Functional dissection of the eukaryotic-specific tRNA-interacting factor of lysyl-tRNA synthetase

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Running title: tRNA-interacting factors of eukaryotic aaRSs
In the cytoplasm of higher eukaryotic cells, aminoacyl-tRNA synthetases have polypeptide chain extensions appended to conventional prokaryotic-like synthetase domains. The supplementary domains, refered to as tRNA-interacting factors (tIFs), provide the core synthetases with potent tRNA-binding capacities, a functional requirement related to the low concentration of free tRNA prevailing in the cytoplasm of eukaryotic cells. Lysyl-tRNA synthetase is a component of the multi-tRNA synthetase complex. It exhibits a lysine-rich N-terminal polypeptide extension that increases its catalytic efficiency. The functional characterization of this new type of tRNA-interacting factor has been conducted. Here we describe the systematic substitution of the thirteen lysine or arginine residues located within the general RNA-binding domain of hamster LysRS made of 70 residues. Our data show that three lysine and one arginine residues are major building blocks of the tRNA-binding site. Their mutation into alanine led to a reduced affinity for tRNA\textsubscript{3}Lys or for a minimalized tRNA mimicking the acceptor-ΨC stem-loop of tRNA\textsubscript{3}Lys, and to a decrease in catalytic efficiency similar to that observed after a complete deletion of the N-terminal domain. Moreover, covalent continuity between the tRNA-binding and core domain is a prerequisite to provide LysRS with a tRNA-binding capacity. Thus, our results suggest that the ability of LysRS to promote tRNA\textsubscript{Lys} networking during translation or to convey tRNA\textsubscript{3}Lys into HIV-1 viral particles rests on the addition in evolution of this tRNA-interacting factor.

\textit{Keywords}: protein-RNA interactions / tRNA-interacting factor / channeling / tRNA packaging / lysyl-tRNA synthetase
Introduction

The aminoacyl-tRNA synthetases are responsible for the interpretation of the genetic code in terms of amino acids. This family of twenty enzymes aminoacylates the corresponding tRNA species, and thus provide the essential link between anticodons and amino acids (1). The rules that govern the accurate pairing between tRNAs and aminoacyl-tRNA synthetases have been especially scrutinized in bacterial systems (reviewed in (2-4)). Numerous crystal structures of tRNA:synthetase complexes have now been described and contribute to our understanding of the RNA:protein recognition problem (5). Although the mode of tRNA binding differs from one synthetase to another, nucleotides from the acceptor stem, the anticodon stem-loop domain, and the dihydrouridine stem, located in the inner, concave side of the L-shaped tRNA molecule, generally provide the basis for cognate tRNA:synthetase interaction. On particular occasions, a non canonical appended domain of the synthetase makes contacts with outer regions of the convex side of tRNA. The coiled-coil domains appended at the N-terminus of *T. thermophilus* SerRS, at the N-terminus of the α-subunit of *T. thermophilus* PheRS, or at the C-terminus of *T. thermophilus* ValRS interact with the variable arm and ΨC loop of tRNA\textsuperscript{Ser} or with the dihydrouridine and ΨC loop of tRNA\textsuperscript{Phe} or tRNA\textsuperscript{Val} (6-8). The N-terminal domain of *S. cerevisiae* or *T. thermophilus* ArgRS recognizes the D-loop of tRNA (9, 10). The C-terminal domain of *T. thermophilus* TyrRS makes contacts with the anticodon stem and the long variable arm of tRNA\textsuperscript{Tyr} (11). The C-terminal junction domain of *S. aureus* IleRS contacts the anticodon stem of tRNA (12).

One of the major differences that characterizes aminoacyl-tRNA synthetases from higher eukaryotes (from Drosophila to human) as compared with their prokaryotic homologues, is the presence of polypeptide chain extensions appended to the N- or C-terminus of the protein (13). MetRS, GlyRS, HisRS, TrpRS, and bifunctional GluProRS share a motif of ~50 amino acid residues (14) that folds into a coiled-coil conformation (15, 16). This polypeptide extension is a tRNA-interacting factor (tIF) that acts as a *cis*-acting factor for aminoacylation (17). One of the
components of the multi-synthetase complex, the protein p43, has the potential to bind tRNA non specifically (18, 19) and to play the role of a trans-acting tIF (20). The crystal structure of the C-terminal moiety of p43 identified a putative OB-fold-based tRNA-binding site (21). An homologous domain is appended to the C-terminus of human TyrRS (22) but its involvement in tRNA binding has not yet been established. The N-terminal eukaryotic-specific domains of ~70 amino acid residues appended to mammalian AspRS, AsnRS and LysRS share sequence similarities (23) and participate in tRNA binding (24-26). These supplementary tRNA binding modules appended to eukaryotic aminoacyl-tRNA synthetases decrease dissociation constants for their cognate tRNAs. Owing to the scarcity of non acylated tRNA in the cytoplasm of higher eukaryotic cells (discussed in (26)), these tIFs are thought to be required for tRNA cycling during translation (27).

