Identity Elements for Specific Aminoacylation of a tRNA by Mammalian Lysyl-tRNA Synthetase Bearing a Nonspecific tRNA-Interacting Factor†

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ABSTRACT: Mammalian lysyl-tRNA synthetase (LysRS) has an N-terminal polypeptide chain extension appended to a prokaryotic-like synthetase domain. This extension, termed a tRNA-interacting factor (tIF), possesses a RNA-binding motif [KxxxK(K/R)xxK] that binds nonspecifically the acceptor TΨC stem−loop domain of tRNA and provides a potent tRNA binding capacity to this enzyme. Consequently, native LysRS aminoacylates a RNA minihelix mimicking the amino acid acceptor stem−loop domain of $tRNA_3^{Lys}$. Here, examination of minihelix recognition showed that mammalian LysRS aminoacylates RNA minihelices without specificity of sequence, revealing that none of the nucleotides from the acceptor TΨC stem−loop domain are essential determinants of $tRNA^{Lys}$ acceptor identity. To test whether the tIF domain reduces the specificity of the synthetase with regard to complete tRNA molecules, aminoacylation of wild-type and mutant noncognate tRNAs by wild-type or N-terminally truncated LysRS was examined. The presence of the UUU anticodon of $tRNA^{Lys}$ appeared to be necessary and sufficient to transform yeast $tRNA^{Asp}$ or $tRNA_i^{Met}$ into potent lysine acceptor tRNAs. Thus, nonspecific RNA−protein interactions between the acceptor stem of tRNA and the tIF domain do not relax the tRNA specificity of mammalian LysRS. The possibility that interaction of the full-length cognate tRNA with the synthetase is required to induce the catalytic center of the enzyme into a productive conformation is discussed.

Aminoacyl-tRNA synthetases are responsible for interpreting the genetic code. They esterify amino acids to the 3′-end of the appropriate tRNAs (*1*). The fidelity of protein synthesis is dependent, in part, on the accuracy of aminoacyl-tRNA synthesis. The 20 aminoacyl-tRNA synthetases have a common overall structural organization. The catalytic domain contains the conserved class-defining sequence motifs. A second nonconserved domain is appended to the N- or C-terminus of the active site domain. In general, the two-domain architecture of tRNA synthetases mirrors the two domains (the acceptor TΨC stem−loop domain and the anticodon D stem−biloop domain) of the L-shaped tRNA molecule (*2*).

Aminoacyl-tRNA synthetases attach a single amino acid to the 3′-end of the adequate tRNA species. Formation of a discrete tRNA−protein complex involves recognition signals on the tRNAs termed identity determinants. Because nucleotide sequences responsible for adequate pairing between a synthetase and a tRNA eventually relate an anticodon to an amino acid, they have been termed a second genetic code (*3*). A wealth of structural and biochemical data have provided a detailed knowledge of the identity problem (*4*−*6*). The anticodon of the tRNA molecule may or may not contribute an essential identity signal. However, even if the anticodon is an important contributor to the identity of a tRNA, specific aminoacylations of minihelix substrates were observed. For instance, sequence-specific aminoacylation of RNA minihelices mimicking the acceptor TΨC stem−loop domain of $tRNA^{Met}$ by *Escherichia coli* MetRS has been observed (*7*) despite the fact that the CAU anticodon has a prominent role in $tRNA^{Met}$ identity (*8*). Similarly, although the GUC anticodon is the strongest identity determinant of yeast $tRNA^{Asp}$, RNA minihelices with the G73 discriminator base are efficiently and specifically aminoacylated by yeast AspRS (*9*).

Compared with bacterial LysRS,[1] the mammalian enzyme possesses a polypeptide chain extension of ∼60 amino acid residues appended to the N-terminus of the protein (*10*, *11*). We previously reported that this eukaryote-specific N-terminal domain has the properties of a tRNA-interacting factor (tIF) and participates in tRNA binding (*12*). The native enzyme has the capacity to bind and to aminoacylate a minihelix mimicking the acceptor TΨC stem−loop domain of $tRNA_3^{Lys}$. The functional tRNA binding capacity of mammalian LysRS is lost upon removal of this domain (2 order of magnitude increase in K_d). The RNA-binding motif with the sequence KxxxK(K/R)xxK has been identified within the N-terminal domain of LysRS; it provides nonspecific tRNA binding properties to the enzyme (*13*). A similar RNA binding site was found within the N-terminal extensions of other eukaryotic class IIb enzymes (*14*) and may be responsible for the high rate of tRNA mischarging observed with yeast AspRS (*15*).

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[1] Abbreviations: LysRS, lysyl-tRNA synthetase; tIF, tRNA-interacting factor.

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By contrast with *E. coli* LysRS, the human enzyme shows no preference for the discriminator base at position 73 (*11*). Positions U35 and U36 are major identity determinants of human $tRNA_3^{Lys}$ (*16*). To determine whether the general tIF appended to mammalian LysRS decreases its tRNA aminoacylation specificity, we constructed a series of RNA substrates designated to search for identity determinants within the acceptor TΨC stem−loop structure of tRNA. We found that mammalian LysRS aminoacylates RNA minihelices with low efficiency but without specificity of sequence. Full-length noncognate tRNAs bearing the corresponding minihelix sequences were also poor substrates for LysRS as compared with $tRNA_3^{Lys}$. Introduction of the UUU anticodon into chimeric tRNAs was sufficient to trigger aminoacylation by mammalian LysRS.

MATERIALS AND METHODS

Enzymes. Native LysRS from hamster was expressed in yeast and purified as described previously (*10*). Its N-terminally truncated derivative (LysRS-ΔN) was obtained by elastase treatment of the native enzyme (*17*).

