Single atom modification (O → S) of tRNA confers ribosome binding

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ABSTRACT

Escherichia coli tRNA^{Lys}_{SUU}, as well as human tRNA^{Lys3}_{SUU}, has 2-thiouridine derivatives at wobble position 34 (s²U³⁴). Unlike the native tRNA^{Lys}_{SUU}, the full-length, unmodified transcript of human tRNA^{Lys3}_{UUU} and the unmodified tRNA^{Lys3}_{UUU} anticodon stem/loop (ASL^{Lys3}_{UUU}) did not bind AAA- or AAG-programmed ribosomes. In contrast, the completely unmodified yeast tRNA^{Phe} anticodon stem/loop (ASL^{Phe}_{GAA}) had an affinity ($K_d = 136 \pm 49$ nM) similar to that of native yeast tRNA^{Phe}_{GmAA} ($K_d = 103 \pm 19$ nM). We have found that the single, site-specific substitution of s²U₃₄ for U₃₄ to produce the modified ASL^{Lys}_{SUU} was sufficient to restore ribosomal binding. The modified ASL^{Lys}_{SUU} bound the ribosome with an affinity ($K_d = 176 \pm 62$ nM) comparable to that of native tRNA^{Lys}_{SUU} ($K_d = 70 \pm 7$ nM). Furthermore, in binding to the ribosome, the modified ASL^{Lys3}_{SUU} produced the same 16S P-site tRNA footprint as did native *E. coli* tRNA^{Lys}_{SUU}, yeast tRNA^{Phe}_{GmAA}, and the unmodified ASL^{Phe}_{GAA}. The unmodified ASL^{Lys3}_{UUU} had no footprint at all. Investigations of thermal stability and structure monitored by UV spectroscopy and NMR showed that the dynamic conformation of the loop of modified ASL^{Lys3}_{SUU} was different from that of the unmodified ASL^{Lys}_{UUU}, whereas the stems were isomorphous. Based on these and other data, we conclude that s²U₃₄ in tRNA^{Lys}_{SUU} and in other s²U₃₄-containing tRNAs is critical for generating an anticodon conformation that leads to effective codon interaction in all organisms. This is the first example of a single atom substitution (U₃₄ → s²U₃₄) that confers the property of ribosomal binding on an otherwise inactive tRNA.

Keywords: 2-thiouridine; anticodon conformation; anticodon stem/loop analogs; nucleoside modifications; ribosome; translation; tRNA^{Lys}

INTRODUCTION

One of the most striking and characteristic properties of tRNA is the presence of naturally occurring nucleoside modifications, especially in the anticodon stem and loop domain (Sprinzl et al., 1998). In fact, it is estimated that in *Escherichia coli*, the amount of genetic information devoted to the enzymes necessary for nucleoside modifications is at least four times as much as the tRNA genes themselves (Björk, 1995). Although the importance of these ubiquitous nucleoside modifications in tRNA structure and function is widely acknowledged, it is the subtle effect of modifications that is most commonly reported. For instance, modified nucleosides of *E. coli* tRNA^{Phe} favorably affect ribosome binding by about twofold (Harrington et al., 1993). Recently, von Ahsen et al. (1997) reported that the unmodified anti-

codon stem/loops (ASLs) of *E. coli* tRNA^{Phe}, tRNA^{Asn}, and tRNA^{Thr} and yeast tRNA^{Phe} were bound efficiently by 30S ribosomal subunits, the latter with an affinity comparable to native yeast tRNA^{Phe}. However, the unmodified ASLs of *E. coli* tRNA^{Lys}, tRNA^{Gln}, and tRNA^{Pro} could not bind the appropriately programmed ribosomes, even at very high Mg²⁺ concentrations (50 mM).

