Efficient aminoacylation of tRNA$^{\text{Lys,3}}$ by human lysyl-tRNA synthetase is dependent on covalent continuity between the acceptor stem and the anticodon domain

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ABSTRACT

In this work, we probe the role of the anticodon in tRNA recognition by human lysyl-tRNA synthetase (hLysRS). Large decreases in aminoacylation efficiency are observed upon mutagenesis of anticodon positions U35 and U36 of human tRNA$^{\text{Lys,3}}$. A mini-helix derived from the acceptor-ΨC stem–loop domain of human tRNA$^{\text{Lys,3}}$ was not specifically aminoacylated by the human enzyme. The presence of an anticodon-derived stem–loop failed to stimulate aminoacylation of the mini-helix. Thus, covalent continuity between the acceptor stem and anticodon domains appears to be an important requirement for efficient charging by hLysRS. To further examine the mechanism of communication between the critical anticodon recognition elements and the catalytic site, a two piece semi-synthetic tRNA$^{\text{Lys,3}}$ construct was used. The wild-type semi-synthetic tRNA contained a break in the phosphodiester backbone in the D loop and was an efficient substrate for hLysRS. In contrast, a truncated variant that lacked nucleotides 8–17 in the D stem–loop displayed severely reduced catalytic efficiency. The elimination of key tRNA tertiary structural elements has little effect on anticodon-dependent substrate binding but severely impacts formation of the proper transition state for catalysis. Taken together, our studies provide new insights into human tRNA structural requirements for effective transmission of the anticodon recognition signal to the distal acceptor stem domain.

INTRODUCTION

The relationship between aminoacyl-tRNA synthetases and tRNAs establishes the genetic code whereby specific amino acids are esterified to cognate anticodon-bearing tRNAs during translation. All tRNAs have similar secondary and tertiary structures. Synthetase interaction with the cognate tRNA is based on the presence of identity elements within the tRNA that allow for specific binding and catalysis (1). Both the bacterial and yeast recognition systems have been extensively studied. In general, tRNA identity elements are contained primarily within the acceptor stem and anticodon domains (1). These two domains are located at opposite ends of the L-shaped tRNA tertiary structure (2). Precisely how communication between the critical anticodon recognition elements and the catalytic site located ~76 Å away occurs is in general an open question for the synthetases, and undoubtedly involves a complex set of protein–RNA interactions outside the primary recognition sites (1).

Based on both primary sequence and structural data the aminoacyl-tRNA synthetases have been divided into two major classes each containing 10 members (3). The two classes of synthetases have been further organized into distinct subclasses. Class II aminoacyl-tRNA synthetases are characterized by a highly degenerate set of sequence elements known as motifs 1, 2 and 3. The enzymes that belong to subclass Ib (asparaginyl-, aspartyl- and lysyl-tRNA synthetases) are further distinguished by an N-terminal oligonucleotide-binding (OB) fold that recognizes anticodons with uridine at the second position (U35) (4). The specific molecular interactions between this N-terminal domain and the anticodon have been characterized using biochemical and structural methods for yeast aspartyl-tRNA synthetase (AspRS) (5–7) and both Escherichia coli and Thermus thermophilus lysyl-tRNA synthetases (LysRS) (8–10). Moreover, in the case of E.coli LysRS, mutagenesis of anticodon nucleotides 35 and 36 resulted in ≥100-fold decrease in in vitro aminoacylation efficiency, whereas mutagenesis of U34 to G had a less dramatic effect (17-fold) on activity (11).

In the case of yeast AspRS, in addition to the anticodon, the acceptor stem contains important recognition elements that are known to make specific contacts with the enzyme (5,7). In particular, mutations at the so-called ‘discriminator’ base located at position 73 result in decreases in in vitro catalytic efficiency that are similar in magnitude to those observed upon anticodon mutagenesis (6). In this system, a minihelix derived from the acceptor stem domain is a substrate for aminoacylation, albeit with a $k_{\text{cat}}$ that is reduced ~9000-fold relative to full-length tRNA$^{\text{Asp}}$ (12). Attempts to further stimulate minihelix$^{\text{exp}}$ aminoacylation by addition of a separate anticodon stem–loop were unsuccessful (12). Thus, in this subclass Ib yeast system, covalent attachment of the anticodon domain to the acceptor domain appears to be a requirement for effective communication between these two key sites.

