73 discriminator of $tRNA^{Asp}$ interact with three side chains of the enzyme. We examined lysyl-tRNA synthetase, a close structural homologue of the aspartyl enzyme. Different substitutions were introduced into the *Escherichia coli* enzyme (A73 discriminator) to make its loop more like that of the human enzyme (G73 discriminator). Our data show that the loop of motif 2 of the lysine enzyme makes tRNA functional contacts, as predicted from the structural comparison. And yet, the *E. coli* enzyme with the "humanized" loop sequence had the same quantitative kinetic preference for A73 versus G as the wild-type enzyme. We conclude that discriminator base selectivity in the lysine enzyme requires residues in addition to or other than those in the loop of motif 2. Thus, even tRNA synthetases that are close structural homologues may use the same RNA binding element to make functional contacts with places (in the acceptor stem) that are idiosyncratic to each synthetase−tRNA pair.

We report here an investigation of the molecular basis for a nucleotide-specific interaction that enables tRNAs to be distinguished from each other in the aminoacylation reaction. In the canonical tRNA structure, the 3′-acceptor end terminates in the sequence $N^{73}CCA_{3'-OH}$. The identity of N73 was originally proposed to be a discriminator that grouped tRNAs according to the chemical nature (charged, hydrophobic, etc.) of the amino acid which was attached (*1*). Subsequently, the importance of the discriminator base in determining the efficiency of aminoacylation was demonstrated for many systems (*2*, *3*). X-ray structural analysis of synthetase cocrystals with $tRNA^{Asp}$, $tRNA^{Ser}$, and $tRNA^{Gln}$ revealed specific interactions that provided a rationale for the functional significance of the discriminator base in these systems (*4*−*6*). However, the applicability of these results for understanding N73 interactions across the entire family of aminoacyl-tRNA synthetases (aaRSs)[1] remains to be investigated. It is this question that was studied here, by choosing a system that is closely related to one of those ($tRNA^{Asp}$) for which detailed structural information is available.

On the basis of sequence and structure similarities of their catalytic domains, the 20 aaRSs are divided into two classes, designated class I and class II (*7*−*10*). Almost invariably, class I aaRSs initially aminoacylate the 2′-OH, while class II aaRSs charge the 3′-OH of A76 (*8*, *11*). The class I active site consists of a nucleotide-binding (Rossman) fold that has the 15-amino acid signature sequence that ends in the tetrapeptide "HIGH" and has the "KMSKS" pentapeptide (*7*, *12*, *13*). The class II catalytic domain is built around an antiparallel β-sheet surrounded by α-helices and embodies three conserved signature motifs, motifs 1−3 (*6*, *9*, *14*−*16*).

The class II synthetases are usually homodimeric (*14*). Motif 1 contributes to dimer formation, and motifs 2 and 3 are main components of the active site (*11*). Motif 2 includes two antiparallel β-strands connected by a variable loop, and motif 3 consists of a strand helix. The β-strands of motif 2 and the adjacent β-strand of motif 3 form the ATP binding pocket and stabilize the aminoacyl adenylate. The loop of motif 2, however, is involved in tRNA recognition.

In the crystal structure of yeast aspartyl-tRNA synthetase (AspRS) complexed with its cognate tRNA (*5*, *17*), four residues of the motif 2 loop (Glu327, Asn328, Ser329, and Thr331) make direct contacts with G73 in the single-stranded acceptor end of the tRNA. These are the only base-specific contacts between G73 and amino acid residues of the bound enzyme (Ser423 and Thr424 bond with the G73 phosphate oxygen). In particular, Glu327, Ser329, and Thr331 make direct contacts with the guanine base through their side chains, while the backbone carbonyl of Asn328 bonds to N2 of guanine.

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[1] Abbreviations: aaRS, aminoacyl-tRNA synthetase; AlaRS, alanyl-tRNA synthetase; AspRS, aspartyl-tRNA synthetase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LysRS, lysyl-tRNA synthetase; PP_i, pyrophosphate; SerRS, seryl-tRNA synthetase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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The significance of these contacts was demonstrated in several ways. For example, the aminoacylation efficiency was reduced more than 100-fold with a G73 → A substitution in $tRNA^{Asp}$ (*18*). Also, small RNA helices that recreate the acceptor stem of $tRNA^{Asp}$ are efficiently charged, provided they encode G73 (*19*). Finally, Glu327 → Gln, Glu327 → Ala, and Ser329 → Ala single point mutations substantially reduce the aminoacylation efficiency (with tRNA as the substrate variable), thus pointing to the importance of these motif 2 loop amino acids for tRNA functional contacts (*20*).