We showed previously that native mammalian LysRS has the ability to form a stable tRNA:protein complex with human tRNA$_{3}$Lys, whereas a N-terminally truncated derivative has lost this property (26). The presence of this extension decreases the $K_d$ and $K_m$ values for tRNA, and therefore should facilitate tRNA aminoacylation under the conditions of suboptimal tRNA concentration prevailing in vivo. These in vitro data provided a rational explanation to the phenotype of growth retardation observed for yeast cells that express an allele of the yeast KRS1 gene with a deletion of its eukaryotic-specific N-terminal extension (28). Other studies have also involved human LysRS as the possible vector of tRNA$_{3}$Lys for packaging into the HIV viral particles. In retroviruses, initiation of reverse transcription is primed by a cellular tRNA that is selectively encapsidated into the virion. In the case of HIV-1, the primer tRNA is tRNA$_{3}$Lys. It forms an extended network of template/primer interactions with the viral primer binding site (PBS) but also with viral sequences located upstream of the PBS (29). Accordingly, tRNA$_{3}$Lys is efficiently packaged into HIV-1 viral particles, albeit not selectively (30). The other major tRNA species is tRNA$_{1,2}$Lys. Primer/template annealing is not responsible for incorporation of tRNA$_{Lys}$ into the virion, which suggested that a viral protein may be involved in selection of the primer tRNA for packaging (31).
Recent data have shown that LysRS, or a proteolytically truncated derivative, is selectively packaged in HIV-1 viral particles and may thus be the carrier of tRNALys (32).

To understand the role of the eukaryotic-specific N-domain of LysRS in tRNA cycling in translation and thereby in tRNA capture into HIV-1 viral particle, we wanted to determine the molecular basis for the potent tRNA binding properties of mammalian LysRS. To evaluate the contribution of the many lysine residues present in the tIF of LysRS, we determined the kinetic and tRNA-binding capacities of a series of LysRS mutants with single lysine to alanine changes. We eventually delineated the residues that contribute to the function of the eukaryotic-specific tIF of class-II aminoacyl-tRNA synthetases.
Experimental Procedures

Mutant construction

Plasmid pYeDP10/CKRS (2µ, URA3) contains the wild-type hamster LysRS cDNA expressed under the control of the PGK promoter (33). The lysine-rich region of the N-domain of LysRS, from Met1 to Lys40, is encoded by a BgII-SacI fragment of 135 nucleotides. The 13 lysine or arginine codons encoding residues at positions 8, 10, 16, 19, 23, 24, 25, 27, 30, 31, 35, 38, and 40 were substituted with GCU codons corresponding to the major tRNA<sub>Ala</sub> species in yeast, tRNA<sub>Ala</sub>. After removal of wild-type BgII-SacI fragment, cDNAs carrying a single of these mutations were constructed by assembling 6 overlapping oligonucleotides. The ligation mixture was used to transform E. coli HB101 cells. The sequence of the constructs was checked by DNA sequencing.

The diploid yeast strain CCdYK01 (his3/his3, leu2/leu2, ura3/ura3, trp1/trp1, KRS1/krs1::TRP1) (33) was transformed to Ura<sup>+</sup> with plasmid pYeDP10 encoding the various hamster LysRS mutants by the lithium chloride method (34). Sporulation of diploid cells in the nitrogen-deficient starvation medium (1% potassium acetate, 0.1% yeast extract, 0.05% dextrose) and random spore analysis were performed according to standard procedures (35). Briefly, sporulated cells were incubated overnight at 28 °C with lyticase at 750 units/ml (Sigma), supplemented with 1.5% IGEPAL (Sigma), incubated 15 min on ice and subjected to sonication (2-fold 30 sec) to release spores from their asci. After washing and resuspension in H<sub>2</sub>O, spore colonies were grown on selective medium. Haploid cells Trp<sup>+</sup> and Ura<sup>+</sup> in phenotype were selected, and analyzed by Western blotting for the lack of the endogenous yeast LysRS and the presence of plasmid-encoded hamster LysRS as described (33).

Protein overexpression and purification

Wild-type and mutant LysRS was expressed in yeast and purified according to a purification scheme adapted from (33). All purification steps were conducted at 4 °C with a BioCAD Workstation (Applied Biosystems), except otherwise stated. Briefly, recombinant yeast cells were grown at 28 °C in 1.5 l of YPG medium to an A<sub>600</sub> of 8.
Cells were washed, resuspended in extraction buffer (1 ml/g of cells) an lysed in an Eaton Press. After a two-fold dilution with extraction buffer containing protease inhibitors (5 mM diisopropyl fluorophosphate; 1 mM PMSF; 2 µg/ml chymostatine; 2 µg/ml antipaine), cell debris were removed by centrifugation. Nucleic acids were removed by precipitation with Polymin P at 0.2%, and the clear supernatant was applied to a 50 ml S Sepharose FF column (Amersham Biosciences). LysRS was eluted by a linear gradient (20 column vol.) of potassium phosphate from 50 to 300 mM (pH 7.5) containing 1 mM EDTA, 10% glycerol and 10 mM β-mercaptoethanol. Fractions containing LysRS were dialyzed (30 mM potassium phosphate pH 7.5, 1 mM EDTA, 10% glycerol and 10 mM β-mercaptoethanol), applied to a Mono Q HR 5/5 column equilibrated in 50 mM potassium phosphate pH 7.5, 1 mM EDTA, 10% glycerol and 10 mM β-mercaptoethanol and washed with 5 column volumes of the same buffer containing protease inhibitors (5 mM DiFP; 1 mM PMSF; 2 µg/ml chymostatine; 2 µg/ml antipaine), and eluted at room temperature by a linear gradient (40 column vol.) of potassium phosphate from 50 to 150 mM. Fractions containing LysRS were dialyzed against 25 mM potassium phosphate pH 7.5, 2 mM dithiothreitol (DTT), 55% glycerol, and stored at -20 °C at a protein concentration of ~ 2-10 mg/ml.

The N terminally truncated derivative (LysRS-ΔN) was obtained by elastase treatment of the native enzyme (36). The N-terminal polypeptide extension of LysRS (N-LysRS) was expressed in E.coli and purified as described (26).