RNA Substrate Preparation. The DNA templates were assembled from synthetic oligonucleotides and cloned into *Hin*dIII−*Bam*HI sites of pUC18 (oligonucleotide sequences used in this study available upon request). Linearization of plasmids carrying RNA genes was performed with *Bst*NI or *Fok*I. Digestion was followed by in vitro transcription with T7 RNA polymerase as previously described (*18*).

Aminoacylation Assay. Aminoacylation of RNA minihelix substrates was performed at 25 °C in 20 mM imidazole-HCl buffer (pH 7.5), 0.5 mM DTT, 12 mM $MgCl_2$, 2 mM ATP, and 150 μM ^{14}C-labeled lysine (309 Ci/mol). The reaction was conducted in the presence of 50 μM RNA substrates and 2.5 μM enzyme appropriately diluted in 10 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol, and 4 mg/mL bovine serum albumin. At different time intervals, 20 μL aliquots of the reaction mixture were transferred onto pieces of Whatman 3MM paper presoaked with ice-cold 5% trichloroacetic acid (TCA) and 1 mM [^{12}C]lysine. After incubation for 1 h on ice, filters were washed five times for 10 min with ice-cold 5% trichloroacetic acid containing 1 mM lysine and washed once with 95% ethanol to remove free [^{14}C]lysine. Filters were dried, and the amount of radiolabeled aminoacyl-tRNA was measured by liquid scintillation counting.

For the determination of k_{cat}/K_m values, the RNA substrate concentrations ranged from 10 to 85 μM for minihelix substrates and from 0.1 to 1 μM for chimeric tRNAs. Aminoacylation reactions were performed for 10−30 min. When the initial rates of tRNA charging increased linearly with an increasing tRNA concentration (to 85 μM), indicating that this concentration is much lower than the Michaelis constant K_m for the tRNA, accurate values for the kinetic parameters could not be obtained. The slope of the linear plot of the initial rate versus tRNA concentration gave a good approximation of the catalytic efficiency (k_{cat}/K_m).

Data for aminoacylation of minihelices are the result of at least three independent determinations. Standard errors were in the range of 20−30% of the values.

Analysis of Aminoacylated Substrates by Acid−Urea Polyacrylamide Gel Electrophoresis. After being incubated for 30 min, aminoacylation mixtures were subjected to two extractions with phenol equilibrated in 0.1 M NaOAc (pH 4.5), and nucleic acids were recovered by precipitation with ethanol. The pellets were dissolved in 0.1 M NaOAc (pH 5.0) and analyzed by electrophoresis on an acid−urea polyacrylamide gel as described previously (*19*). After electrophoresis, the gel was incubated in 3% acetic acid for 1 h, and in Amplify solution (Amersham Biosciences) for 30 min, dried, and subjected to autoradiography.

RESULTS

Lysylation of Noncognate Acceptor Minihelices by LysRS. The discriminator nucleotide A73 is a strong identity element of $tRNA^{Lys}$ in *E. coli* (*20*, *21*). By contrast, the nucleotide at position 73 is generally a G among eukaryotic $tRNA^{Lys}$, but U and A are also found among natural substrates (Figure 1A). Mutagenesis of G73 to any other nucleotide showed that N73 is not an important recognition element for human LysRS (*11*). LysRS of mammalian origin possesses an auxiliary tIF appended to the N-terminus of the protein and is able to aminoacylate a minihelix substrate (Acc_3^{Lys}) derived from the acceptor TΨC stem−loop domain of $tRNA_3^{Lys}$ (*12*). The capacity of LysRS to aminoacylate Acc_3^{Lys} depended on the presence of the N-terminal tIF. To investigate the basis of $tRNA^{Lys}$ recognition by LysRS of mammalian origin, we first wanted to determine if any nucleotide determinant is located within the acceptor TΨC stem−loop domain of tRNA. We took advantage of the ability of the native enzyme to aminoacylate an Acc_3^{Lys} RNA minihelix (Figure 2A), and we synthesized a series of minihelices that, collectively, allowed us to assess the contribution of all conserved nucleotides.

Among the 35 nucleotides of Acc_3^{Lys}, 25 are strongly conserved in all eukaryotic lysine tRNAs (Figure 1A). If the 13 invariant nucleotides that are present at the same position in all tRNA species are excluded, 12 nucleotides are highly conserved (more than 95% of the occurrences) and were good candidates for representing identity elements. Thus, the essential targets for our studies were guanines at positions 1 and 69−71, cytosines at positions 2, 3, and 72, pyrimidines at positions 4 and 68 in the acceptor stem, as well as guanines at positions 51 and 59, and cytosine at position 63 in the TΨC stem−loop domain. The aim of this study was also to address the problem of accuracy of tRNA aminoacylation by eukaryotic aminoacyl-tRNA synthetases in a cellular context. Therefore, to analyze in vitro the specificity of the aminoacylation of RNA minisubstrates by LysRS, we chose a series of RNA minihelices derived from naturally occurring tRNAs from mammals and yeast. Indeed, we previously observed that hamster LysRS can be overproduced in hamster or human cells without impairing either the viability or the growth rate of the cells (ref *10* and unpublished results). Moreover, the mammalian enzyme is able to functionally replace a null allele of the yeast KRS1 gene (*10*). These results suggested that tRNA aminoacylation by LysRS is highly specific, even if the mammalian enzyme possesses an N-terminally appended, nonspecific tRNA-binding domain.

