



A Structural Basis for Restricted Codon Recognition Mediated by 2-thiocytidine in tRNA Containing a Wobble Position Inosine

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Abstract

Three of six arginine codons (CGU, CGC, and CGA) are decoded by two *Escherichia coli* tRNA^{Arg} isoacceptors. The anticodon stem and loop (ASL) domains of tRNA^{Arg1} and tRNA^{Arg2} both contain inosine and 2-methyladenosine modifications at positions 34 (I₃₄) and 37 (m²A₃₇). tRNA^{Arg1} is also modified from cytidine to 2-thiocytidine at position 32 (s²C₃₂). The s²C₃₂ modification is known to negate wobble codon recognition of the rare CGA codon by an unknown mechanism, while still allowing decoding of CGU and CGC. Substitution of s²C₃₂ for C₃₂ in the *Saccharomyces cerevisiae* tRNA^{Ile}_{IAU} anticodon stem and loop domain (ASL) negates wobble decoding of its synonymous A-ending codon, suggesting that this function of s²C at position 32 is a generalizable property. X-ray crystal structures of variously modified ASL^{Arg1}_{ICG} and ASL^{Arg2}_{ICG} constructs bound to cognate and wobble codons on the ribosome revealed the disruption of a C₃₂-A₃₈ cross-loop interaction but failed to fully explain the means by which s²C₃₂ restricts I₃₄ wobbling. Computational studies revealed that the adoption of a spatially broad inosine-adenosine base pair at the wobble position of the codon cannot be maintained simultaneously with the canonical ASL U-turn motif. C₃₂-A₃₈ cross-loop interactions are required for stability of the anticodon/codon interaction in the ribosomal A-site.

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Introduction

Transfer RNA (tRNA) functions in protein translation by transporting amino acids to the ribosome for protein synthesis. During this process, the tRNA anticodon recognizes and binds the complementary triplet code on messenger RNA (mRNA). The accuracy and efficiency with which tRNA executes this role is significantly enhanced by at least ninety-three currently known naturally occurring posttranscriptional nucleoside modifications, representing a wide array of chemistries and physicochemical properties [1–3]. The most chemically complex and best studied of these modifications occur

within the tRNA anticodon stem and loop (ASL) domain. Among these, modifications to positions 34 and 37, which are respectively within and immediately 3'-adjacent to the anticodon, are the most common [1,4]. The presence of modified nucleosides in the tRNA ASL has been shown to affect its thermal stability and structural conformation [5–10], increase its ribosomal binding affinity [5,6,11–13], enhance specificity of codon decoding [10,11,14–18], maintain the translational reading frame [2,19–21], and promote correct tRNA and mRNA translocation through the ribosome [13]. Modifications have also been demonstrated in some instances to be requirements for

prestructuring the ASL into the canonical tRNA U-turn capable of binding the ribosomal A site [8,10,22–25].

In *Escherichia coli* (*E. coli*), six possible mRNA codons specify the amino acid arginine. They are decoded by five different tRNA isoacceptors, each with their own distinct modification profile [4]. Of particular note are the tRNA^{Arg1}_{ICG} and tRNA^{Arg2}_{ICG} isoacceptors, which share identical ASL primary sequences. The ASLs differ only in one posttranscriptional modification. Only tRNA^{Arg1}_{ICG} possesses the rare modification 2-thiocytidine (s²C) at position 32, upstream of the anticodon (Fig. 1A). Both ASLs have a 2-methyladenosine at position 37 (m²A₃₇) and an inosine at position 34 (I₃₄) (Fig. 1A and B; Fig. 1D) [26,27]. Outside of the ASL region, tRNA^{Arg2} also possesses an additional nucleoside, A₂₀, in its dihydrouridine loop [4,26,27]. A₂₀, which is present in the other three *E. coli* tRNA^{Arg} isoacceptors as well, has been implicated as an essential aminoacylation determinant [4,26–28]; its deletion or mutation in tRNA^{Arg2} results in a 370-fold decrease in aminoacylation activity, suggesting that constitutively, tRNA^{Arg1} is likely to be aminoacylated at a lower efficiency than tRNA^{Arg2} [29]. It is also possible that A₂₀ in tRNA^{Arg2} may be an antideterminer with regard to the biosynthesis of s²C₃₂.

The rare 2-thiocytidine modification at position 32 of tRNA^{Arg1} merits special attention, as the implications of

this unusual cytidine thiolation are only just beginning to be explored [1,30,31]. Although s²C is observed in species in both the Archaea and Bacteria kingdoms, it occurs much more rarely than other thiolated tRNA nucleosides, such as the relatively common 2-thiouridine at position 34 (s²U₃₄) or methylthioadenosine derivatives at position 37 [32,33]. In *E. coli*, the s²C modification at position 32 is observed only in the members of the tRNA^{Arg} family (except tRNA^{Arg2}) and in tRNA^{Ser2}_{GCU} [1,4,34]. In all known instances, s²C₃₂ is found within a common consensus sequence element, C₃₂U₃₃NC₃₅NA₃₇A₃₈ [4,35], characterized by a conserved cytidine at the second position of the anticodon, a modified purine at position 37, an adenosine at position 38, and the invariant U₃₃. The cytidine at position 35 has, such as A₂₀, been shown to be a key determinant for aminoacylation [27–29]. In all known tRNA structures, C₃₂ and A₃₈ interact via a conserved bifurcated hydrogen bond between the O2 of C₃₂ and the N6 of A₃₈ that forms a noncanonical mismatch base pair at the junction between the stem and loop regions of the ASL [36].