In contrast to the yeast AspRS system, previous investigations of another subclass Ib yeast synthetase, namely human LysRS...
tRNA Lys,3, assays contained 12.5 nM LysRS and tRNA concentrations of 0.25–8 µM. Concentrations of the inhibitors tested were as follows: 0–8 µM 3′-oxidized tRNAlys,3; 0–60 µM U35G tRNAlys,3; 0–100 µM minihelixlys,3; 0–20 µM UUU anticodon stem–loop; 0–100 µM U35G anticodon stem–loop. Dixon plots were produced and the K_i values were determined from replots of the slopes versus 1/[tRNA] (15).

T7 RNA polymerase was purified according to Grodberg and Dunn (16) from E.coli strain BL-21/pAR 1219, which was a gift from Dr F. William Studier.

Ribonucleic acids

Unmodified wild-type human tRNAlys,3 was prepared by in vitro transcription from FokI-linearized plasmid pLYSF119 as previously described (13). Mutant tRNAlys,3 variants as well as minihelixlys,3 derived from the acceptor-TΨC stem–loop domain of tRNAlys,3 were prepared by overlap extension PCR mutagenesis (17) and verified by sequencing (18). The gene for 3′-59mer tRNAlys preceded by a T7 RNA polymerase promoter was overexpressed in a BL21 strain. 

RESULTS

We have previously shown that in vitro transcripts of human tRNAlys,3 are substrates for both the N-terminal truncated and full-length forms of hLysRS. Both forms of hLysRS were able to efficiently aminoacylate both native and unmodified E.coli tRNAlys,3 indicating that the base modifications are not critical for cross-species aminoacylation (13). In the present study, we used partially purified native human tRNAlys in aminoacylation assays to examine the effect of base modifications on cognate charging by hLysRS. The k_0 cat/K_m shows a 10-fold decrease for the unmodified transcript compared to the native transcript.
human tRNA\textsubscript{Lys}, indicating that the base modifications make a modest contribution to recognition of human tRNA\textsubscript{Lys} by hLysRS.

**Anticodon mutations**

Figure 1 shows the structure of unmodified human tRNA\textsubscript{Lys,3} and the individual mutations at the last two positions of the anticodon that were prepared and tested using hLysRS-ΔN65. The results show that mutations at either of these two positions have a significant effect on aminoacylation efficiency, with decreases of 150- to >3000-fold at position 35, and 11- to 180-fold at position 36. The most severe effect was observed upon U35G substitution, which resulted in a complete loss of aminoacylation activity. In general, purine substitutions at both positions resulted in more severe decreases than pyrimidine substitutions (Fig. 1 and Table 1). As shown in Table 1, individual kinetic parameters were derived from Lineweaver–Burk plots and are the average of two or three determinations carried out at 30°C with an average standard deviation of ±52%. ND indicates that the individual kinetic parameters could not be reliably determined because of the high $K_m$ compared to tRNA concentrations routinely used in the assay. No entry indicates there was no detectable activity.

### Table 1. Kinetic parameters determined from *in vitro* aminoacylation of human tRNA\textsubscript{Lys,3} variants using hLysRS-ΔN65

<table>
<thead>
<tr>
<th>tRNA\textsubscript{Lys,3} variant</th>
<th>$k_{\text{cat}}$ (s\textsuperscript{-1})</th>
<th>$K_m$ (µM)</th>
<th>$k_{\text{cat}}/K_m$ (relative)</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.38</td>
<td>3.4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>U35A</td>
<td>ND</td>
<td>ND</td>
<td>0.0003</td>
<td>-3000</td>
</tr>
<tr>
<td>U35C</td>
<td>0.0079</td>
<td>7.8</td>
<td>0.0065</td>
<td>153</td>
</tr>
<tr>
<td>U35G</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>&gt;3000</td>
</tr>
<tr>
<td>U36A</td>
<td>0.0068</td>
<td>9.5</td>
<td>0.0055</td>
<td>182</td>
</tr>
<tr>
<td>U36C</td>
<td>0.046</td>
<td>3.7</td>
<td>0.093</td>
<td>11</td>
</tr>
<tr>
<td>U35G</td>
<td>0.012</td>
<td>16</td>
<td>0.0056</td>
<td>179</td>
</tr>
</tbody>
</table>

Individual kinetic parameters were derived from Lineweaver–Burk plots and are the average of two or three determinations carried out at 30°C with an average standard deviation of ±52%. ND indicates that the individual kinetic parameters could not be reliably determined because of the high $K_m$ compared to tRNA concentrations routinely used in the assay. No entry indicates there was no detectable activity.