Minihelices Acc_2^{Arg}, Acc_e^{Met}, and Acc^{Asp} have a limited set of modifications as compared with Acc_3^{Lys}, as far as conserved residues are concerned. The conserved pyrimidine

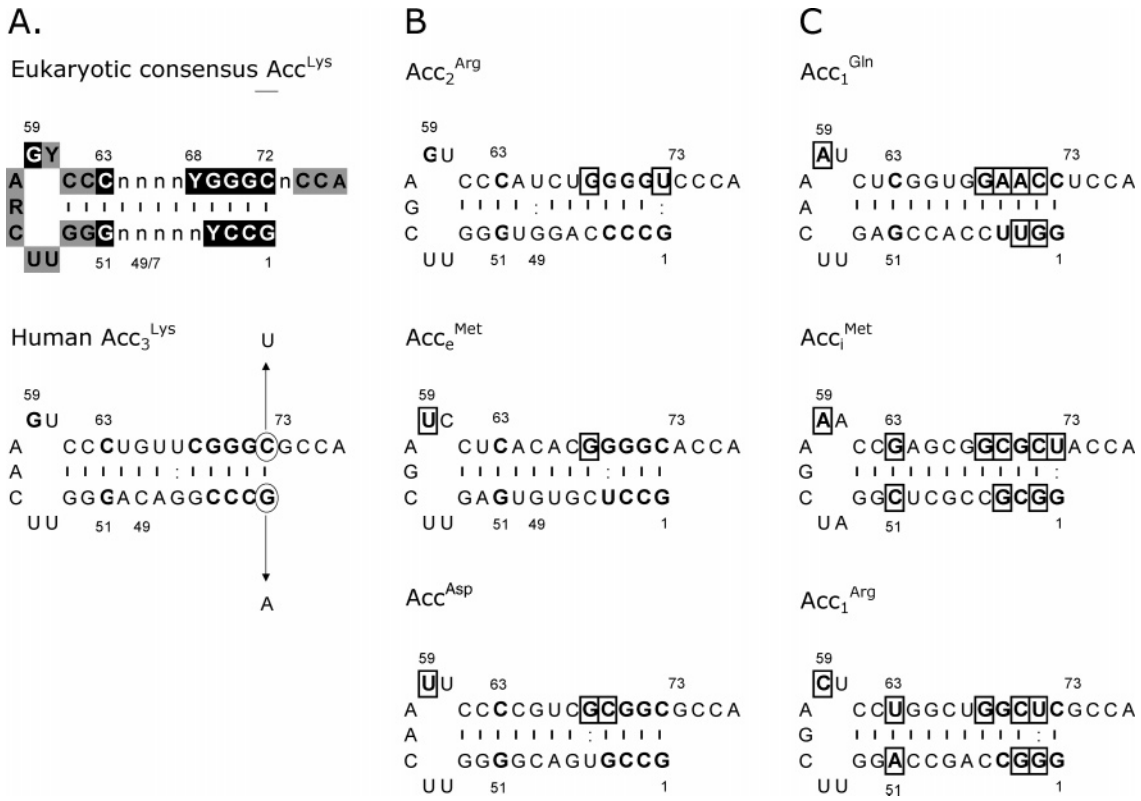

FIGURE 1: Nucleotide sequences of acceptor TΨC stem−loop minihelices (Acc) derived from the different tRNAs used in this study. (A) Nucleotides that are conserved in eukaryotic $tRNA^{Lys}$ (more than 95% of the occurrences) are indicated with black boxes (shaded boxes for invariant residues). The structure of the acceptor TΨC stem−loop region of human $tRNA_3^{Lys}$ is shown. Nucleotides that are conserved in eukaryotic $tRNA^{Lys}$ are shown with bold letters. Arrows indicate substitutions introduced into the Acc_3^{Lys}A1U72 mutant. (B) Nucleotide sequences of the acceptor TΨC stem−loop minihelices derived from beef $tRNA_2^{Arg}$, rabbit elongator $tRNA_e^{Met}$, and yeast $tRNA^{Asp}$. Nucleotide positions that differ from the consensus sequence of Acc^{Lys} are boxed. (C) Nucleotide sequences of the acceptor TΨC stem−loop minihelices derived from human $tRNA_1^{Gln}$, yeast initiator $tRNA_i^{Met}$, and human $tRNA_1^{Arg}$. To improve the efficiency of in vitro transcription, the U1A72 pair of yeast Acc^{Asp} and the A1 nucleotide of yeast Acc_i^{Met} were replaced with the G1C72 pair and the G1 nucleotide, respectively.

at position 68 is replaced with a G in the three minihelices. Acc_e^{Met} and Acc^{Asp} also introduce a G59U mutation into the TΨC loop. C72U and G69C mutations are also introduced into Acc_2^{Arg} and Acc^{Asp}, respectively. These three RNA minihelices were synthesized by in vitro transcription and were tested in aminoacylation assays with LysRS (Figure 2A). Surprisingly, Acc^{Asp} was aminoacylated as efficiently as Acc_3^{Lys}. Moreover, the initial rates of aminoacylation of Acc_e^{Met} and Acc_2^{Arg} by LysRS were 3- and 6-fold higher than that of Acc_3^{Lys}, respectively. This resulted in 6.5- and 8.9-fold increases in the catalytic efficiency (k_{cat}/K_m) for aminoacylation of Acc_e^{Met} and Acc_2^{Arg}, respectively, compared with that of Acc_3^{Lys} (Table 1). Thus, conserved nucleotides at positions 59, 68, 69, and 72 of $tRNA^{Lys}$ are not crucial for aminoacylation of minihelices. It is not clear why Acc_e^{Met} and Acc_2^{Arg} were better substrates than Acc_3^{Lys}. It may be noticed that these two minihelices contain a G at position 49, a nucleotide that is present in more than 60% of the $tRNA^{Lys}$ sequences, but a C in $tRNA_3^{Lys}$.