Because the lack of ribosomal binding by the unmodified ASLs of tRNA^{Lys} and tRNA^{Gln} was previously attributed to the lack of naturally occurring nucleoside modifications (von Ahsen et al., 1997), we were interested in examining the role of nucleoside modifications in the ASL structure and function. The uridine at wobble position 34 in *E. coli* tRNA^{Lys}, tRNA^{Glu}, and tRNA^{Gln} are modified with a sulfur at position 2 (s²). In addition, the s²U₃₄ of *E. coli* tRNA^{Lys} and tRNA^{Glu} are modified with a methylaminomethylene group (mnm) at base position 5 (mnm⁵s²U₃₄, Fig. 1), whereas human tRNA^{Lys} is modified with a methoxycarbonylmethyl group (mcm) at position 5 (mcm⁵s²U, Fig. 1B). However, the five-position derivative of s²U₃₄ in tRNA^{Gln} is yet to be un-

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FIGURE 1. Nucleotide sequences of human tRNA^{Lys3} anticodon stem/loops and structures of modified uridines. **A:** Nucleotide sequences of the anticodon stem/loops (ASLs) of native human tRNA^{Lys3}_{SUU} and that of the chemically synthesized tRNA^{Lys} ASLs used in the experiments, ASL^{Lys3}_{SUU} and ASL^{Lys3}_{UUU}. **B:** Chemical structures of mcm⁵s²U (modified U₃₄ in human tRNA^{Lys3}_{SUU}), mnm⁵s²U (modified U₃₄ in *E. coli* tRNA^{Lys}_{SUU}), and s²U in ASL^{Lys3}_{SUU}. Modified nucleosides: ψ: pseudouridine; mcm⁵s²U: 5-methoxycarbonylmethyl-2-thiouridine; mnm⁵s²U: 5-methylaminomethyl-2-thiouridine; ms²t⁶A: 2-methylthio-N⁶-threonylcarbamoyladenine; s²U: 2-thiouridine. Human and *E. coli* tRNA^{Lys}_{SUU} anticodon stem-loop domains have identical sequences except for the three base pairs at the top of the stem (Agris et al., 1997).

ambiguously identified. The s²U modification strongly influences nucleoside structure and conformation and plays an important role in RNA duplex stabilization (Sierzputowska-Gracz et al., 1987; Houssier et al., 1988; Agris et al., 1992; Smith et al., 1992; Kumar & Davis, 1997). Both of these physicochemical contributions would be expected to be important during codon-anticodon interaction. Here we report that a single atom modification (U₃₄ → s²U₃₄) plays a critical role in the ribosomal binding of tRNA^{Lys}_{SUU} species and possibly other 2-thiouridine-containing tRNAs.

RESULTS

Ribosomal binding of native tRNAs, unmodified tRNAs, and ASLs

The unmodified anticodon stem and loop of *E. coli* tRNA^{Lys}_{UUU} (ASL^{Lys}_{UUU}) and that of tRNA^{Gln}_{UUG} (ASL^{Gln}_{UUG}) did not bind the appropriately programmed ribosomes, presumably because of lack of modified nucleosides (von Ahlsen et al., 1997). However, rather than requiring one or more modified nucleosides, perhaps these particular ASLs failed to bind because their smaller sequences lacked a required structural element. Therefore, we compared the ribosome binding of the unmodified transcripts of the tRNAs to that of fully

modified, native tRNAs. In vitro transcribed, full-length *E. coli* tRNA^{Gln}_{UUG} and tRNA^{Glu}_{UUC} and human tRNA^{Lys3}_{UUU} all failed to bind the appropriately programmed 30S ribosomal subunits (Fig. 2A,B). Under the same conditions, native *E. coli* tRNA^{Lys}_{SUU} effectively bound poly(A)-programmed ribosomes (Fig. 2A), *E. coli* tRNA^{Glu}_{SUC} bound randomly polymerized poly(AG)-programmed ribosomes (Fig. 2B), and yeast tRNA^{Phe}_{GmAA} bound poly(U)-programmed ribosomes (Fig. 2C). Thus, the lack of ribosomal binding by unmodified *E. coli* lysine and glutamine ASLs (von Ahlsen et al., 1997) was not due to the size of the RNA used in these experiments. A similar negative result was observed for the transcript of tRNA^{Lys}_{UUU} when ribosomes were programmed with randomized poly(AG) containing both the AAG and AAA codons (data not shown). Thus, the inability of unmodified ASLs and tRNA transcripts of lysine, glutamine, and glutamic acid to bind the ribosome was neither due to the sequence length nor a codon preference. Because of these results and the fact that fully modified tRNA^{Lys}_{SUU}, tRNA^{Gln}_{SUG}, and tRNA^{Glu}_{SUC} have in common s²U derivatives at the wobble position 34, we postulated that s²U₃₄-containing tRNAs are dependent on nucleoside modifications for ribosome binding.