On the basis of sequence relationships, the class II aaRSs can be subdivided. Class IIa includes the histidyl-, prolyl-, seryl-, and threonyl-tRNA synthetases, and class IIb consists of the aparaginyl-, aspartyl-, and lysyl-tRNA synthetases (*11*). Crystal structures of all three class IIb enzymes have been determined (*15*−*17*, *21*). These structures confirm the close relationships between enzymes that were suggested by their sequence similarities. For instance, the catalytic domains of *Thermus thermophilus* AspRS and lysyl-tRNA synthetase (LysRS) are comprised of 361 and 271 residues, respectively. In an alignment of the sequences of these catalytic domains, 46 of these respective residues are identical. In a structural alignment, 120 α-carbons in the respective catalytic domains of *T. thermophilus* AspRS and LysRS can be superimposed with an rms deviation of 0.885 Å (*21*). [Likewise, 290 of the 338 α-carbon atoms in the catalytic domain of *T. thermophilus* asparaginyl-tRNA synthetase can be superimposed with an rms deviation of 1.37 Å on their counterparts in yeast AspRS (*16*).] Among other parts of the structure, the close similarity of the lysine and aspartate enzymes is evident in the motif 2 region. For example, the 32 amino acids of the strand−loop−strand motif of the *Escherichia coli* lysine enzyme can almost be superimposed on their counterparts in yeast AspRS.

Yeast AspRS is the only class IIb enzyme structure that was determined with its bound tRNA completely resolved (*14*, *17*). Given the close relatedness of the aspartyl- and lysyl-tRNA synthetases, we imagined that discriminator base recognition by the lysine enzyme might closely follow that of AspRS. That is, we thought that specific residues in the loop of motif 2 of LysRS might provide the critical contacts with N73, as they do in AspRS. For these experiments, we took advantage of *E. coli* LysRS and its requirement for A73 (*22*, *23*). The idea was to determine whether that recognition could be manipulated from a preference for A73 to a preference for G (Figure 1; *24*). A G73 is found in human $tRNA^{Lys}$ (Figure 1; *24*). In particular, we were encouraged to pursue this kind of approach because of evidence which shows that synthetase−tRNA contacts coadapt during the course of evolution (*25*, *26*). The loop of motif 2 of the human enzyme is closely similar to that of the *E. coli* enzyme. Thus, substitution of the human sequences into the motif 2 loop of the *E. coli* enzyme might compensate for the A73 → G change that occurred during the course of evolution.

MATERIALS AND METHODS

Mutant Construction, Protein Expression, and Purification. The plasmid pQB263 (Cubist Pharmaceuticals, Cambridge, MA) was used as a source for the *E. coli* LysRS (*lysU*) gene. [To construct pQB263, the *lysU* insert derived from pMAK705 originally described by Chen et al. (*27*) was cloned into pQB169 (*26*).] A 1.57 kb *Nde*I−*Not*I PCR fragment of plasmid pQB263 containing the *E. coli lysU* gene was cloned into the pET-3a-based expression vector pM131 (*28*) to generate plasmid pBAS6. A C-terminal six-histidine affinity tag was appended to the enzyme by the substitution of a *Bss*HII−*Not*I fragment of the *lysU* gene for a PCR-amplified fragment encoding the six-histidine terminus. The resulting plasmid (pBAS39) was used for expression and purification of *E. coli* LysRS. (The six-histidine tagged synthetase possessed normal LysRS enzymatic activity.)

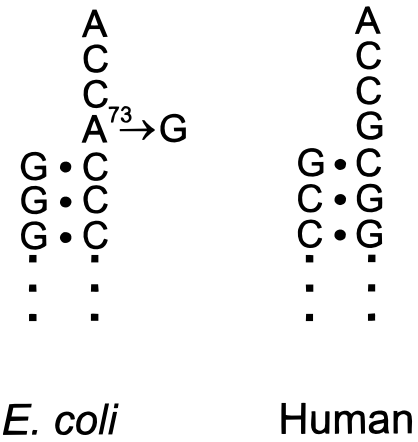

FIGURE 1: Sequences and base pairing of the *E. coli* and human $tRNA^{Lys}$ acceptor ends with the N73 discriminator base (*24*).

To construct mutations in the motif 2 loop, a *Mlu*I site was introduced into the *lysU* gene in the motif 2 coding region. This new site (that did not change the encoded amino acid) facilitated the generation of motif 2 loop mutations by PCR in one step using a single mutagenic primer. Mutant *Mlu*I−*Not*I fragments were cloned into the pBAS39 expression vector by exchange with the corresponding fragment in *lysU*. The resulting plasmids were verified by DNA sequencing.