A second set of RNA minihelices introduced a large number of modifications within the acceptor arm of $tRNA_3^{Lys}$: seven conserved residues were exchanged in Acc_1^{Gln}, eight in Acc_1^{Arg}, and nine in Acc_i^{Met}. As shown in Figure 2B, these three constructs were efficiently aminoacylated by LysRS, with a k_{cat}/K_m similar to (Acc_1^{Gln}) or only 6.6-fold lower than (Acc_1^{Arg}) that of Acc_3^{Lys} (Table 1). The later substrate (Acc_1^{Arg}) contains an A51·U63 base pair within the TΨC stem, instead of a G·C base pair which is highly conserved among $tRNA^{Lys}$ sequences. This G51·C63 base pair may correspond to a minor identity element of $tRNA^{Lys}$.

Because all the constructs used above contained a G at position 1 to improve the efficiency of the in vitro transcription reaction, we also constructed a mutant of Acc_3^{Lys} with a G1·C72 to A1·U72 change. This mutation had an only limited effect on the aminoacylation efficiency of Acc_3^{Lys} (Figure 2C and Table 1). Collectively, all the permutations that were tested (Figure 2) suggested that native mammalian LysRS does not strongly distinguish tRNAs according to nucleotides from the acceptor TΨC stem−loop domain of tRNA.

Because the yield of [^{14}C]lysine incorporation was very low compared with that of a standard $tRNA^{Lys}$ aminoacylation assay, we wanted to ascertain that lysyladenylates synthesized by LysRS effectively reacted with the 3′-end of cognate or noncognate Acc minihelices. Indeed, it has been reported in bacterial (*22*) and yeast (*23*) systems that adenylates poorly bound to the enzyme may diffuse outside the catalytic center of the synthetase and therefore may react with nucleophilic amino acid residues of the protein. First, in the absence of a RNA minihelix in the Acc aminoacylation reaction, no radioactivity was recovered in the TCA-precipitable fraction (Figure 2C). Therefore, [^{14}C]lysine recovery was dependent on the presence of RNA. Second, the reaction mixture was treated for 1 h at 37 °C in 1 M Tris-HCl (pH 8.0) before TCA precipitation, a condition designated to hydrolyze the ester bond formed between the

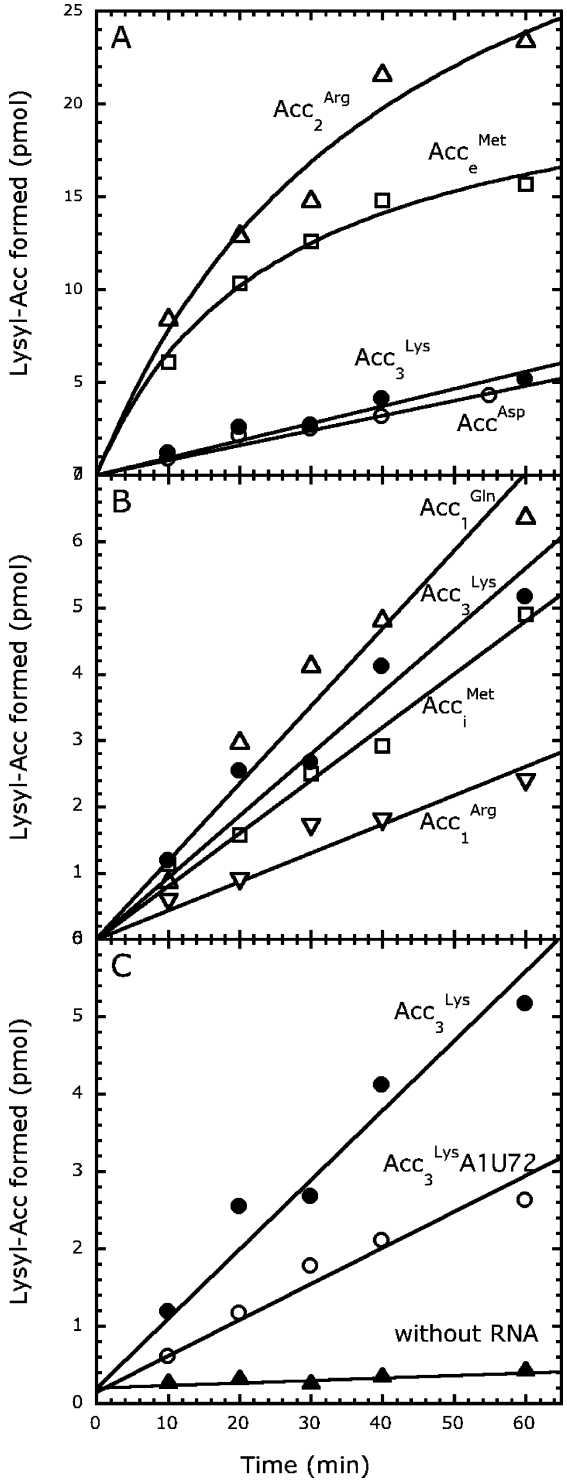

FIGURE 2: Time course of aminoacylation of RNA minihelices by LysRS. The reactions were conducted at 25 °C with the indicated RNA substrates (50 μM) and 2.5 μM LysRS as described in Materials and Methods.

carboxylic group of the amino acid and the 3′-terminal hydroxyl group of tRNA. This chemical deacylation treatment resulted in a complete loss of [^{14}C]lysine recovery. Finally, aminoacylation of RNA minihelices was directly visualized by acid−urea gel electrophoresis (Figure 3). Thus, the eukaryotic native LysRS species does aminoacylate RNA minihelices without specificity of sequence. The acceptor minihelices from the natural tRNAs that were tested (Table 1) can be aminoacylated by LysRS with catalytic efficiencies