**Aminoacylation of minihelix\textsuperscript{Lys,3}**

Acceptor-TΨC stem–loop-derived minihelices are substrates for many aminoacyl-tRNA synthetases despite the fact that they lack the anticodon and D stem–loop domains (21). Even in cases where the anticodon is a major recognition element, minihelices have been shown to be specifically aminoacylated. However, very few human synthetases have been tested for minihelix aminoacylation to date. We prepared the minihelix corresponding to the acceptor-TΨC stem–loop domain of human tRNA\textsubscript{Lys,3} (Fig. 2) and tested it in aminoacylation assays with hLysRS. The minihelix was not detectably aminoacylated by the human enzyme even when high concentrations of RNA (200 µM) and hLysRS (1 µM) were included in the assay. The minihelix weakly inhibited aminoacylation of tRNA\textsubscript{Lys,3} (data not shown). Based on inhibition experiments we estimate that the $K_i$ is ≥100 µM; however, due to the relatively weak binding, it was only possible to determine a lower limit for the $K_i$ value. Thus, the lack of charging even at high minihelix concentrations may, in part, be attributed to a lower binding affinity.

We next determined whether the addition of an RNA stem–loop mimicking the anticodon domain of tRNA\textsubscript{Lys,3} (Fig. 2) could stimulate minihelix\textsuperscript{Lys,3} aminoacylation. We first established that the wild-type anticodon was a good inhibitor of tRNA\textsubscript{Lys,3} aminoacylation, with a $K_i$ of 5 µM (Table 2), indicating that binding to LysRS was similar to the full-length tRNA. Moreover, the specificity of anticodon binding is maintained in the context of the RNA stem–loop, since a U35G anticodon stem–loop variant is a significantly weaker inhibitor with a $K_i$ of 33 µM (Table 2). Nevertheless, we were unable to observe any detectable minihelix\textsuperscript{Lys,3} aminoacylation even upon addition of 100 µM anticodon stem–loop domain.

**Aminoacylation of semi-synthetic tRNA\textsuperscript{Lys,3}**

Aminoacylation experiments with the minihelix\textsuperscript{Lys,3} and anticodon stem–loops suggest that covalent continuity is required for communication between the critical anticodon elements and the site of aminoacylation. To elucidate whether the entire tRNA structure is required for the anticodon to trigger aminoacylation of the acceptor stem domain, semi-synthetic tRNA constructs were prepared. The first semi-synthetic...
DISCUSSION

A previous in vitro study of E. coli tRNA^{Lys} recognition found that E. coli LysRS required the discriminator base and the anticodon for efficient aminoacylation (11). Moreover, a 140-fold difference in catalytic efficiency was observed between unmodified and fully modified E. coli tRNA^{Lys} (13). Multiple alignments of 21 class II LysRS sequences previously indicated that the catalytic domain is one of the most highly conserved domains among synthetases (13). Nevertheless, in contrast to the E. coli enzyme, our earlier in vitro work with hLysRS showed it to be insensitive to the identity of the discriminator base (13). The fact that the human enzyme could efficiently aminoacylate both native and unmodified E. coli tRNA^{Lys} also suggested that this enzyme is relatively insensitive to base modifications. The human tRNA^{Lys,3} isoacceptor is extensively modified, containing 13 base modifications. In this work, using a crude preparation of native human tRNA^{Lys}, we observed only a 10-fold contribution of base modifications to aminoacylation catalytic efficiency of hLysRS. Thus, modified bases are not essential for hLysRS recognition, but contribute modestly to overall catalytic efficiency.

In this study, we also probed anticodon recognition by hLysRS. It is well established that the subclass Ib synthetases recognize the anticodon via an N-terminal OB-fold domain (4).
Figure 3. Scheme showing preparation of semi-synthetic tRNAs. A chemically synthesized 5′-oligonucleotide (17mer, indicated in larger font and italics) is annealed to an in vitro transcribed 3′-59mer fragment. The solid black line in the folded L-shaped representation indicates a break in the sugar–phosphate backbone in the wild-type construct prepared with the 5′-17mer. The truncated semi-synthetic variants are shown by numbered arrows indicating the length of each fragment from the 5′-end. The 5′-7mer is also boxed. Dotted lines in the L-shaped representation indicate tertiary interactions and are based on the known structure of yeast tRNA^Phe^ (2).