Protein concentration was determined by using calculated absorption coefficients of 0.547 and 0.600 A_{280} units·mg^{-1}·cm^{-2}, respectively for LysRS (and LysRS mutants) and LysRS-ΔN.

**Gel retardation assay**

Plasmids ptRNA^{lys}_3 and pAcctRNA^{lys}_3 (26) were linearized with FokI and BstNI, respectively, and subjected to in vitro transcription with T7 RNA polymerase purified from the strain BL21/pAR1219 generously provided by Prof. W. Studier (Brookhaven National Laboratory).

^{32}P-labeled tRNAs were synthesized in a reaction mixture (50 µl) containing 1 µg
of template DNA, 40 mM Tris-HCl pH 8.0, 6 mM MgCl₂, 1 mM spermidine, 5 mM DTT, 0.01% Triton X-100, 1 mM each CTP, UTP and GTP, 10 µM [α-³²P]ATP (200 Ci/mmol), 50 units T7 RNA polymerase. After incubation at 37 °C for 1 hour, the transcripts were purified by electrophoresis on a denaturing 12% polyacrylamide gel (mono:bis, 19:1), recovered from the gel by soaking in H₂O, precipitated with ethanol and resuspended in H₂O. The RNA transcripts were renatured by heating at 90 °C for 2 min and cooling at room temperature for 20 min in the presence of 5 mM MgCl₂.

Protein-RNA interactions were analyzed using a band shift assay. Homogeneous wild-type LysRS and LysRS mutants were incubated at increasing concentrations with radiolabeled-RNA (25,000 cpm per point) in a 11 µl volume containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 10% glycerol and BSA at 0.1 mg/ml. After incubation at 25 °C for 20 min, the mixture was placed on ice and loaded on a 6% polyacrylamide gel (mono:bis, 29:1) containing 5% glycerol in 0.5 × TBE pH 8.0 at 4 °C. After electrophoresis, the gel was fixed, dried and subjected to autoradiography. Free and bound tRNA was quantified by densitometry measurements.

**tRNA₃Lys expression and purification**

For aminoacylation assays, human tRNA₃Lys was produced in *E. coli* JM101Tr transformed with plasmid pBSTK3 (37). Cells were grown in 1.5 l of 2×TY medium supplemented with ampicillin. Total tRNA fraction (500 A₂₆₀ units; 300 pmol tRNA₃Lys/A₂₆₀ unit) was prepared as described by Meinnel et al. (38) and tRNA₃Lys was partially purified on a SOURCE 15Q column (160 A₂₆₀ units; 600 pmol tRNA₃Lys/A₂₆₀ unit).

**tRNA aminoacylation assay**

Initial rates of tRNA aminoacylation were measured at 25 °C in 0.1 ml of 20 mM Imidazole-HCl buffer (pH 7.5), 100 mM KCl, 0.5 mM DTT, 12 mM MgCl₂, 2 mM ATP, 180 µM ¹⁴C-labeled lysine (NEN; 16.66 Ci/mol) and saturating amounts of tRNA (26). Human tRNA₃Lys expressed in *E. coli* (lysine acceptance of 600 pmol/A₂₆₀) was used as tRNA substrate. The incubation mixture contained catalytic amounts (1-2 nM) of
enzymes appropriately diluted in 10 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol, containing bovine serum albumin at 4 mg/ml. One unit of activity is the amount of enzyme producing 1 nmol of lysine-tRNA\(^{\text{Lys}}\)/min, at 25 °C. For the determination of \(K_m\) values for tRNA, tRNA\(^{\text{Lys}}\) concentrations of 0.2–50 μM were used. Michaelian parameters were obtained by non-linear regression of the theoretical Michaelis-Menten equation to the experimental curve using the KaleidaGraph 3.0.8 software (Abelbeck Software).

**Lysine Activation assay**

The isotopic \([^{32}\text{P}]\text{PP}_i\)-ATP exchange reaction was conducted as described previously (39). The assay mixture contained, in a final volume of 0.1 ml, 20 mM imidazole-HCl pH 7.5, 10 mM MgCl\(_2\), 0.1 mM EDTA, and 2 mM each of ATP, \([^{32}\text{P}]\text{pyrophosphate (2.5 Ci/mol)},\) and lysine. The reaction was started by the addition of limiting amounts of enzymes (4 nM) appropriately diluted in 10 mM Tris-HCl, pH 7.5, containing 10 mM 2-ME and BSA at 4 mg/ml. After 10 min at 25 °C, the reaction was stopped by the addition of 2.5 ml of a solution containing 100 mM pyrophosphate, 50 mM sodium acetate pH 4.5, 0.35% perchloric acid and 0.4% Norit. Samples were filtered through Whatmann n°1 filters, washed extensively with water and counted in a liquid scintillator. A unit of enzyme activity is defined as the amount of enzyme required to form 1 nmol of \([^{32}\text{P}]\text{ATP/min}.