Table 1: Kinetic Constants for Lysylation of RNA Substrates by LysRS

RNA substrate[a]	initial rate of aminoacylation: min^{-1}	initial rate of aminoacylation: relative	k_{cat}/K_m (s^{-1} μM^{-1})
beef Acc_2^{Arg}	$(4.5 \pm 1.2) \times 10^{-3}$	5.77	7.1×10^{-6}
rabbit Acc_e^{Met}	$(2.5 \pm 0.5) \times 10^{-3}$	3.20	5.2×10^{-6}
yeast Acc^{Asp}	$(9.7 \pm 2.5) \times 10^{-4}$	1.24	9.1×10^{-7}
human Acc_1^{Gln}	$(9.0 \pm 2.2) \times 10^{-4}$	1.15	5.1×10^{-7}
human Acc_3^{Lys}	**$(7.8 \pm 2.0) \times 10^{-4}$**	**1.0**	**8.0×10^{-7}**
yeast Acc_i^{Met}	$(7.5 \pm 2.2) \times 10^{-4}$	0.96	nd
human Acc_3^{Lys}A1U72	$(4.0 \pm 0.8) \times 10^{-4}$	0.51	4.2×10^{-7}
human Acc_1^{Arg}	$(3.7 \pm 0.9) \times 10^{-4}$	0.47	1.2×10^{-7}
human $tRNA_3^{Lys}$	$(1.65 \pm 0.15) \times 10^{2}$	2.1×10^{5}	2.07

[a] Values for $tRNA_3^{Lys}$ were determined previously (*12*).

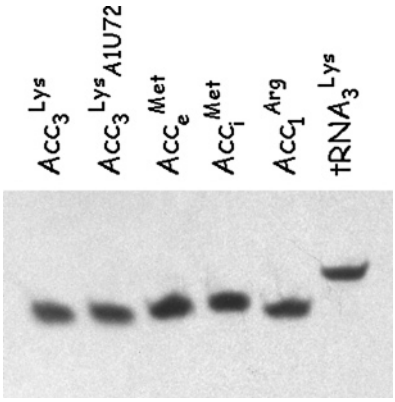

FIGURE 3: Control of aminoacylation of minihelices by acid−urea polyacrylamide gel electrophoresis. Aminoacylation of minihelices was conducted as described in Materials and Methods. Fractions of [^{14}C]lysine-labeled RNA minihelices or $tRNA_3^{Lys}$ (containing approximately 5000 cpm) were subjected to acid−urea polyacrylamide gel electrophoresis and visualized by fluorography.

close to that of human Acc_3^{Lys} (7-fold lower for Acc_1^{Arg} to 9-fold higher for Acc_2^{Arg}).

We showed previously that the N-terminally appended tRNA-interacting factor of LysRS interacts with the amino acid acceptor stem of $tRNA_3^{Lys}$ and that its removal results in the inability of the truncated enzyme to aminoacylate a minihelix mimicking the $tRNA_3^{Lys}$ acceptor arm (*12*). Similarly, the initial rate of aminoacylation of Acc_i^{Met}, a noncognate minihelix, by the N-terminally truncated LysRS was decreased more than 4-fold as compared with that of the full-length enzyme and thus was barely detectable (data not shown). Thus, additional contacts provided by the N-terminal extension of the native enzyme largely contribute to the aminoacylation of the noncognate minihelices. Nevertheless, efficiencies of minihelix aminoacylation were always 5−7 orders of magnitude lower than those of $tRNA^{Lys}$.

Lysylation of Noncognate tRNAs by LysRS. To test if the lack of specificity of aminoacylation of RNA minihelices by native eukaryotic LysRS is also observed for aminoacylation of complete tRNA molecules, we determined whether yeast $tRNA^{Asp}$ and $tRNA_i^{Met}$ are efficient substrates for native LysRS. These two tRNAs were selected because (i) their corresponding minihelices were good substrates as compared with Acc_3^{Lys} (Table 1) and (ii) yeast $tRNA^{Asp}$ possesses a uridine at position 35 in the anticodon and $tRNA_i^{Met}$ a uridine at position 36, which are present in the $tRNA_3^{Lys}$ anticodon. Because the three nucleotides of the anticodon of $tRNA^{Lys}$

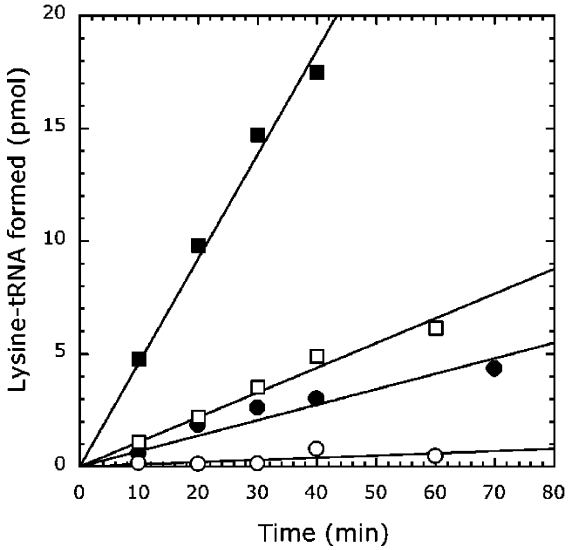

FIGURE 4: Time course of aminoacylation of noncognate tRNAs by eukaryotic LysRS. Aminoacylation of yeast $tRNA^{Asp}$ (■ and □) and yeast $tRNA_i^{Met}$ (● and ○) was conducted at 25 °C, with 50 μM tRNA substrates and 2.5 μM LysRS (■ and ●) or LysRS-ΔN (□ and ○).