To test this hypothesis, heptadecamer ASLs corresponding to the human tRNA^{Lys3}_{SUU} sequence were produced with and without s²U₃₄ by automated oligo-

FIGURE 2. Binding of tRNAs and anticodon/stem loops (ASLs) to programmed 30S ribosomal subunits. Ribosome binding assays were carried out as described under Materials and Methods by using ^{32}P -labeled tRNAs or ASLs and appropriately programmed 30S ribosomal subunits. **A:** Binding of lysine tRNAs and ASLs to poly(A)-programmed 30S ribosomal subunits. ■ native *E. coli* tRNA^{Lys}_{SUU}; □ human tRNA^{Lys3}_{UUU} transcript; ● ASL^{Lys3}_{SUU}; and ○ ASL^{Lys3}_{UUU}. **B:** Binding of *E. coli* tRNA^{Glu}_{SUC} (■), and *E. coli* tRNA^{Glu}_{UUC} (◇) transcript to poly(AG)-programmed 30S ribosomal subunits, and *E. coli* tRNA^{Gln}_{UUG} (△) transcript to poly(AC)-programmed 30S ribosomal subunits. **C:** Binding of (■), native yeast tRNA^{Phe}_{GmAA}, and (□), ASL^{Phe}_{GAA} to poly(U)-programmed 30S ribosomal subunits. G_m: 2'-O-methylguanosine.

nucleotide synthesis and then assayed for ribosome binding. Human and *E. coli* tRNA^{Lys}_{SUU} ASLs have identical sequences except for the three base pairs at the top of the stem (Agris et al., 1997). Surprisingly, we found that the ASL^{Lys3}_{SUU} (Fig. 1A), singularly modified with just s²U at position 34, was able to bind poly(A)- and poly(AG)-programmed ribosomes almost as effectively as native *E. coli* tRNA^{Lys}_{SUU} (Fig. 2A). In fact, the K_d for the interaction of ASL^{Lys3}_{SUU} with poly(A)-programmed 30S ribosomal subunits (176 ± 62 nM) was comparable to that of native *E. coli* tRNA^{Lys}_{SUU} (70 ± 7 nM). As expected, the unmodified human ASL^{Lys3}_{UUU} (Fig. 1A) did not bind AAA- or AAG-programmed ribosomes (Fig. 2A). In contrast, unmodified yeast ASL^{Phe}_{GAA} bound poly(U)-programmed 30S ribosomal subunits as effectively ($K_d = 136 \pm 49$ nM) as native tRNA^{Phe}_{GmAA} ($K_d = 103 \pm 19$ nM) (Fig. 2C).

16S P-site footprints by tRNAs and ASLs

To determine if the ASL^{Lys3}_{SUU} bound the ribosome at the same site as fully modified tRNA^{Lys}_{SUU}, we conducted a chemical footprinting analysis of the ASL and tRNA on 16S rRNA. Chemical protections were analyzed for five of the commonly recognized 16S P-site nucleotides (A532, G926, A794, C795, and G1338; Moazed & Noller, 1990). The ASL^{Lys3}_{SUU} produced the same footprint on 16S rRNA as did the native *E. coli* tRNA^{Lys}_{SUU} (Fig. 3A,B). Both ASL and tRNA protected all five P-site nucleosides. Of the five 16S rRNA nucleosides that were probed, A532, G926, and G1338 were intrinsically more reactive to chemical probes than A794 and C795. Hence, they were more easily recognized as being protected in the presence

of ASL or tRNA (Fig. 3B). The reduced chemical reactivities and the weaker protections of A794 and C795 have also been documented by others (Moazed & Noller, 1990). As expected, the unmodified ASL^{Lys3}_{UUU} provided no substantial protection of P-site nucleotides from either kethoxal (Fig. 3A) or dimethyl sulfate (Fig. 3B) chemical probes. In contrast, the unmodified yeast ASL^{Phe}_{GAA} produced the same footprint as did native yeast tRNA^{Phe}_{GmAA}, the ASL^{Lys3}_{SUU}, and native *E. coli* tRNA^{Lys}_{SUU}. Thus, as shown by both filter binding and chemical probing, for some tRNAs such as tRNA^{Lys}_{SUU}, but not all tRNAs, the nucleoside modifications are critical for ribosomal P-site binding.