For determination of kinetic parameters in the \(\text{PP}_i\)-ATP exchange reaction, the concentration of ATP in the assay was varied from 25 µM to 5 mM, and that of lysine from 10 µM to 2 mM. Michaelian parameters were deduced as described above.
Results

Rationale for mutations in the eukaryote-specific N-domain of LysRS

Mammalian LysRS is a dimer of $2 \times 68$ kDa. A N-terminal deletion of 50 amino acid residues removes the eukaryotic-specific appended domain and results in a dimer of $2 \times 62$ kDa. This polypeptide extension precedes helix H1 from the anticodon-binding domain of bacterial LysRS (Fig. 1). The truncated derivative of LysRS, LysRS-$\Delta N$, displayed a 50-fold lower apparent affinity for tRNA$^{3\text{Lys}}$ and a 4-fold increase in $K_M$ for tRNA$^{3\text{Lys}}$ in the aminoacylation reaction, as compared with the native enzyme (26). To ascertain that the removal of the N-domain of LysRS did not perturb the catalytic center of the enzyme, kinetic parameters for lysine and ATP in the ATP-PP$\_i$ exchange reaction were determined. The $K_M$ of the wild-type and N-terminally truncated LysRS for ATP ($384 \pm 20$ and $371 \pm 12 \mu$M) and lysine ($157 \pm 9$ and $152 \pm 7 \mu$M) and their $k_{cat}$ values of ATP formation ($26 \pm 1$ and $27 \pm 2$ s$^{-1}$) were identical. This result extends our earlier observation showing that LysRS and LysRS-$\Delta N$ also display identical kinetic parameters for ATP and lysine in the tRNA$^{3\text{Lys}}$ aminoacylation reaction (26).

We have previously shown that interactions between tRNA and N-domain of LysRS are essentially nonspecific: similar band shifts have been observed with cognate or noncognate tRNAs. This result has suggested that formation of RNA-protein complexes involves electrostatic interactions between side chains of basic amino acid residues from the N-domain of LysRS and tRNA phosphate-sugar backbone. In the N-domain of hamster LysRS, 12 lysine or 2 arginine residues are located between positions 8 to 45 (Fig. 1), and were good candidates to build the RNA-protein interface. Because no structural data is available for this domain, we sought to identify positions in this RNA-binding motif that would influence its function. For this purpose, we performed systematic substitution of these basic residues with alanines. These substitutions were K8A, K10A, K16A, K19A, K23A, R24A, R25A, K27A, K30A, K31A, K35A, K38A, and K40A.

Expression and purification of LysRS variants

Incorporation of additional sequences such as an His-tag in LysRS impairs its
catalytic parameters (26). Thus, wild-type and mutant LysRS were isolated by standard chromatography procedures. Proteins were expressed in yeast from a multicopy plasmid (2 µ, URA3) that encoded each of the cDNAs under the control of a PGK promoter. The endogenous yeast LysRS possesses a N-domain related to that of the hamster enzyme (Fig. 1) and efficiently aminoacylates mammalian tRNA$^{\text{Lys}}$. We showed that expression of hamster LysRS in S. cerevisiae functionally replaces a null-allele of the yeast KRS1 gene (33). Thus, wild-type and mutant LysRS were expressed in yeast in a null background. The diploid yeast strain CCdYK01 (KRS1/krs1::TRP1) (33) was transformed with plasmids encoding the various hamster LysRSs. After sporulation and random spore analysis, haploid strains (Trp$^+$ and Ura$^+$ in phenotype) bearing a disrupted yeast KRS1 chromosomal allele and rescued by plasmid-encoded hamster LysRSs, were selected. The expression of the mammalian enzymes and the lack of the yeast enzyme in these strains was verified by Western blot analysis using antibodies directed to the mammalian or yeast LysRS (result not shown). This observation already showed that the thirteen LysRS variants are functional and are able to rescue yeast cells lacking endogenous LysRS.

Wild-type and mutant LysRS were purified to homogeneity using a standardized isolation scheme on S-Sepharose and Mono-Q columns. The binding of wild-type LysRS on S-Sepharose essentially rests on its N-domain: it is eluted at a potassium phosphate concentration of 140 mM whereas LysRS-$\Delta$N does not bind at a salt concentration of 25 mM. Among the different mutants, the six LysRS derivatives K16A, K19A, K23A, R24A, K27A, and K31A were eluted early in the gradient (95 to 110 mM), while other mutants were eluted at salt concentrations ranging from 120 to 140 mM. This observation suggested that the six lysine residues listed above are especially exposed to the solvent and are good candidates for interacting with tRNA.

**Single mutations have $K_\text{m}$ effects for tRNA aminoacylation**

Because the N-domain of LysRS is a key element to tRNA binding, its removal is accompanied by a 3-fold decrease in catalytic efficiency ($k_{\text{cat}}/K_\text{M}$) in the aminoacylation reaction of tRNA$^{\text{Lys}}$, resulting essentially from a 4-fold increase in $K_\text{M}$ (Table 1). The
change in kinetic parameters for tRNA$_3^{Lys}$ aminoacylation triggered by the thirteen mutant enzymes was investigated. Partially purified human tRNA$_3^{Lys}$ (lysine acceptance of 600 pmol/A$_{260}$) expressed in E. coli was used to determine the steady-state kinetic parameters in the aminoacylation reaction. While all mutant enzymes exhibited similar $k_{cat}$ values of Lys-tRNA$_{Lys}$ formation comprised between 3.76 and 4.83 s$^{-1}$, as compared with 4.85 s$^{-1}$ for wild-type, moderate but reliable variations of the $K_M$ values for tRNA$_3^{Lys}$ were determined (Table 1). Mutant K23A had a $K_M$ value that was increased more than 3-fold as compared with wild-type. Thus, it is worth noting that the single mutation of lysine-23 into alanine induced kinetic changes comparable to that observed for a complete deletion of the N-domain. Similarly, the four single mutants K19A, R24A, R25A, and K27A also displayed a significant increase in $K_M$ (2.1 to 3.6-fold). Conversely, the $K_M$ and $k_{cat}$ values for the eight other mutants (lysine into alanine at positions 8, 10, 16, 30, 31, 35, 38, or 40) were not significantly affected. These results suggested that the tRNA binding motif of the tIF of LysRS may be built around the conserved basic residues located between positions 19 to 27 (Fig. 1).