Table 2: Kinetic Constants for Lysylation of Wild-Type[a] and Hybrid tRNA Substrates by LysRS and LysRS-ΔN

	LysRS		LysRS-ΔN	
tRNA	k_{cat}/K_m (s^{-1} μM^{-1})	relative k_{cat}/K_m	k_{cat}/K_m (s^{-1} μM^{-1})	relative k_{cat}/K_m
human $tRNA_3^{Lys}$	2.07	100	0.67	100
yeast $tRNA_i^{Met}$	1.9×10^{-6}	0.00009	3.8×10^{-7}	0.00006
yeast $tRNA^{Asp}$	4.7×10^{-6}	0.0002	1.18×10^{-6}	0.0002
yeast $tRNA_i^{Met}U$[b]	0.042	2	0.036	5.4
yeast $tRNA^{Asp}U$[b]	0.032	1.5	0.025	3.7
yeast $tRNA^{Asp}K$[c]	0.061	2.9	0.036	5.4

[a] Values for $tRNA_3^{Lys}$ were determined previously (*12*). [b] $tRNA_i^{Met}U$ and $tRNA^{Asp}U$ are chimeric $tRNA_i^{Met}$ and $tRNA^{Asp}$ that possess the UUU anticodon of $tRNA_3^{Lys}$, respectively. [c] $tRNA^{Asp}K$ is a chimeric $tRNA^{Asp}$ that possesses a $tRNA_3^{Lys}$ anticodon D stem−loop domain.

are strong identity elements in lysine-specific aminoacylation in prokaryotes and eukaryotes (*16*, *20*, *21*), we surmised that these two tRNAs might be less efficiently distinguished by native LysRS. Aminoacylation of these tRNAs was conducted as described above for acceptor minihelices. Yeast $tRNA^{Asp}$ and $tRNA_i^{Met}$ could be aminoacylated by LysRS (Figure 4), but their initial rates of aminoacylation fell in the range of those observed for aminoacylation of RNA minihelices. The removal of the N-terminal tIF of LysRS had a significant effect on the aminoacylation efficiency of $tRNA^{Asp}$ and $tRNA_i^{Met}$ with decreases of ∼4−7-fold (Figure 4). These two tRNAs exhibited a $(0.4-1.1 \times 10^6)$-fold reduction in the catalytic efficiency for lysylation compared with $tRNA_3^{Lys}$ (Table 2). Therefore, addition of the anticodon D loop domain of tRNA to Acc^{Asp} or Acc_i^{Met} did not improve the aminoacylation capacity of either. This result suggested that no additional productive contact is formed between the anticodon D stem−loop domains of these tRNAs and LysRS, as compared with the acceptor minihelices alone, which resulted in their efficient discrimination.

Addition of an UUU Anticodon to $tRNA^{Asp}$ and $tRNA_i^{Met}$ Improves Their Lysylability. We investigated whether the addition of the UUU anticodon of $tRNA_3^{Lys}$ to $tRNA^{Asp}$ or $tRNA_i^{Met}$ could stimulate lysylation of these noncognate tRNAs. $tRNA_i^{Met}U$ and $tRNA^{Asp}U$ are mutants of yeast $tRNA_i^{Met}$ and $tRNA^{Asp}$ carrying the UUU anticodon (Figure 5). These chimeric tRNAs exhibited 22000- and 6800-fold increases in the catalytic efficiency for lysylation compared with that of wild-type tRNAs, respectively, for $tRNA_i^{Met}U$ and $tRNA^{Asp}U$ (Table 2). The k_{cat}/K_m values of the mutants were only ∼50-fold reduced compared with that of $tRNA_3^{Lys}$ (as compared with an $\sim 10^6$-fold reduction for wild-type $tRNA^{Asp}$ and $tRNA_i^{Met}$). Thus, addition of the anticodon sequence of $tRNA_3^{Lys}$ alone leads to a strong increase in the aminoacylation efficiency by LysRS as compared with acceptor minihelices or wild-type $tRNA^{Asp}$ and $tRNA_i^{Met}$.

We next determined whether the replacement of the complete anticodon D stem−loop structure of $tRNA^{Asp}$ with the corresponding $tRNA_3^{Lys}$ domain could further improve the aminoacylation efficiency of $tRNA^{Asp}$ as compared with that of $tRNA^{Asp}U$ (Figure 5). The ability of $tRNA^{Asp}K$, containing the anticodon D stem−loop structure of $tRNA_3^{Lys}$, to be aminoacylated by LysRS was comparable with that of $tRNA^{Asp}U$ (with an only slight 2-fold increase in k_{cat}/K_m), suggesting that the UUU anticodon alone is necessary to confer on $tRNA^{Asp}$ a much higher level of aminoacylation by LysRS.

Because none of the conserved residues of the acceptor arm is essential for aminoacylation of Acc_3^{Lys} by native LysRS (Table 1) and because the UUU anticodon of $tRNA_3^{Lys}$ is sufficient to confer on $tRNA^{Asp}$ an aminoacylation efficiency comparable to that of $tRNA^{Asp}K$, we concluded that the anticodon is the major and potent discriminant of $tRNA_3^{Lys}$ identity in the mammalian lysylation system, despite the presence of the nonspecific tIF appended to eukaryotic LysRS that interacts with the acceptor domain of tRNA.

However, these chimeric tRNAs still exhibit a significantly lower catalytic efficiency than $tRNA_3^{Lys}$. One possible explanation could be that the chimeric tRNA molecules do not fold into ternary structures identical to a genuine $tRNA^{Lys}$ and thus cannot be aminoacylated as efficiently as $tRNA_3^{Lys}$. An alternative possibility would be that, in the presence of a cognate anticodon, LysRS does display some extent of specificity toward the tRNA acceptor domain. To test for the involvement of the tIF domain in acceptor domain discrimination, we also measured the catalytic efficiency of LysRS-ΔN toward $tRNA_i^{Met}U$, $tRNA^{Asp}U$, and $tRNA^{Asp}K$ (Table 2). The k_{cat}/K_m values of the mutants were only ∼20-fold reduced compared with that of $tRNA_3^{Lys}$, as compared with an ∼50-fold reduction when full-length LysRS was used. Thus, the contribution of the tIF domain to the tRNA discrimination problem appears to be only minor.