The enzymes were purified from strain BL21(DE3) (*29*). Cultures were grown at 37 °C in 2 L of Lennox L broth (American Bioanalytical, Natick, MA) containing 100 μg/mL ampicillin to an optical density (at 600 nm) of 0.4−0.6. The cells were then induced by the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside and incubated for an additional 3 h. The cells were harvested and resuspended in 10 mL of lysis buffer [20 mM sodium phosphate (pH 7.4), 500 mM NaCl, 20 mM imidazole, 10 mM β-mercaptoethanol, and 10% glycerol]. Following the addition of 10 μL of a saturated 2-propanol solution of phenylmethanesulfonyl fluoride, the cells were lysed with a French press (12 000 psi) and the lysate was centrifuged for 30 min at 100000*g*. The supernatant was diluted to a final volume of approximately 30 mL in lysis buffer and incubated (30 min at 4 °C with gentle mixing) with 5 mL of Ni-NTA agarose (Qiagen, Santa Clarita, CA). Following incubation, the resin was washed six times in lysis buffer. Each wash entailed gently centrifuging the resin (approximately 1 min at 1000*g*), carefully removing the supernatant, and resuspending the resin in 40 mL of fresh buffer. Bound six-histidine tagged LysRS was eluted by the addition of 5 mL of lysis buffer containing 250 mM imidazole to the resin followed by gentle mixing at 4 °C for 5−10 min. The resin was centrifuged as described above, and elution was performed twice. The eluted

protein was concentrated with a Centriprep 30 concentrator (Amicon, Beverly, MA) and stored in 50% glycerol at −20 °C. Enzyme concentrations were determined by the Bradford protein assay (*30*) using bovine serum albumin as a standard and by active site titration (*31*). Typically, 15−20 mg of the pure enzyme was obtained per liter of cell culture.

Transfer RNA Preparation and Mutant Construction. A pUC18-based plasmid containing the wild-type *E. coli* $tRNA^{Lys}$ gene in front of the T7 RNA polymerase promoter was generously provided by M. Saks (University of Oregon, Eugene, OR). A *Bst*NI site at the 3′-end of the gene allowed generation of a linear template that provided the correct 3′-CCA terminus in the tRNA by in vitro runoff transcription (*32*). The G73 → A mutation in the *E. coli* tRNA was introduced by overlap extension PCR mutagenesis using the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The resulting mutant plasmid was verified by DNA sequencing. In vitro transcription reaction mixtures contained 0.1 μg/μL *Bst*NI-linearized plasmid in 40 mM tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) buffer (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 20 mM dithioerythritol, ATP, CTP, GTP, and UTP (4 mM each), 0.5 unit/μL ribonuclease inhibitor, and >20 units/μL T7 RNA polymerase. The transcription reactions were stopped by phenol/chloroform extraction, and the tRNA transcripts were purified by denaturing electrophoresis (12% polyacrylamide/8 M urea) followed by electroelution and ethanol precipitation. The purified tRNA was resuspended in 100 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 7.5). Prior to the assay being carried out, the tRNA was denatured by heating to 80 °C followed by slow cooling to 55 °C, at which time 0.1 M $MgCl_2$ was added (2 mM final concentration) with subsequent cooling to room temperature. Concentrations were determined by absorbance at 260 nm ($\epsilon_{260} = 6.9 \times 10^5$) and were verified by aminoacylation plateau levels (ca. 1500 pmol/A_{260}).

Enzyme Assays. Lysyl adenylate synthesis was assessed with the lysine-dependent ATP−pyrophosphate (PP_i) exchange assay at 37 °C (*34*). The reaction mixture contained 100 mM Tris-HCl (pH 7.5), 10 mM KF, 7 mM β-mercaptoethanol, 5 mM $MgCl_2$, 1 mM ATP, 2 mM $NaPP_i$, 2 mM lysine, 0.1 mg/mL bovine serum albumin, and approximately 0.01 μCi/μL [^{32}P]$NaPP_i$. Reactions were initiated with the addition of LysRS (usually 50 nM final concentration). As previously outlined (*34*), aliquots were removed at various times and the reactions quenched in a mixture containing 0.2 M $NaPP_i$, 7% perchloric acid, and 3% activated charcoal. The charcoal mixture was filtered and washed with a solution of 10 mM $NaPP_i$ and 0.5% perchloric acid on a vacuum manifold using a glass fiber filter. The filters were then measured by scintillation counting to determine ^{32}P levels in charcoal-adsorbed ATP.