**tRNA binding is significantly perturbed by single mutations**

To determine the effect of single mutations in the N-domain of LysRS on binding of tRNA$_3^{Lys}$, we measured the dissociation constant $K_d$ in a gel mobility shift assay using radiolabeled human tRNA$_3^{Lys}$ obtained by *in vitro* transcription. Free and bound tRNA species were quantified by densitometry measurements. Earlier we showed that wild-type LysRS forms a stable complex with tRNA$_{Lys}$ while LysRS-ΔN did not (26). Mutants K8A, K10A, and K16A, that exhibited kinetic parameters similar to wild-type, interacted with tRNA$_3^{Lys}$ with an apparent dissociation constant of 100 ± 10 nM (Fig. 2), a value very similar to that determined for wild-type LysRS (75 nM) under the same assay conditions. By contrast, the three mutants K23A, R24A, and K27A, that exhibited the largest increases in $K_M$ values for tRNA$_{Lys}$, also displayed a significant 2-fold decrease in affinity towards tRNA$_3^{Lys}$ ($K_d$ ~200 nM). An exception is mutant R25A that showed a 2.2-fold increase in $K_M$ for tRNA but a $K_d$ similar to wild-type. Its behavior will be further addressed in the discussion. Other mutants displayed
intermediate $K_d$ values.

Because the effect of single mutations on the $K_d$ values for tRNA were of modest amplitude, we sought to compare the RNA-binding potentials of wild-type and mutant LysRS toward a minimized substrate. Indeed, in the lysine system, the anticodon is a major, if not unique identity determinant for aminoacylation of tRNA$^{3\text{Lys}}$ by LysRS from mammalian origin, either in vitro (40) or in vivo (41). Assuming that the mode of tRNA binding in mammals is similar to that described in bacteria (42, 43), anticodon interacts with the β-barrel, N-terminal domain of LysRS, a domain that is present in all our mutant enzymes. In contrast, we reported previously that wild-type LysRS, but not LysRS-$\Delta N$, binds and aminoacylates RNA minihelices derived from the acceptor-TΨC stem–loop of tRNA$^{3\text{Lys}}$. Thus, assuming that the N-domain of LysRS preferentially interacts with the acceptor arm of tRNA$^{3\text{Lys}}$, we expected that mutations in the N-domain of LysRS would reveal sharper differences when focusing on the binding of the acceptor arm alone.

To investigate this possibility, we prepared an in vitro transcribed, $^{32}$P-labeled amino acid acceptor minihelix (Acc$^{3\text{Lys}}$) of tRNA$^{3\text{Lys}}$. This minimalized substrate was used in a gel-mobility shift assay to examine its association with mutant enzymes (Fig. 3). Wild-type LysRS is known to bind this minimalized substrate with an apparent $K_d$ of $0.50 \pm 0.05 \mu M$ (26). Mutants K8A, K10A, K16A, and K40A bound this stem-loop structure with a $K_d$ similar to wild-type. In contrast, the four mutants K19A, K23A, R24A, and K27A produced a much weaker complex, with $K_d$ increased more than 5-fold (Fig. 3). Thus, mutations within the N-domain of LysRS mainly alter interactions of wild-type LysRS with the acceptor-TΨC stem–loop structure of tRNA$^{3\text{Lys}}$.

**The N-domain of LysRS is not intimately related to the core enzyme**

Having shown that the tRNA binding site of the eukaryotic-specific N-domain of LysRS accommodates the four basic residues K19, K23, R24, and K27, we asked whether the N-domain and the core enzyme had to be physically linked to provide wild-type LysRS with RNA-binding properties. Previously, we found that the isolated core and N-domains of LysRS bind tRNA weakly, but act synergistically in the wild-
type protein to build a high affinity binding site for tRNA (26). We reasoned that the sum of three weak interactions between the acceptor arm of tRNA$_3^{\text{Lys}}$ and the N-domain of LysRS, the anticodon arm of tRNA$_3^{\text{Lys}}$ and the core domain of LysRS, and between the N- and core-domains of LysRS might be sufficient to produce a stable complex. Thus, provided that the two domains of LysRS interact, we expected that the tRNA binding capacity of the core enzyme might be enhanced after mixing with its N-domain. Alternatively, if covalent continuity is required to associate the two domains of LysRS, no enhancement of tRNA binding should be observed in the presence of the two separate domains.

To test this possibility, we compared the tRNA-binding potential of wild-type, dimeric LysRS ($K_d$ of about 150 nM, expressed as monomer concentration), of N-terminally truncated, dimeric LysRS (LysRS-ΔN; $K_d$ of about 6 µM, expressed as monomer concentration), and of the monomeric N-domain of LysRS (N-LysRS; $K_d$ of about 20 µM) with that obtained with a 1:1 mixture of N-LysRS and LysRS-ΔN (Fig. 4). LysRS-ΔN (1 µM, dimer concentration) was preincubated with N-LysRS (2 µM, monomer concentration) in the tRNA-binding assay buffer for 30 min at 4°C. The mixture was serially diluted, immediately incubated with tRNA and subjected to gel-mobility shift assay. The tRNA-binding capacity revealed by the mixture of LysRS-ΔN with N-LysRS was not significantly improved as compared with LysRS-ΔN alone (Fig. 4). Increasing the concentration of N-LysRS up to 10 µM in the preincubation mixture did not cause more effect. Therefore, the covalent link between N-LysRS and LysRS-ΔN is required to confer on the native enzyme a robust tRNA-binding propensity.
Discussion