DISCUSSION

Identity Determinants of Mammalian $tRNA^{Lys}$. As compared with the prokaryotic enzyme, mammalian LysRS displays an additional N-terminal domain that binds the acceptor TΨC stem−loop structure of tRNA (*12*). The eukaryote-specific appended domain contributes a tRNA-interacting factor that triggers the formation of a stable RNA−protein complex. The native enzyme binds $tRNA_3^{Lys}$ and a RNA minihelix mimicking its acceptor arm with K_d values of 75 and 500 nM, respectively, that differ by less than 1 order of

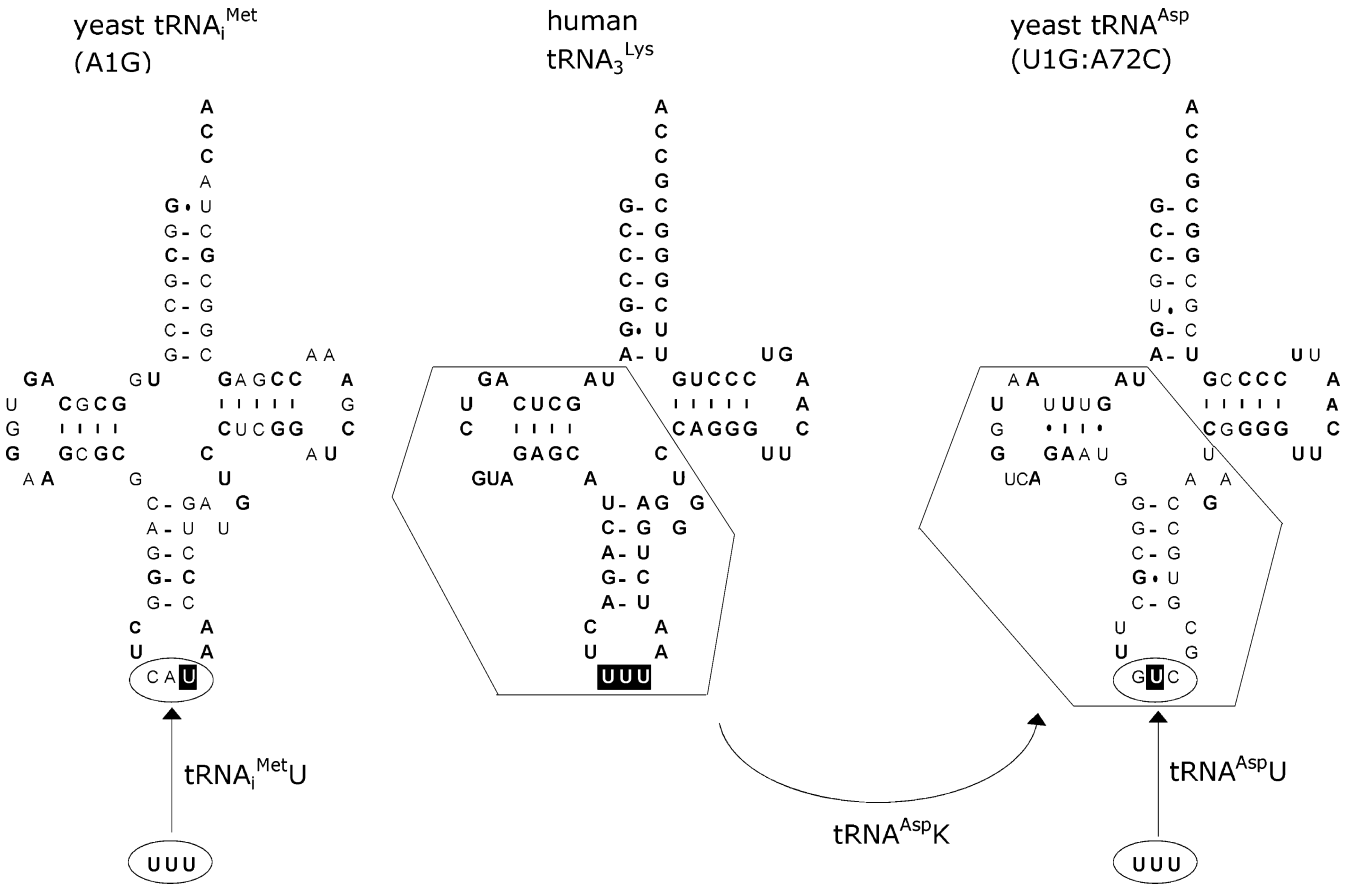

FIGURE 5: Sequence and cloverleaf structure of human tRNA$_3^{Lys}$ and of chimeric yeast tRNA$_i^{Met}$ and tRNA^{Asp}. Nucleotides that are conserved with tRNA$_3^{Lys}$ are indicated in bold letters. Nucleotides conserved with the anticodon of tRNA$_3^{Lys}$ are boxed in black. The UUU anticodon of tRNA$_3^{Lys}$ was introduced into tRNA$_i^{Met}$ and tRNA^{Asp} to give tRNA$_i^{Met}$U and tRNA^{Asp}U, respectively. tRNA^{Asp}K was constructed by insertion of the anticodon D stem−loop domain of tRNA$_3^{Lys}$ into tRNA^{Asp}.

magnitude (*13*). The removal of the appended domain did not induce a significant perturbation of the catalytic site of the enzyme, as deduced from the catalytic parameters measured in the lysine activation reaction (*13*). However, the capacity of the N-terminally truncated mammalian LysRS to bind and to aminoacylate tRNA^{Lys} or Acc^{Lys} was significantly weakened (*12*). In this study, we showed that LysRS aminoacylates a series of minihelices mimicking the acceptor TΨC stem−loop domain of several noncognate tRNAs. The best of these substrates (Acc$_2^{Arg}$) is aminoacylated with an efficiency 60-fold higher than that of Acc$_1^{Arg}$ (Table 1), suggesting that some identity determinants, nucleotide G49 and the G51·C63 base pair, are located in the acceptor domain of tRNA. However, because all minihelices that were tested were very poor substrates, these nucleotides could have only a role of a minor identity element.