Consistent with the predictions of the Wobble Hypothesis, the presence of inosine (a structural analog of guanosine) in wobble position 34 enables tRNA^{Arg1}_{ICG} and tRNA^{Arg2}_{ICG} to decode not only their cognate codon CGC but also the wobble codons CGU and CGA [37]. Both *E. coli* and *Saccharomyces cerevisiae* (*S. cerevisiae*) decoding of CGA have been noted to be inherently inefficient in the presence of I₃₄ [38–40]. The roles of the s²C₃₂ and m²A₃₇ modifications in tRNA^{Arg1}_{ICG} and tRNA^{Arg2}_{ICG}, however, are only partially understood.

The structural and functional properties of 2-thiocytidine were initially predicted to mimic those observed for the similarly modified pyrimidine 2-thiouridine (s²U), which occupies the wobble position of several tRNA species. The s²U modification has been shown to increase the degree of base stacking between U₃₄ and U₃₅ [41], an effect attributed to the greater polarizability of sulfur in comparison to oxygen [42,43]. The larger van der Waals radius of sulfur also significantly promotes the adoption of a C3'-endo sugar pucker and an *anti*-conformation of the N-glycosidic (X) dihedral angle in both U₃₄ and its 3'-neighbor at position 35 [14,44–46].

Given that precedent and with the observation that a change from C₃₂ to s²C₃₂ alters the accessibility of the tRNA^{Arg1} anticodon loop to nuclease S1 in a pattern more indicative of an initiator tRNA than an elongator [47], it seemed that the structure or conformation of the stacked bases at positions 31, 32, and 33 in the canonical U-turn of tRNA^{Arg1} might be altered in the presence of s²C₃₂. The less electronegative sulfur was also predicted to disrupt the conserved C₃₂-A₃₈ cross-loop base pair, a stable interaction known to affect the conformation of the ASL domain [36,41,48]. However, although the presence of s²C₃₂ in *E. coli* ASL^{Arg1}_{ICG} was shown

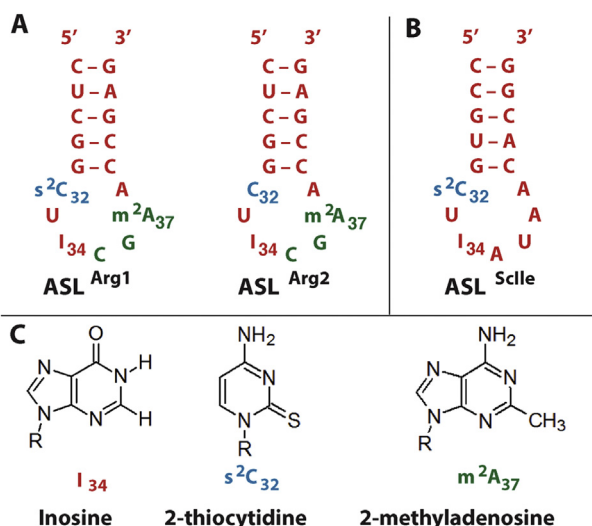


Fig. 1. Anticodon stem and loop (ASL) domain constructs. (A). The primary sequences, secondary structures, and sites of modifications of the constructs for *E. coli* ASL^{Arg1} and ASL^{Arg2}. (B). The primary sequence, secondary structure, and sites of modifications of *S. cerevisiae* ASL^{Ile}. *S. cerevisiae* tRNA^{Ile} has the modification I₃₄ but C₃₂ is not naturally modified. The s²C₃₂ (blue) was introduced into two of the ASL^{Ile} constructs under investigation. (C). The atomic structures of the modified nucleosides inosine (I), 2-thiocytidine (s²C), and 2-methyladenosine (m²A).

spectroscopically to decrease thermodynamic stability and increase base stacking of the RNA, nuclear magnetic resonance (NMR) solution structures of variously modified ASL^{Arg1} and ASL^{Arg2}_{ICG} constructs were found to be nearly identical. Unusually, they were all observed to adopt a 5'-UNCG-3' tetraloop conformation in solution rather than the expected prestructured U-turn motif [14].