Aminoacylation assays were performed at 37 °C in a reaction mixture consisting of 100 mM HEPES (pH 7.5), 1 mM 1,4-dithiothreitol, 10 mM $MgCl_2$, 2.5 mM ATP, 20 μM lysine, 0.02 μCi/μL [^{3}H]lysine, and 0.5−24 μM tRNA. Reactions were initiated with the addition of LysRS (typically 500 nM final concentration). As previously described (*33*), aliquots were removed at appropriate time intervals, spotted onto trichloroacetic acid-soaked Whatman (grade 3) filter pads, washed, and measured by liquid scintillation counting. Kinetic parameters (k_{cat} and K_M) for aminoacylation and PP_i exchange were determined by direct fitting of the Michaelis−Menten equation by nonlinear regression analysis (*35*).

RESULTS

Motif 2 Loop Mutants in Lysine Activation and tRNA Aminoacylation. Yeast AspRS is a dimer of identical 564-amino acid polypeptides (*17*). There are two major domains: a C-terminal catalytic domain that contains the three canonical motifs that define the class II enzymes and an N-terminal anticodon binding domain (*17*). In the AspRS−$tRNA^{Asp}$ structure, three motif 2 loop side chains make contacts with G73 in the tRNA acceptor stem. The carboxyl of Glu327 hydrogen bonds to N2 of G73, and the hydroxyl groups of Ser329 and Thr331 bond with N1 and O6 of G73, respectively (Figure 2, top) (*5*).

E. coli LysRS is a dimer of identical 504-amino acid polypeptides (*15*). In the C-terminal catalytic domain, the loop of motif 2 (12 amino acids) is essentially the same size as that of the yeast aspartyl enzyme [13 amino acids; for class II enzymes as a whole, the motif 2 loop varies from about 8 to 20 amino acids (*8*)]. The motif 2 sequences of human and *E. coli* LysRS are closely similar. In particular, throughout the entire 32-amino acid strand−loop−strand sequence, 25 residues are identical or have a conservative replacement. In the loop itself, only three contiguous residues are different, where the human enzyme has the sequence Asp-Leu-Thr (DLT) and the *E. coli* enzyme has the sequence Ser-Val-Arg (SVR) (Figure 2, bottom). These residues correspond to positions 267−269, respectively, in the sequence of *E. coli* LysRS.

The aminoacylation reaction occurs in two steps: condensation of amino acid with ATP to form the aminoacyl adenylate, followed by transfer of the aminoacyl group from the adenylate to the tRNA. In the class II aspartyl-, asparaginyl-, and seryl-tRNA synthetases, the strands of motif 2 form part of the ATP binding site (*16*, *20*, *36*). While no structure is yet available for the class II alanyl-tRNA synthetase (AlaRS), both strand and loop residues in motif 2 are important for transactions involving the aminoacyl adenylate (*37*). For these reasons, we were first interested in determining whether substitutions in the loop of motif 2 of LysRS would affect aminoacyl adenylate synthesis. Were adenylate synthesis to be affected, conclusions regarding the effect of substitutions on the tRNA-dependent step of aminoacylation would be more difficult to resolve or interpret.

Aminoacyl adenylate synthesis was followed by the lysine-dependent ATP−PP_i exchange assay. Three individual mutations replaced an amino acid in the loop of motif 2 of the *E. coli* enzyme with its counterpart from the human enzyme. These substitutions were S267D, V268L, and R269T, respectively. In addition, we constructed a protein containing the triple mutation SVR → DLT. Each of these four enzymes was stably overexpressed and readily purified.

The four mutant enzymes had ATP−PP_i exchange activities that were indistinguishable from that of the wild-type *E. coli* enzyme. An example of the time dependence of the exchange reaction for the five proteins is shown in Figure 3. Because the activities of the five enzymes are quantitatively similar, S267, V268, and R269 most likely make neither direct nor indirect contacts with the aminoacyl