The two nucleotides, U35 and U36, from the anticodon of tRNA$_3^{Lys}$ have been shown to be major identity determinants (*16*). Accordingly, when the UUU anticodon was transferred to yeast tRNA^{Asp} or tRNA$_i^{Met}$, each became a good substrate of LysRS even if the aminoacylation efficiencies remained 50-fold lower than that of tRNA$_3^{Lys}$ (Table 2). The transfer of the complete anticodon D stem−loop structure of tRNA$_3^{Lys}$ into yeast tRNA^{Asp} did not further improve the efficiency of the chimeric molecule.

Role of the tRNA Molecule in Promoting Aminoacylation. Noteworthy is the fact that binding of a tRNA molecule or of a minihelix mimicking the acceptor domain is not sufficient to warrant its aminoacylation. Indeed, even though mammalian LysRS, with the contribution of its N-terminal tIF domain, binds noncognate tRNAs as strongly as tRNA^{Lys} (*12*), efficient esterification of lysine to the 3′-end of a tRNA molecule is only achieved with tRNAs that present the cognate anticodon. We showed previously that eukaryotic LysRS is able to aminoacylate a RNA minihelix. However, the k_{cat}/K_m value for aminoacylation of Acc^{Lys} is decreased more than 10^6-fold relative to the k_{cat}/K_m for tRNA$_3^{Lys}$. This reduction essentially resulted from a large decrease in k_{cat} (65000-fold reduction in k_{cat}, as compared with a modest 40-fold increase in K_m) (*12*), which suggested that a poor enzyme−transition state complementarity is obtained with a minihelix substrate. In the study presented here, we also show that LysRS aminoacylates minihelix substrates without specificity of sequence and with efficiencies comparable to that of Acc^{Lys}.

Binding of the anticodon domain of tRNA is not sufficient to stimulate charging of an acceptor minihelix by LysRS (*12*, *16*), and efficient aminoacylation requires covalent continuity between the two domains of the tRNA molecule (*16*). However, as shown here with chimeric tRNAs, binding of a UUU anticodon to the anticodon binding domain of the synthetase is not sufficient to confer a full aminoacylation capacity on a tRNA. The geometries of the tRNA molecule or nucleotide elements located outside the anticodon are also required for optimal catalysis. Thus, a specific anticodon−synthetase interaction seems to be important for the improvement of the enzyme−transition state complementarity in the aminoacylation reaction.

The especially low levels of aminoacylation by LysRS observed with noncognate tRNAs and with RNA minihelices, even if their binding constants are in the same range as for $tRNA^{Lys}$, the natural substrate, suggest that a complete docking of the tRNA molecule on the enzyme is a critical requirement for catalysis. One possible explanation would be that a specific interaction between the anticodon of tRNA and the anticodon-binding domain of the synthetase could be a prerequisite for induction of a conformational change that would switch the active site of the enzyme into a productive conformation in the aminoacylation reaction.

Role of the tRNA−Synthetase Interaction in Promoting Aminoacylation. The requirement of the tRNA molecule in the activation of the catalytic center of a synthetase is particularly exemplified by the tRNA-dependent aminoacyl-adenylate formation catalyzed by the three class I aminoacyl-tRNA synthetases, ArgRS (*24*, *25*), GluRS, and GlnRS (*26*, *27*). In these three cases, tRNA binding triggers a conformational change that induces a transition from an inactive to an active state (*28*−*30*). It is noteworthy that the synthesis of lysyl-tRNA can be achieved by two unrelated class I and class II enzymes and that class I LysRS also requires tRNA for lysine activation (*31*).

Class II lysyl-tRNA synthetase is a dimeric enzyme. The crystal structure of bacterial LysRS, from *E. coli* (*32*) or *Thermus thermophilus* (*33*), revealed that dimerization is sustained by interactions between the catalytic domains, but also between the N-terminal anticodon-binding domain of one subunit and the catalytic domain of the other subunit. The dimer interface between the catalytic domains of class II tRNA synthetases has been shown to contribute productive conformations to the catalytic domains (*34*, *35*). The C-terminal catalytic domain of *Bacillus stearothermophilus* LysRS expressed in *E. coli* was a dimer and retained its catalytic activity (*36*). However, addition of the N-terminal anticodon binding domain in trans resulted in a significant stabilization of the transition state of the catalytic domain in the lysine activation reaction. Altogether, these data indicate that domain−domain communication in LysRS is important for induction of the catalytic center of the enzyme in a productive conformation.

The crystal structures of yeast AspRS, a class IIb enzyme closely related to LysRS, obtained in the presence or absence of $tRNA^{Asp}$ revealed that tRNA binding induces structural changes in the catalytic site of the enzyme (*37*, *38*). A scenario of tRNA binding has been proposed according to which the synthetase first binds the anticodon moiety of the tRNA molecule and then the tRNA−enzyme interaction propagates to the active site, inducing conformational changes. Structural data obtained on cocrystals of LysRS with the tRNA molecule provided information only about the interaction of the anticodon with the N-terminal β-barrel domain of the enzyme (*33*). These data suggested that only the first step of tRNA recognition has been trapped in the crystals. These results are consistent with the hypothesis that productive binding of the tRNA in a conformation suited for aminoacylation may require a significant rearrangement of the enzyme via an induced fit mechanism. Association of a minihelix Acc^{Lys} with LysRS would not induce the cascade of events leading to an activated enzyme and to the correct positioning of A76 in the active site. Thus, the tRNA may have a dual function, as a substrate but also as an activator of the enzyme.

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