UV and NMR spectroscopies

The dramatic restoration of ribosomal binding activity by a single, simple base modification underscores the importance of the 2-thio group in tRNA^{Lys}_{SUU} and prompted us to examine possible structural differences between the unmodified ASL^{Lys3}_{UUU} and the ASL^{Lys3}_{SUU}. Thermal denaturations, monitored by both UV spectroscopy and the imino proton chemical shifts of NMR spectra (Fig. 4A,B), failed to detect any significant differences in the stability of the ASLs. The ASLs had similar denaturation profiles and identical melting points ($T_m = 48 \pm 1$ °C). These results were not surprising considering that the techniques employed principally monitored the stabilities of ASL stems and not the loops. However, structural and dynamic differences between the two ASL loops became apparent in analysis of NMR spectra of nonexchangeable protons (Fig. 5). Examination of the DQF-COSY spectra of ASL^{Lys3}_{UUU} and ASL^{Lys3}_{SUU} revealed that H5–H6 cross-peaks arising

FIGURE 3. Footprinting of tRNAs and ASLs at the 16S rRNA P-site. **A:** Chemical probing experiments were conducted with kethoxal, as described in Materials and Methods, with poly(U)- (lanes 1–3) or poly(A)-programmed (lanes 4–7) 30S ribosomal subunits plus native yeast tRNA^{Phe}_{GmAA} (lane 2), ASL^{Phe}_{GAA} (lane 3), *E. coli* tRNA^{Lys}_{SUU} (lane 5), ASL^{Lys3}_{SUU} (lane 6), or ASL^{Lys3}_{UUU} (lane 7). **B:** Dimethyl sulfate protection experiments were conducted with poly(A)-programmed (lanes 1–4) 30S ribosomal subunits plus *E. coli* tRNA^{Lys}_{SUU} (lane 2), ASL^{Lys3}_{SUU} (lane 3), or ASL^{Lys3}_{UUU} (lane 4), as described in Materials and Methods.

from pyrimidines in the first four base pairs of the stem (U₂₇, C₂₈, C₄₀, and U₄₁) were not affected by the presence of s²U₃₄ in the loop (Fig. 5A,B). However, the remaining six pyrimidines of the modified ASL^{Lys3}_{SUU}, including five in the loop (C₃₂, U₃₃, s²U₃₄, U₃₅, and U₃₆), produced more than six signals. These results suggest that the loop region of ASL^{Lys3}_{SUU}, unlike that of the unmodified ASL^{Lys3}_{UUU}, was engaged in a slow conformational equilibrium involving two or more species. We have yet to fully describe that equilibrium.

However, there is no doubt that the presence of s²U has changed the loop.

DISCUSSION

Using site-directed incorporation of a modified nucleoside into synthetic ASLs, we have identified s²U₃₄ as a modification important for ribosomal P-site binding of tRNA^{Lys}_{SUU}. The dramatic rescue of ribosomal binding by the modification of U₃₄ to s²U₃₄ in the ASL^{Lys} may

FIGURE 4. Thermal denaturation analyses of ASL^{Lys3}_{SUU} and ASL^{Lys3}_{UUU}. **A:** UV thermal denaturation profiles of the ASLs (~2 μM) were obtained as described in Materials and Methods in 10 mM phosphate buffer, pH 7.2, containing 100 mM NaCl and 0.1 mM EDTA. **B:** Imino proton NMR spectra of ASL^{Lys3}_{SUU} and ASL^{Lys3}_{UUU} as a function of temperature. The spectra were collected on ~0.1 mM ASLs in 10 mM phosphate buffer, pH 6.0, containing 0.1 mM EDTA.

FIGURE 5. **A:** Comparison of the 500-MHz DQF-COSY spectra of ASL^{Lys3}_{UUU} and ASL^{Lys3}_{SUU} in the region showing cross peaks between H5–H6 protons. Boxes placed behind the cross-peaks in the ASL^{Lys3}_{UUU} spectrum and in the corresponding positions in ASL^{Lys3}_{SUU} spectrum facilitate comparison. **B:** Shaded boxes indicate stem pyrimidine H5–H6 cross-peaks that were not affected by the s²U-modification. Cross-peaks observed outside the boxes in the ASL^{Lys3}_{SUU} spectrum were from the loop pyrimidines of ASL^{Lys3}_{SUU} and due to a dynamic equilibrium of the loop this ASL. A small impurity of ASL^{Lys3}_{UUU} (<10%) in the ASL^{Lys3}_{SUU} sample was responsible for weak cross-peaks inside open boxes in the ASL^{Lys3}_{SUU} spectrum.