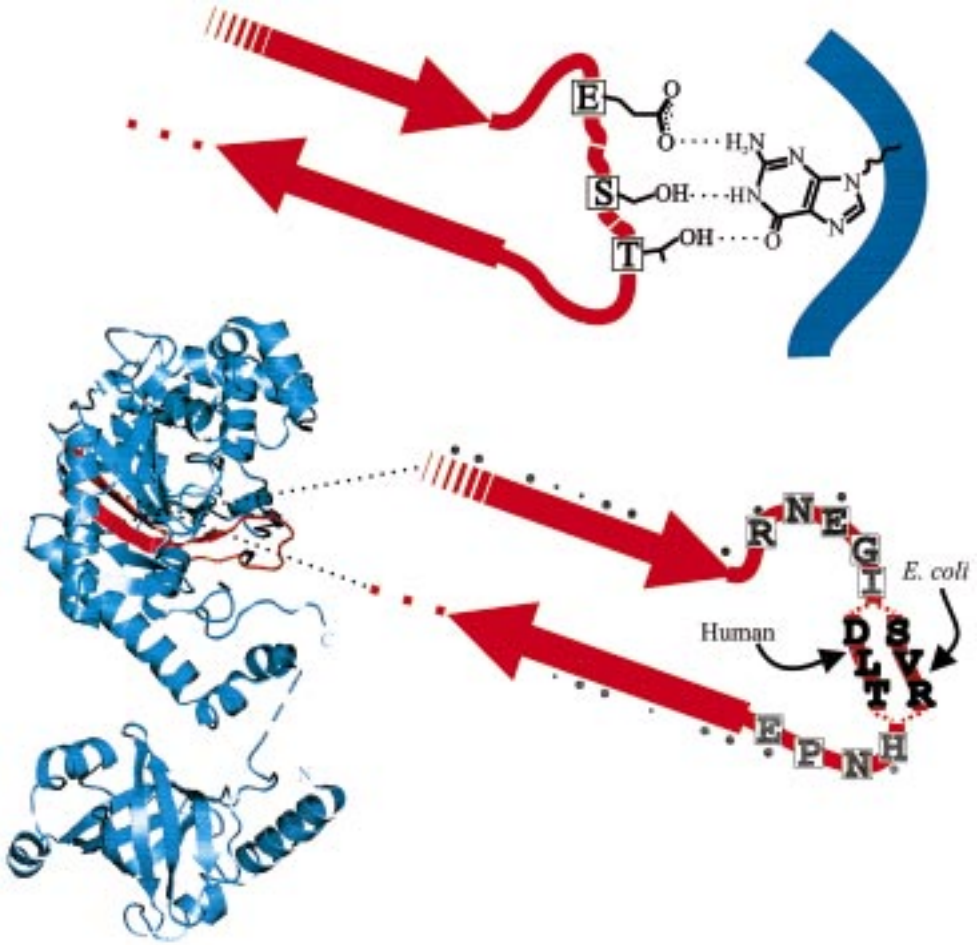

FIGURE 2: (Top) Schematic representation of amino acid side chain contacts in the AspRS motif 2 loop with unpaired base G73 of the $tRNA^{Asp}$ acceptor stem. Motif 2 consists of two antiparallel β-strands (red arrows) connected by a variable loop (*5*). (Bottom) The structure of *E. coli* LysRS (*15*). A dashed line represents a seven-residue peptide not resolved in the electron density map which connects the catalytic (C-terminal) and anticodon-binding (N-terminal) domains. In a schematic diagram on the right, the motif 2 loop sequences of *E. coli* and human LysRS are compared. The loops only differ by a tripeptide segment in the central region (black lettering). Conservative substitutions (·) and regions of identity (●) between yeast AspRS and human and *E. coli* LysRS are denoted.

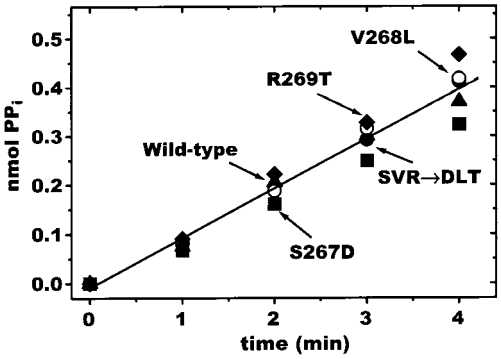

FIGURE 3: Comparison of wild-type and mutant *E. coli* LysRS amino acid activation activities at pH 7.5 and 37 °C. The pyrophosphate exchange activity of the wild-type enzyme is the same as that of the triple mutant enzyme and each of the enzymes possessing point mutations. The activity was assayed as described in Materials and Methods.

adenylate, ATP, or lysine. Any effect of these mutations on tRNA aminoacylation can, therefore, be ascribed to tRNA functional contacts.

Next we investigated the aminoacylation of $tRNA^{Lys}$ with the four mutant enzymes. The SVR → DLT triple mutation reduced aminoacylation efficiency more than 17-fold (Figure 4, top). Thus, this substitution disrupts a tRNA functional contact. Because the enzyme was stably produced in *E. coli*, and because it has the full adenylate synthesis activity of the wild-type enzyme, we imagine that the effect of the triple substitution is highly localized and does not perturb the overall structure of the protein.