The eukaryotic-specific N-terminal domain of LysRS is an RNA-binding domain that acts as a cis-acting tIF (26). The removal of the N-domain of LysRS is accompanied by a 3.9-fold increase in the $K_M$ value for tRNA$_{3}^{\text{Lys}}$, as compared with wild-type LysRS, and by a two-orders-of-magnitude increase in $K_d$. Basically, the four mutants K19A, K23A, R24A, and K27A recapitulated the aminoacylation properties of the deletion mutant LysRS-$\Delta$N. The results of tRNA$_{3}^{\text{Lys}}$ and Acc-tRNA$_{3}^{\text{Lys}}$ binding assays, together with the effect of single substitutions on the retention on Mono-Q columns and on the aminoacylation of tRNA$_{3}^{\text{Lys}}$ (Fig. 5), collectively demonstrate that the tRNA binding site of the tIF of LysRS is likely to accommodate these four basic residues.

The 3D-structure of the core domain of mammalian LysRS is likely to be a close structural homologue of T. thermophilus (45% identities) or E. coli (43% identities) LysRS. The level of sequence identity is similar between the human LysRS and the bacterial enzymes, or between the T. thermophilus and E. coli LysRS (46% identities) from the other hand, two enzymes that display analogous fold. In the crystal structure of the two bacterial enzymes (42, 44), there are two major domains. The C-terminal catalytic domain is built around an antiparallel $\beta$-sheet and contains the three canonical sequence motifs that typify the class-II aminoacyl-tRNA synthetases. The N-terminal $\beta$-barrel domain interacts with the anticodon stem-loop region of tRNA. Base-specific contacts involve the three anticodon bases. Helix H1 establishes water-mediated interactions with the tRNA backbone in the anticodon stem region. The extra N-terminal domain specific to eukaryotic LysRS, although appended to the anticodon binding domain of the synthetase, improves docking of the CCA end of tRNA in the active site of the enzyme (26). Hydrodynamic properties of the isolated N-domain are characteristic of an elongated molecule. In the crystal structure of bacterial LysRSs, the N-terminal residue is separated by $\sim$50 Å from the entrance of the active site crevice of the same monomer, but also by only $\sim$60 Å from the active site of the other monomer. Thus, stabilization of binding of the tRNA acceptor arm on one monomer of
the dimeric synthetase may result from an additional interaction with the N-domain of the same monomer or from cross-subunit interaction with the N-domain from the other monomer. Future experiments can address this conundrum.

The 3D-structure of the N-domain of LysRS has not yet been solved, neither as an isolated domain, nor associated within the wild-type protein. The PSIRED method of secondary structure prediction (45) suggests that amino acid residues 19 to 36 of the N-domain of LysRS have a high probability to fold into an \( \alpha \)-helical conformation. This hypothesis is further supported by the observation that a synthetic tricosapeptide representing residues 30 to 52 of the N-domain of yeast AspRS forms an \( \alpha \)-helix in solution in the presence of equimolar amounts of octadecaphosphate (46). As shown in Figure 6, the sequence of the tricosapeptide contains the conserved sequence motif KxxxK(K/R)xK identified in this work as the tRNA binding site of the N-domain of LysRS. The finding that an octadecaphosphate induced the \( \alpha \)-helical conformation of the tricosapeptide had suggested that the lysine-rich region of the extension of yeast AspRS can provide a structural motif involved in non-specific nucleic acid/protein interactions. The sequence SKxxLKKxxK is the most conserved signature sequence of N-terminal extensions of class IIb synthetases (23). This observation indicated that this motif may be responsible for the strong tRNA-binding capacity of wild-type AspRS from yeast. Collectively, these data support the conclusion according to which the sequence-motif KxxxK(K/R)xK has an \( \alpha \)-helical conformation and builds the non-specific tRNA binding site of mammalian LysRS and of other eukaryotic synthetases that possess it. It is worth noting that the four basic residues K19, K23, R24, and K27 identified in this work as the major components of the eukaryotic specific RNA binding motif of hamster LysRS are clustered on one side, and at one extremity of the predicted \( \alpha \)-helix (Fig. 6). Thus, the RNA-binding motif of mammalian LysRS is separated by 44 residues from the N-terminus of helix H1, located in the anticodon binding domain of the synthetase, and is carried by a long \( \alpha \)-helix (Fig. 6), two features that support the possibility of an interaction with the acceptor stem of tRNA\(^{\text{Lys}}\).
Apart from the tRNA-binding residues identified in this work (K19, K23, R24, and K27), the predicted α-helix also contains basic residues R25, K30, K31, and K35. Among these residues, substitution of lysine 31 into alanine increased $K_m$ for tRNA$_{\text{Lys}}$ and $K_d$ for native tRNA but caused a weaker increase in $K_d$ for the minimalized substrate (Fig. 5). In yeast LysRS, lysine 31 is replaced by a valine (Fig. 1). Thus, although located at the border of tRNA-binding site (Fig. 6), we chose not to include this residue in the RNA-binding motif, strictly speaking. Concerning arginine 25, while its replacement into alanine had no effect on RNA binding (either tRNA or AcctRNA), the increase in $K_m$ value was similar to that observed for K19A and K27A mutants (Fig. 5). These data can be explained taking into account its location on the opposite side of the putative helix, as compared with K19, K23, R24, and K27 (Fig. 6). In accordance with the finding that mutation R25A did not change the molarity of elution of LysRS on S-Sepharose (Fig. 5), we may assume that the side of the helix that contains arginine 25 is turned toward the core of the protein. Thus, although arginine 25 is not included in the tRNA-binding motif, an incorrect positioning of the N-domain of LysRS relative to the catalytic domain may account for the $K_m$ effect observed for the R25A mutant.