<table>
<thead>
<tr>
<th>tRNA^Lys_3_variant</th>
<th>Temperature (°C)</th>
<th>( k_{\text{cat}} ) (s(^{-1}))</th>
<th>( K_m ) (µM)</th>
<th>( k_{\text{cat}} / K_m ) (relative)</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>30</td>
<td>2.0</td>
<td>3.4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.7</td>
<td>3.7</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3′-59mer</td>
<td>30</td>
<td>1.5</td>
<td>5.1</td>
<td>0.63</td>
<td>1.6</td>
</tr>
<tr>
<td>+ 5′-17mer</td>
<td>30</td>
<td>0.88</td>
<td>8.9</td>
<td>0.22</td>
<td>4.5</td>
</tr>
<tr>
<td>+ 5′-14mer</td>
<td>20</td>
<td>0.00026</td>
<td>1.5</td>
<td>0.00037</td>
<td>2700</td>
</tr>
</tbody>
</table>

Individual kinetic parameters were derived from Lineweaver–Burk plots and are the average of two or three determinations with an average standard deviation of ±50%. For the 3′-59mer + 5′-14mer or 5′-7mer optimal activity in the aminoaacylation assay was observed at 20°C. The aminoaacylation efficiencies of these two constructs are thus reported relative to the wild-type (full-length) tRNA\(^{\text{Lys}\_3}\), which was also assayed at this temperature.
Although minihelix Asp alone is aminoacylated by yeast system where minihelix aminoacylation has been reported (24,28). The yeast AspRS system is the only subclass IIb aminoacylation were observed upon anticodon addition of valyl-tRNA synthetase, small increases (2–3-fold) in minihelix systems studied, E.coli synthetases from domain. Most of this work has been carried out using class I have been made in some systems to stimulate charging of an involved. To gain some insights into this question, attempts complex set of RNA–protein interactions are likely to be not well understood for most aminoacylation systems, and a mechanistic details of how communication is achieved of aminoacylation and the anticodon are ~76 Å apart (2). The greater anticodon dependence than the prokaryotic system. and our results suggest that the eukaryotic system has an even human tRNA Lys,3 is not charged and that the anticodon stem–Ψ that a minihelix derived from the acceptor-T that at 2 µM, with the exception of the 3'-59mer alone, which was present at 2 µM.

The N-terminal anticodon binding domain shows only moderate sequence conservation (8%) among the 21 LysRS sequences previously examined (13). Our results show that the effect of mutations at U36 of human tRNA\(^{\text{Lys}}\) is similar to the decreases in aminoacylation observed in the E.coli tRNA\(^{\text{Lys}}\) system (11). On the other hand, mutagenesis of U35 to G or A has a larger effect on aminoacylation in the human system than in the E.coli system. To date, no major recognition elements have been identified in the acceptor stem of human tRNA\(^{\text{Lys}}\), and our results suggest that the eukaryotic system has an even greater anticodon dependence than the prokaryotic system.

In the three-dimensional L-shaped tRNA structure, the site of aminoacylation and the anticodon are ~76 Å apart (2). The mechanistic details of how communication is achieved between the anticodon and the amino acid attachment site are not well understood for most aminoacylation systems, and a complex set of RNA–protein interactions are likely to be involved. To gain some insights into this question, attempts have been made in some systems to stimulate charging of an acceptor stem-derived minihelix with the anticodon stem–loop domain. Most of this work has been carried out using class I synthetases from E.coli or yeast (24–28). For two of the systems studied, E.coli isoleucyl-tRNA synthetase and yeast valyl-tRNA synthetase, small increases (2–3-fold) in minihelix aminoacylation were observed upon anticodon addition (24,28). The yeast AspRS system is the only subclass IIb system where minihelix aminoacylation has been reported (12). Although minihelix\(^{\text{Lys}}\) alone is aminoacylated by yeast AspRS, a stem–loop mimicking the anticodon of tRNA\(^{\text{Lys}}\) was not capable of stimulating the aminoacylation further (12).

In our experiments with the human lysine system, we find that a minihelix derived from the acceptor–TΨC stem–loop of human tRNA\(^{\text{Lys}}\) is not charged and that the anticodon stem–loop fails to trigger minihelix\(^{\text{Lys}}\) aminoacylation. These data suggest that covalent continuity between these two key elements is required for aminoacylation by hLysRS. In support of the notion that covalent continuity is sufficient for at least low levels of aminoacylation in the lysine system, yeast and rabbit liver LysRS were reported to weakly aminoacylate a substrate consisting of 5'-G(U\(_{35}\))CCA-3' (29). In the case of the yeast enzyme, which displayed higher activity than the rabbit enzyme in these previous studies, the 3'-U in the single-stranded substrate may mimic N73 of the full-length yeast tRNA\(^{\text{Lys}}\) and the 5’-region of the oligomer may mimic the UUU anticodon. This result suggests that simply by maintaining covalent continuity between the two distal elements, the lysine anticodon trinucleotide is able to trigger acceptor stem aminoacylation.