We then attempted to deconvolute the strong effect of the triple substitution by investigating the effect of each of the point mutations. All of these enzymes were defective in aminoacylation activity (Figure 4, bottom). However, none of them had an activity reduced as much as that of the triple mutant. The smallest effect was seen with the S267D-substituted enzyme, and the largest with the R269T replacement. Thus, the more severely reduced activity of the triple mutant is due in large part to additive effects from each of the point substitutions.

A73 versus G Specificity. Having established a role for the three residues in the loop of motif 2 for tRNA functional contacts, and not for adenylate synthesis, we asked whether these residues might provide the basis for discrimination between A73 (as in *E. coli*) and G73 (as in eukaryotes, including humans) (*24*). Previously, it was found that a simple peptide swap between the class I human and bacterial tyrosyl-tRNA synthetases was sufficient to switch recognition of the first base pair of the acceptor stem from G1·C72 to

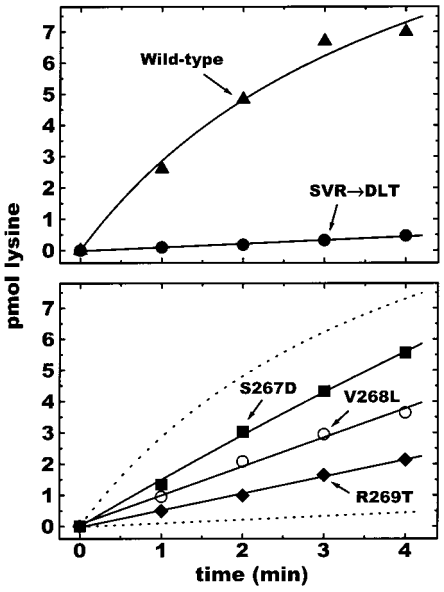

FIGURE 4: Comparison of wild-type and mutant *E. coli* LysRS aminoacylation activities at pH 7.5 and 37 °C. (Top) Charging of $tRNA^{Lys}$ (A73) by *E. coli* LysRS containing a human motif 2 loop (SVR → DLT) compared to charging with the wild-type enzyme. (Bottom) Charging of $tRNA^{Lys}$ (A73) by *E. coli* LysRS mutants containing single (*E. coli* → human) substitutions in the loop of motif 2. The lysylation activities of the wild-type enzyme and of the triple mutant enzyme are shown (dashed line) for comparison. The activity was assayed as described in Materials and Methods.

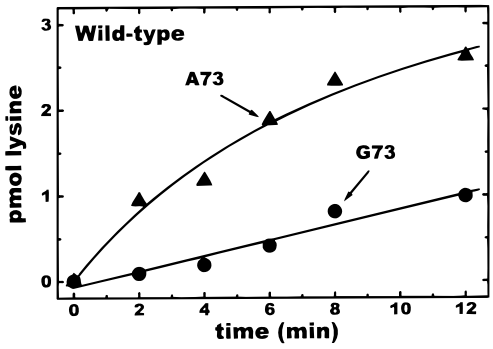

FIGURE 5: N73 discrimination by wild-type *E. coli* LysRS. Lysylation of $tRNA^{Lys}$ with either an A or a G at position 73 at pH 7.5 and 37 °C. The activity was assayed as described in Materials and Methods.

C1·G72 (*26*). Similarly, a peptide swap between isoleucyl- and methionyl-tRNA synthetases succeeded in switching specificity for a single nucleotide in the anticodon loop (*38*). Thus, by analogy with these systems, we thought that one or more of the substitutions made in the loop of motif 2 of LysRS might switch the specificity from A to G.

For this purpose, a G73 mutant of *E. coli* $tRNA^{Lys}$ was constructed and tested with the wild-type enzyme and each of the four mutant enzymes. We found that, with the wild-type enzyme, the rate of aminoacylation of G73 $tRNA^{Lys}$ was reduced about 5.4-fold relative to that of wild-type *E. coli* $tRNA^{Lys}$ (Figure 5; see below). [This reduction for the mutant substrate is about the same as that reported earlier by Tamura et al. (*23*).] Thus, a discriminator base substitution, while significant, is less deleterious than that seen with other class II enzymes, such as the alanyl-, aspartyl-, and seryl-tRNA synthetases (*18*, *39*, *40*).