result from stabilization of the anticodon interaction with the codon through stronger H-bonding. As early as 1974, Gassen and coworkers (Vormbrock et al., 1974) suggested that the lowered pK_a for the N³ imino proton of s²U resulted in a hydrogen bond to adenosine that was stronger for s²U than U. More recently, the s²U-stabilized interaction of codon with anticodon was demonstrated with a model system consisting of complementary pentamers (Kumar & Davis, 1997). Furthermore, in experiments in which the anticodon of tRNA^{Glu} was bound to the complementary anticodon of tRNA^{Phe}_{GmAA}, the presence of s²U₃₄ stabilized the interaction by about 20 °C (Houssier et al., 1988). However, the rescue of ribosomal binding by s²U₃₄ may also be the result of the altered, dynamic anticodon conformation we observed in NMR experiments (Fig. 5). We previously reported that 2-thiouridines are significantly constrained to an *anti*, C3' endo, gauche⁺ conformation (Agris et al., 1992). These same constraints are transferred to the conformation of the 3'-adjacent nucleotide (Agris et al., 1992; Smith et al., 1992), a result now confirmed by others (Kumar & Davis, 1997). In fact, tRNA^{Lys3}_{SUU} appears to have a modification-dependent and unconventional anticodon structure (Agris et al., 1997) that is responsible for unique behaviors during translation (Jacks et al., 1988; Heurgue-Hamard et al., 1996). Based on these observations and our current data, we believe that the physiological role of s²U₃₄ in lysine tRNAs, and perhaps all other s²U-containing tRNAs, is to alter anti-

codon loop conformation and dynamics, facilitating ribosome-mediated interaction with the codon.

The importance of a proper anticodon loop conformation as seen here has also been reported by our lab for a DNA analog of yeast tRNA^{Phe} ASL (dASL^{Phe}). We had shown previously that for a functional dASL^{Phe}, specific nucleoside modifications were needed to keep the anticodon loop of the dASL in an "open loop conformation" (Dao et al., 1994). In fact, a similar requirement for a proper loop conformation is also observed for a functional "U-turn" in the unmodified ASL of yeast tRNA^{Phe} (Ashraf et al., manuscript in prep.). Thus, a proper "open" anticodon loop conformation may be a required feature of all tRNAs.

The results presented here are the first demonstration of a single atom modification (O → S) in tRNA that is critical for ribosomal binding. Thiolated human ASL^{Lys3}_{SUU} binds the ribosome, yet unmodified *E. coli* ASL^{Lys3}_{UUU}, the unmodified human tRNA^{Lys3}_{UUU} transcript, and the corresponding ASL do not. In addition, the unmodified transcripts of tRNA^{Glu}_{UUC} and tRNA^{Gln}_{UUG}, as well as the unmodified ASL^{Glu}_{UUG} (von Ahlsen et al., 1997), do not bind the ribosome. These results demonstrate the importance of the s²U₃₄ nucleoside modification in the in vitro P-site binding of many s²U₃₄-containing tRNAs, regardless of genus. In addition, *asuE* (*trmU*) mutants of *E. coli* (D. Söll, pers. comm.) and *sin3/sin4* mutants of *Schizosaccharomyces pombe* (Heyer et al., 1984; Grossenbacher et al.,

1986) deficient in the synthesis of s²U grow very poorly, suggesting ribosomal binding of s²U-containing tRNAs is modification dependent in vivo, as well as in vitro.

Wobble base uridines are almost always 5-position derivatized, as well as 2-thiolated (Fig. 1). The 5-methylaminomethyl-modification (Fig. 1), but not 2-thiolation, of U₃₄ is a determinant for glutamyl-tRNA synthetase (Agris et al., 1973; Sylvers et al., 1993) and probably for glutamyl- and lysyl-tRNA synthetases (Sen & Ghosh, 1976; Rould et al., 1991; Cusack et al., 1996). Thus, synthetase recognition and ribosome binding could be separate functions of the two modifications of 5-position derivatized s²U₃₄ in the many tRNAs in which it is found. This is not the case with the lysidine modification of C₃₄ in the minor tRNA^{Ile2}_{LAU} iso-acceptor of *E. coli*, which specifies both aminoacyl-tRNA synthetase recognition and codon selection (Muramatsu et al., 1988). We propose that 2-thiolation of U₃₄ evolved to facilitate ribosomal binding/codon-recognition, whereas the 5-position modifications of s²U₃₄ became determinants for aminoacyl-tRNA synthetase.