Several pieces of evidence suggest that the N-domain of LysRS is essentially structurally independent of the core enzyme. Indeed, upon mixing the N-domain with the core enzyme, no increase in RNA-binding capacity was observed (Fig. 4). Thus, if the helical region of the N-domain, including arginine 25, does interact with the core domain of LysRS, this association is likely to be weak. This result is in agreement with previous works showing that wild-type LysRS can be readily modified by controlled elastase treatment to give a truncated, yet active dimer that lost polyanion-binding properties (36). Because the 3D-structure of the core domains of AspRS and LysRS are closely related (44, 47), and taking into account the similarities observed for the extensions of yeast AspRS and LysRS (23), the domain organization of the two class II enzymes of eukaryotic origin should be very similar. The finding that the N-domain could not be depicted in the electron density map of crystallized yeast AspRS (47) also suggests that the tertiary structure of the extension is not stabilized by protein-protein
interactions with the catalytic domain.

The tIFs of eukaryotic aminoacyl-tRNA synthetases are believed to be involved in tRNA cycling during translation (26). We previously investigated the biological significance of the N-terminal polypeptide extension of LysRS from S. cerevisiae with in vivo approaches (28). A yeast KRS1 mutant allele with a deletion encompassing amino acid residues 11 to 68 is able to complement a deletion of the wild-type KRS1 allele, but a phenotype of growth retardation has been observed. This result established the biological relevance of this eukaryotic-specific domain but the exact reason of a defective growth was poorly understood. Our data now provide a rational explanation. The extension of that enzyme possesses the characteristic KxxxK(K/R)xxK sequence motif. Wild-type yeast LysRS is also able to form a stable complex with tRNA\textsubscript{Lys} ($K_d$ of 75 nM) but a mutant with a N-terminal deletion of 68 amino acid residues binds tRNA with a very reduced affinity ($K_d > 5 \mu$M) (Francin, unpublished results). Thus, yeast and mammalian LysRS acquired in evolution a supplementary domain that confers on them a potent RNA-binding capacity by decreasing dissociation constant for their cognate tRNA. The data obtained in vivo suggest that this property improves the translational efficiency of these enzymes.

In regard to the putative involvement of human LysRS in the selective packaging of tRNA\textsubscript{3,Lys} in HIV-1 viral particle, our data suggest that the removal of the 27 N-terminal residues of LysRS, containing the tRNA-binding motif, would impair its propensity to bind tRNA\textsubscript{Lys}, and thus may preclude packaging of the primer RNA. While a polypeptide with an apparent $M_r$ of 63,000 has been identified in extracts of HIV-1 viral particles with antibodies directed to human LysRS, its size is significantly shorter than that of wild-type LysRS (apparent $M_r$ of 70,000) (32). Because the capacity of LysRS to be the carrier of tRNA\textsubscript{3,Lys} into the virion is likely to be impaired by the loss of its N-domain, the polypeptide of $M_r$ 63,000 might result from the loss of C-terminal sequences. However the last strand of the antiparallel $\beta$-sheet that builds the active site of class IIb synthetases is located close to C-terminus. Accordingly, the removal of a few C-terminal residues in yeast LysRS (deletion of 15 residues) or AspRS (deletion
of 10 residues), two class IIb enzymes closely related to human LysRS, is accompanied by their inactivation (28, 48). This is at odds with the observation that aminoacylation of tRNA is a prerequisite for packaging (41). The actual LysRS species packaged into the virion remains elusive, but its propensity to carry tRNA$_3^{\text{Lys}}$ into the viral particle should rest on the presence of the tRNA-binding residues identified in this work.
Acknowledgements

We are very grateful to Dr. Frédéric Dardel for providing plasmid pBSTK3 for expression of human tRNA\textsubscript{\text{Lys}} in \textit{E. coli}. M.F. is the recipient of a fellowship from "École Centrale Paris". The excellent technical assistance of Françoise Triniolles is gratefully acknowledged. This work was supported by grants from the "Agence Nationale de Recherche sur le Sida", the "Association pour la Recherche sur le Cancer" and "La Ligue".
Figure legends

Fig. 1. Sequence alignments of the N-terminal regions of LysRS. The N-terminus of hamster LysRS (Cl; *Cricetulus longicaudatus*) from amino acid residues 1 to 90 is aligned with the corresponding LysRS sequences from *H. sapiens* (Hs), *M. musculus* (Mm), *D. melanogaster* (Dm), *C. elegans* (Ce), *L. esculentum* (Le); *S. cerevisiae* (Sc), *T. thermophilus* (Tt) and *E. coli* [lysS (EcS) and lysU (EcU) gene products]. Helix H1 from the anticodon-binding domain of LysRS-S (44) is indicated. The fourteen lysine or arginine residues from the eukaryotic-specific N-domain of hamster LysRS, and the corresponding conserved residues, are highlighted. The five strictly conserved (K/R) residues are marked with an asterisk.

Fig. 2. Binding of mutant LysRSs to tRNA\textsubscript{\textsuperscript{3}Lys}. *In vitro* transcribed, \( ^{32}\text{P} \)-labeled tRNA\textsubscript{\textsuperscript{3}Lys} was incubated with site-directed mutants of hamster LysRS at different concentrations (0 to 0.5 µM, expressed as dimer concentrations). After electrophoresis at 4°C on a 6% native polyacrylamide gel, the mobility shift of tRNA was visualized by autoradiography. In each assay, the bottom band corresponds to the free tRNA species. Concentrations of LysRS at which half of tRNA forms a complex are marked by boxes; apparent \( K_d \) values are indicated at right.