We then investigated each of the mutant enzymes with the G73 $tRNA^{Lys}$ substrate, and compared the activity with

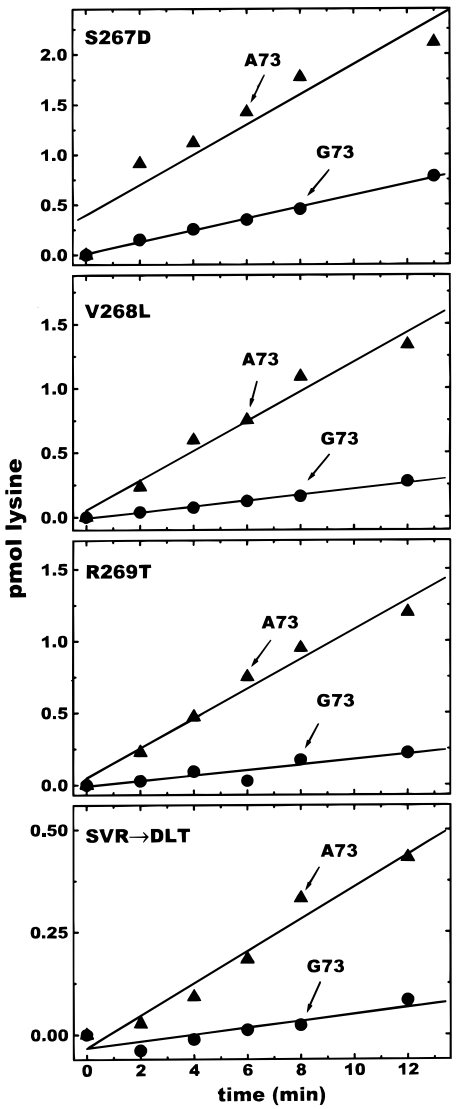

FIGURE 6: N73 discrimination by mutant *E. coli* LysRS enzymes. The relative extent of lysylation of G73 and A73 $tRNA^{Lys}$ is equivalent to that of the wild-type enzyme (see Figure 5) for both the triple mutant enzyme and the enzymes possessing point mutations. The activity was assayed as described in Materials and Methods.

that of the wild-type enzyme. In all cases, the mutant enzyme showed a substantial preference for the wild-type A73-containing substrate (Figure 6). For the triple SVR → DLT mutant protein, the A73 versus G preference is the same as that of the wild-type enzyme (5.4-fold; see below). Thus, even with the motif 2 loop residues from the G73-preferring human enzyme, the *E. coli* protein has the same preference for A73 as does the wild-type enzyme. We conclude that, while the replaced motif 2 loop residues make functional contacts with the tRNA substrate, these contacts by themselves do not provide A73 versus G discrimination.

Kinetic Parameters Affected by Functional Contacts. To clarify the nature of the functional interactions of the motif 2 loop with $tRNA^{Lys}$, we determined kinetic parameters for the aminoacylation of wild-type and G73 $tRNA^{Lys}$ with the wild-type and with the SVR → DLT mutant enzyme. This analysis showed that, for both enzymes, the major effect of the A73 → G substitution in $tRNA^{Lys}$ was in the transition state of catalysis as measured by k_{cat} (Table 1). Little effect of the G73 substitution in $tRNA^{Lys}$ was seen on the K_M for

Table 1: Comparison of Kinetic Parameters of Wild-Type *E. coli* LysRS and *E. coli* LysRS with a Human Motif 2 Loop Replacement (SVR → DLT) at pH 7.5 and 37 °C[a]

	k_{cat} ($\times 10^{-2}$ s^{-1})		K_m (μM)[b]		k_{cat}/K_m (s^{-1} M^{-1})	
activity	wild-type	SVR → DLT	wild-type	SVR → DLT	wild-type	SVR → DLT
tRNA^{Lys} (A73) aminoacylation	7.0 ± 1.0	0.29 ± 0.03	13 ± 4	10 ± 3	5385	290
tRNA^{Lys} (G73) aminoacylation	1.2 ± 0.1	0.075 ± 0.007	12 ± 4	14 ± 2	1000	54
PP_i exchange	350 ± 30	360 ± 32	11 ± 3	9 ± 3	3.2×10^6	4.0×10^6

[a] Values of k_{cat} and K_m were determined as described in Materials and Methods. [b] K_m for A73/G73 tRNA^{Lys} (aminoacylation) or lysine (PP_i exchange).

either the wild-type enzyme or the enzyme bearing the triple mutation in the loop of motif 2. We further confirmed that the mutation specifically affected the tRNA interaction by showing that the K_M for lysine was unaffected by the SVR → DLT substitution. Thus, the manipulated residues of motif 2 are important for functional contacts with tRNA in the transition state of catalysis.