MATERIALS AND METHODS

Native tRNAs, transcripts, ASLs, and 30S ribosomal subunits

Native yeast tRNA^{Phe}_{GmAA}, *E. coli* tRNA^{Lys}_{SUU}, and *E. coli* tRNA^{Glu}_{SUC} were purchased from Sigma Chemical Company. In vitro transcripts of human tRNA^{Lys3}_{UUU}, *E. coli* tRNA^{Glu}_{UUC}, and *E. coli* tRNA^{Gln}_{UUC} were gifts from Drs. Musier-Forsyth and Rodgers and Söll, respectively. ASLs, with and without s²U, were synthesized using an adaptation of standard solid phase chemistry for RNA synthesis and purified by HPLC (Agris et al., 1995; Kumar & Davis, 1997). Sequence and modification of the ASLs were confirmed by MALDI mass spectroscopy (Faulstich et al., 1997), nucleoside composition analysis (Gehrke & Kuo, 1990) and organomercurial-gel electrophoresis (Igloi, 1988). Both mass spectroscopy and HPLC analyses confirmed the purity of ASL^{Lys3}_{SUU} to be greater than 90%. Thermal denaturation and renaturation of tRNA transcripts and ASLs did not alter their abilities to bind the 30S subunits. Small ribosomal subunits (30S) were prepared and activated according to Ericson et al. (1995).

Binding of tRNAs and ASLs to 30S ribosomal subunits

In order to determine the *K_d* of tRNA and ASL binding to the ribosome, 30S subunits (10 pmol) and poly(U), poly(A), poly(AG), or poly(AC) (10 μg) were incubated for 20 min at 37 °C with increasing amounts of ³²P-labeled tRNA or ASL (up to 50 pmol) in 40 μL of CMN buffer (80 mM potassium-cacodylate, pH 7.2, 20 mM MgCl₂, 100 mM NH₄Cl, and 3 mM β-mercaptoethanol). The reaction was incubated for another 20 min on ice, passed through nitrocellulose filters (0.45 μm) and the filter-bound RNA washed twice with ice-cold buffer (100 μL). The filters were then air dried and counted. *K_d*s and

their standard deviations were determined using nonlinear regression analysis on four replicates of each experiment.

Chemical modification and primer extension

Chemical probing and primer extension were accomplished as described by Moazed & Noller (1990) except that the modifications with both dimethyl sulfate and kethoxal were conducted at 20 °C for 30 min in 40 μL of CMN buffer. Ribosomal 30S subunits (10 pmol) programmed with poly(U), poly(A), or poly(AG) (10 μg) were incubated with 50 pmol yeast tRNA^{Phe}_{GmAA} or ASL^{Phe}_{GAA} or 150 pmol of *E. coli* tRNA^{Lys}_{SUU}, human ASL^{Lys3}_{SUU}, or human ASL^{Lys3}_{UUU}.

UV and NMR spectroscopy

Thermal denaturations of tRNA^{Lys} ASLs were monitored with UV and NMR spectroscopies. The UV samples contained ~2 μM (0.3 A₂₆₀ units) RNA in 10 mM sodium phosphate buffer, pH 7.2, 100 mM NaCl and 0.1 mM EDTA. NMR samples contained ~0.1 mM (11.5 A₂₆₀ units) RNA in 10 mM phosphate buffer, pH 6.0, and 0.1 mM EDTA. Double quantum filtered COSY (DQF-COSY) experiments were conducted at 10 °C with a Bruker DRX 500 MHz spectrometer using standard procedures (Rance et al., 1983). The exchangeable and nonexchangeable proton resonances were assigned using standard procedures except for the imino protons of U₃₄, U₃₅, and U₃₆, which were assigned via site-specific incorporation of ¹⁵N3-uridines. Resonances of ASL^{Lys}_{SUU} were assigned by comparison to the spectra of ASL^{Lys}_{UUU}.

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