Fig. 3. Binding of mutant LysRSs to the acceptor-\( \Psi \)C stem–loop of tRNA\textsubscript{\textsuperscript{3}Lys}. *In vitro* transcribed, \( ^{32}\text{P} \)-labeled RNA minihelix mimicking the acceptor arm of tRNA\textsubscript{\textsuperscript{3}Lys} was incubated with site-directed mutants of hamster LysRS at different concentrations (0 to 4 µM, expressed as dimer concentrations). After electrophoresis at 4°C on a 6% native polyacrylamide gel, the mobility shift of RNA was visualized by autoradiography. In each assay, the bottom band corresponds to the free RNA species. Concentrations of LysRS at which half of RNA forms a complex are marked by boxes; apparent \( K_d \) values are indicated at right.

Fig. 4. The covalent continuity between the core and N-domain of LysRS is required for binding tRNA. \( ^{32}\text{P} \)-labeled *in vitro* transcribed tRNA\textsubscript{\textsuperscript{3}Lys} was incubated with the wild-type enzyme (LysRS), with the N-terminally truncated enzyme (LysRS-\( \Delta \)N), with the
isolated N-domain (N-LysRS), or with an equimolar mixture of N-LysRS and LysRS-ΔN, at protein concentrations of 0.03 to 2 µM (expressed as monomer concentrations). The mobility shift of tRNA was visualized by autoradiography. In each assay, the bottom band corresponds to the free tRNA species.

**Fig. 5.** Comparative bargraph representation of the properties of wild-type and mutant LysRS. The affinity of LysRS for S-Sepharose matrix (represented by the molarity of elution, top panel), the $K_M$ value for tRNA in the aminoacylation reaction (represented as $1/K_M$, second panel), and the affinity for tRNA$_{3}{\text{Lys}}$ or for the acceptor-TΨC stem-loop of tRNA$_{3}{\text{Lys}}$ (represented as $1/K_d$, bottom panels) are indicated for the wild-type enzyme (WT), the N-terminally truncated enzyme (ΔN) of for mutant species (identified by the corresponding sites of the mutations).

**Fig. 6.** Secondary structure prediction and 3D-model of the RNA-binding motif of LysRS. The sequence of the N-domain of hamster LysRS, from residues 1 to 60, is indicated with predicted secondary structure elements (E for extended or β-strands; H for α-helices). The sequence of the tricosapeptide of the N-terminal extension of yeast AspRS (Drs-Sc) that folds into an α-helical conformation is shown above. The conserved basic residues are boxed; those building the tRNA binding-motif of hamster LysRS are presented with a white background. An axial (left) or longitudinale (right) view of the putative α-helix is represented.
References

19. Shalak, V., Kaminska, M., Mitnacht-Kraus, R., Vandenabeele, P., Clauss, M.,


Table 1. Kinetic constants\textsuperscript{a} of wild-type and mutant LysRS in tRNA\textsubscript{3}\textsuperscript{Lys} aminoacylation

<table>
<thead>
<tr>
<th>LysRS</th>
<th>$K_M$ (µM) (tRNA\textsubscript{3}\textsuperscript{Lys})\textsuperscript{b}</th>
<th>$k_{cat}$ (s\textsuperscript{-1})</th>
<th>$k_{cat}/K_M$ (s\textsuperscript{-1} µM\textsuperscript{-1})</th>
</tr>
</thead>
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<tr>
<td>wild-type</td>
<td>1.70 ± 0.08 (1)\textsuperscript{c}</td>
<td>4.85 ± 0.15</td>
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<tr>
<td>K8A</td>
<td>1.45 ± 0.08 (0.85)</td>
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<tr>
<td>K10A</td>
<td>1.62 ± 0.15 (0.95)</td>
<td>4.06 ± 0.23</td>
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<td>R24A</td>
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<td>4.83 ± 0.35</td>
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<tr>
<td>R25A</td>
<td>3.78 ± 0.31 (2.22)</td>
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<tr>
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<tr>
<td>K30A</td>
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<td>4.16 ± 0.16</td>
<td>1.56</td>
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<td>K31A</td>
<td>3.15 ± 0.15 (1.85)</td>
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<tr>
<td>ΔN</td>
<td>7.68 ± 0.43 (4.52)</td>
<td>8.10 ± 0.36</td>
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\textsuperscript{a}Standard errors were determined from at least two independent data sets.

\textsuperscript{c}Relative values.

\textsuperscript{b}tRNA\textsubscript{3}\textsuperscript{Lys} acceptance of 600 pmol/A\textsubscript{260}.
Fig. 2

**tRNA₃Lys**

<table>
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<th>LysRS</th>
<th>K₈ₐ</th>
<th>K₁₀ₐ</th>
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<th>K₁₉ₐ</th>
<th>K₂₃ₐ</th>
<th>R₂₄ₐ</th>
<th>R₂₅ₐ</th>
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<tr>
<td>0.5 µM</td>
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</tbody>
</table>

K₉ (nM)
Fig. 3

Acc-tRNA₃<sup>Lys</sup>

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<th>LysRS</th>
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</tr>
<tr>
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</tr>
<tr>
<td>K16A</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>R25A</td>
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</tr>
<tr>
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<tr>
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Kd (µM) 0 0.06 4 µM
Fig. 4
Fig. 5
Fig. 6