DISCUSSION

When compared to other class II enzymes such as yeast AspRS, *E. coli* AlaRS, and *T. thermophilus* seryl-tRNA synthetase (SerRS) (*18*, *39*, *40*), *E. coli* LysRS has a relatively modest preference (5.4-fold) for a specific discriminator base (i.e., A73). An even greater lack of sensitivity for a particular N73 base is found for the human enzyme (*28*). For example, a transcript encoding human tRNA^{Lys} (G73 discriminator) with an A73 replacement is aminoacylated by the human enzyme with an efficiency that is reduced 0.43-fold (relative to that of the transcript for the wild-type sequence). When the *E. coli* is compared with the human enzyme, the difference in discriminator base preference for A73 is about 12-fold (5.4/0.43). [That is, the *E. coli* enzyme has a 12-fold greater preference (as measured by k_{cat}/K_M) for A73 compared to the human enzyme.] This difference amounts to about 1.5 kcal/mol of apparent free energy of transition state stabilization.

Our investigations were motivated by the detailed structural information that is available for the yeast AspRS cocrystal with tRNA^{Asp}, a close homologue of the lysine enzyme. In the aspartyl system, all base-specific contacts with N73 are self-contained within the loop of motif 2 (*5*). We imagined that, if contacts with N73 were made through the residues we changed in the motif 2 loop of the *E. coli* lysyl enzyme, then a difference in specificity that amounted to 1.5 kcal/mol would be great enough to determine experimentally. Thus, by "humanizing" the motif 2 loop of the *E. coli* enzyme, we anticipated a potential change in the preference for A73 from 5.4-fold to a preference for G73 of about 2.3-fold (1/0.43).

Instead, all four of the mutant *E. coli* enzymes with residues from the human protein still manifested a clear preference for A73, and remarkably, the triple mutant had the same 5.4-fold preference for A seen with the wild-type enzyme. This result suggests that residues in the loop of motif 2 are not in themselves sufficient to account for the discriminator base specificity of the *E. coli* and human lysine enzymes. Thus, residues in addition to or entirely independent of those in the loop of motif 2 are responsible for the A versus G discrimination. (Given the virtually identical A versus G discrimination of the wild-type enzyme and the one containing the triple mutation, we question whether the loop of motif 2 has a direct role in N73 functional recognition in this system.) In contrast, simple and highly localized peptide transplants were sufficient to switch base specificity in two studies of class I tRNA synthetases (*26*, *38*). Peptide transplants have also been used successfully to switch base specificities among DNA binding proteins (*41*, *42*).

A recurrent theme in the study of recognition by tRNA synthetases is the role of the k_{cat} parameter. In general, both k_{cat} and K_M parameters (with respect to tRNA) determine the aminoacylation specificity. In contrast to DNA−protein complexes that often have dissociation constants in the nanomolar to picomolar range, synthetase−tRNA complexes typically have dissociation constants that are much higher, i.e., on the order of 1 μM (*43*). The rationale is that the synthetases have to turn over rapidly during protein synthesis. Because equilibrium dissociation constants are usually determined by the kinetic rate constants for complex dissociation, a small dissociation constant prevents rapid enzyme turnover. With a dissociation constant in the micromolar range, the capacity for discrimination between tRNAs by binding interactions alone is limited. Thus, k_{cat} discrimination plays a major role in ensuring the high specificity of aminoacylation that is needed to preserve the accuracy of the genetic code. Indeed, with respect to the functional contacts made by the loop of motif 2 with tRNA^{Lys} studied here, these contacts impact mostly k_{cat}.

Given the evidence presented here that the motif 2 loop of *E. coli* LysRS does not make functional contacts with the N73 discriminator base, our results raise the possibility that this loop may interact with a different, nearby part of the tRNA structure. One possibility is that base pairs that are at the beginning of the acceptor helix, adjacent to the discriminator base (Figure 1), interact with the motif 2 loop of LysRS. In the class II SerRS, the loop of motif 2 is larger and interacts with base pairs that are within the acceptor stem helix (*6*, *44*). However, SerRS is not a member of class IIb, and with a larger loop in motif 2 (20 vs 12 residues in LysRS), it is not clear that this comparison is useful. However, a small adjustment in the position of the motif 2 loop of LysRS might make possible a functional interaction with C74 or with the G1·C72 base pair of tRNA^{Lys} (Figure 1). Indeed, flexibility of the motif 2 loop is seen in the crystal of *E. coli* LysRS (*15*). In the serine system, this loop is ordered upon tRNA binding (*6*). Thus, loop−tRNA contacts may result from an induced fit upon tRNA binding and may be idiosyncratic to the synthetase−tRNA pair. Future experiments can address this possibility.

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