To probe this idea further, we have dissected human tRNA\(^{\text{Lys}}\) in more subtle ways and carried out a kinetic analysis of semi-synthetic tRNA variants (Fig. 3 and Table 3). Simply introducing a break in the sugar–phosphate backbone after C17 does not significantly affect aminoacylation catalytic efficiency. However, when sequence elements in the D stem–loop known to be involved in core tRNA tertiary interactions are deleted, hLysRS catalytic efficiency is severely affected despite the fact that the global tRNA-like fold is maintained (Fig. 5). Most strikingly, the 2700-fold decrease in catalytic efficiency observed with the 3'-59mer + 5'-7mer construct was entirely due to a decrease in \(k_{\text{cat}}\) with binding actually slightly improved relative to the full-length tRNA (Table 3). Thus, the anticodon domain of this semi-synthetic tRNA construct is likely to be properly bound to the synthetase. Specificity of the binding interaction is supported by the fact that a U35→G substitution in the semi-synthetic construct abolishes activity. The inhibition trends observed with stem–loops bearing UUU and UGU anticodon sequences also suggest that hLysRS is able to specifically recognize the major (U35) recognition element in the context of a simple RNA stem–loop (Table 2).

Thus, the primary effect of deleting key tertiary structural elements is on the transition state of the aminoacylation reaction. The similarity in \(K_{m}\) values determined for both full-length and truncated U35G variants is in accord with the critical nature of the interactions between the central anticodon base and the N-terminal OB-fold (9).

The presence of D stem elements and tRNA tertiary structure was also shown to be important for aminoacylation by class I yeast methionyl-tRNA synthetase, although whether deletion of the D arm affected primarily \(k_{\text{cat}}\) or \(K_{m}\) was not reported in this study (26). These researchers concluded that although most of the binding energy is localized in the anticodon region, protein contacts with the D arm are required for optimal

**Figure 4.** Comparison of aminoacylation activity of full-length unmodified tRNA\(^{\text{Lys}}\) and semi-synthetic tRNA constructs. The semi-synthetic tRNAs are indicated by the length of the 5'-oligonucleotide. The inset shows an expanded view of the results obtained with the shorter constructs. All RNAs were assayed at 5 µM, with the exception of the 3'-59mer alone, which was present at 2 µM.

**Figure 5.** Ethidium stained, native 12% polyacrylamide gel showing: full-length tRNA\(^{\text{Lys}}\) (lane 1); 3'-59mer alone (lane 2); 3'-59mer + 5'-17mer (lane 3); + 5'-15mer (lane 4); + 5'-14mer (lane 5); + 5'-10mer (lane 6); + 5'-7mer (lane 7).
catalysis. In the case of another class I enzyme, E. coli glutaminyll-tRNA synthetase, specific amino acids that interact with the inside of the L-shaped tRNA have been identified. This is one of the few systems where a high resolution co-crystal structure of the synthetase complexed with tRNA is known (30). Synthetase mutations at these interaction sites result in relaxed anticodon recognition, thus providing a connection between the acceptor end and the anticodon domain (31).

Subclass IIb yeast AspRS is one of two class II enzymes for which a high resolution X-ray structure in complex with the tRNA has been reported (5). In this case, the acceptor stem contains key recognition elements (of approximately equal importance to the anticodon) and a minihelix is aminoacylated by yeast AspRS. The addition of a separate anticodon domain was not able to stimulate catalysis, presumably due to missing RNA–protein contacts present in the intact L-shaped structure (12). Based on the co-crystal structure, enzyme contacts to backbone functional groups of U11 and U12 in the D stem are observed (7). However, mutations at a neighboring identity element, the G10:U25 base pair, affect primarily the $K_{\text{obs}}$ (7).

The addition of a separate anticodon domain contains key recognition elements (of approximately equal importance to the anticodon) and a minihelix is aminoacylated by yeast AspRS. The addition of a separate anticodon domain was not able to stimulate catalysis, presumably due to missing RNA–protein contacts present in the intact L-shaped structure (12). Based on the co-crystal structure, enzyme contacts to backbone functional groups of U11 and U12 in the D stem are observed (7). However, mutations at a neighboring identity element, the G10:U25 base pair, affect primarily the $K_{\text{obs}}$ (7).

In summary, in this work we show that the anticodon is a critical recognition element for hLysRS and examine the mechanism of communication between the anticodon and the amino acid acceptor stem domain in a human system for the first time. As reported previously for several E. coli and yeast aminoacylation systems, covalent continuity between the acceptor and anticodon domains appears to be an important requirement for efficient charging by the human synthetase. Elimination of tertiary structural elements of tRNA is known (30). Synthetase mutations at these interaction sites result in relaxed anticodon recognition, thus providing a connection between the crucial anticodon recognition elements and the acceptor stem.

REFERENCES