In this study, we describe the mechanism by which the 2-thiocytidine modification at position 32 attenuates wobble recognition of adenosine by inosine. An unrelated yeast ASL was engineered to contain both modifications and demonstrated that the effects observed in modified tRNA^{Arg1} are not unique to this specific tRNA and may be more universally applicable. To better understand the structural basis of that effect, four crystal structures were solved of variously modified ASLs of *E. coli* tRNA^{Arg1} and tRNA^{Arg2}_{ICG} bound to both cognate and synonymous wobble codons in the ribosomal A site. We compared these structures to a previously published crystal structure of a shorter ASL^{Arg}_{ICG} construct, with only the one modified nucleoside, I₃₄, bound to CGA on the ribosome. This CGA-bound ASL^{Arg}_{ICG} construct revealed a significant displacement of the C₃₂ nucleobase, disrupting the classic bifurcated cross-loop C₃₂-A₃₈ hydrogen bond. This suggested the C₃₂-A₃₈ interaction as a structural locus that might be further disrupted upon s²C₃₂ modification. Indeed, previous studies have pointed to other systems in which modification, or changes to the identity, of the nucleoside occupying position 32 (resulting in a weakened interaction between 32 and 38) has had an effect on local conformational flexibility, ribosomal A site affinity, and translational fidelity [36,49–51]. Accordingly, molecular dynamics (MD) simulations were used to model the ribosome-bound structures of various modified ASL^{Arg1} and ASL^{Arg2} constructs and mRNA codons. Results indicated that the addition of the sulfur moiety of s²C₃₂ disrupts the ability of the purine inosine to wobble pair with another purine at the anticodon-codon interface because of stress imparted to the structure by the adoption of a distorted C₃₂-A₃₈ cross-loop hydrogen bond. This altered intraloop interaction sterically hinders formation of the canonical U-turn motif and thus, tRNA^{Arg1} insertion into the ribosomal A site, thereby abolishing CGA, but not CGU or CGC, codon binding.

Results

Thermodynamic and conformational properties of s²C₃₂ and I₃₄ in the ASL are consistent across tRNAs

Previous studies [14] suggested thermodynamic, conformational, and functional consequences of

the combined presence of 2-thiocytidine at position 32 and inosine at position 34 of the tRNA^{Arg1} and tRNA^{Arg2} isoacceptors. To assess those effects in other molecular contexts, four ASL constructs of 5-base pair stems and 7-membered loops of *S. cerevisiae* tRNA^{Ile}_{IAU} were designed to contain various possible combinations of s²C₃₂ and I₃₄: ASL^{Ile}_{AAU} with and without s²C₃₂ and ASL^{Ile}_{IAU} with and without s²C₃₂. The ASL of *S. cerevisiae* tRNA^{Ile}_{IAU} was chosen to test our hypothesis that the C₃₂-A₃₈ cross-loop interaction plays a critical role in s²C₃₂-dependent codon restriction because in addition to naturally occurring I₃₄, it contains an unmodified C₃₂ that participates in a canonical C₃₂-A₃₈ interaction. Furthermore, the second and third anticodon positions differ from *E. coli* ASL^{Arg1} (Fig. 1A and B) [4]. If our hypothesis regarding s²C₃₂-dependent wobble restriction is correct and does not rely on ASL features other than s²C₃₂, I₃₄, and a C₃₂-A₃₈ cross-loop interaction, ASL^{Ile} with an s²C₃₂ modification will bind NNU and NNC codons only (not NNA). *S. cerevisiae* tRNA^{Ile}_{IAU} 34 [4] Standard phosphoramidite and 5'-silyl-2'-acetoxyethyl orthoester (ACE) [52] chemistries were used to synthesize the four differently modified ASL^{Ile} constructs: ASL^{Ile}_{AAU}, ASL^{Ile}_{AAU}:s²C₃₂, ASL^{Ile}_{IAU}, and ASL^{Ile}_{IAU}:s²C₃₂ (Fig. 1C) [53].

The thermodynamic and conformational properties of the modifications to the ASL^{Ile} were assessed using UV-monitored thermal denaturation studies and circular dichroism (CD) spectroscopy (Fig. 2A and B). In agreement with trends observed previously for modified ASL^{Arg}, the addition of either inosine or 2-thiocytidine to ASL^{Ile} resulted in a modest and reproducible reduction in thermal stability (Table 1) [14]. The introduction of 2-thiocytidine at position 32 (s²C₃₂) to ASL^{Ile}_{AAU} also lowered the melt temperature (T_m, the temperature at which half of the ASL hairpins are fully denatured) by 7.2 °C compared with that of the unmodified ASL^{Ile}_{AAU} and resulted in an increase in the Gibbs free energy at 37 °C (ΔΔG₃₇ = −1.96 kcal/mol). The magnitude of this effect on melting temperature and overall stability agrees well with that observed upon addition of s²C₃₂ to unmodified ASL^{Arg}_{ACG} [14]. A destabilization of similar magnitude was observed upon the addition of an inosine at position 34 (I₃₄) to the unmodified ASL; the T_m decreased by 6.1 °C, whereas the ΔΔG₃₇ increased by 1.81 kcal/mol. The doubly modified ASL^{Ile}_{ICG}:s²C₃₂ construct showed the most dramatic decrease in thermodynamic stability and suggested a modest additive destabilizing effect of the two modifications (ΔT_m = 7.8 °C; ΔΔG₃₇ = −2.39 kcal/mol). These trends differed only slightly from those observed for the *E. coli* ASL^{Arg} constructs for which similar increases in Gibbs free energy were noted for the singly and doubly modified ASLs but in which the introduction of inosine by itself had no significant effect on melting temperature [14].

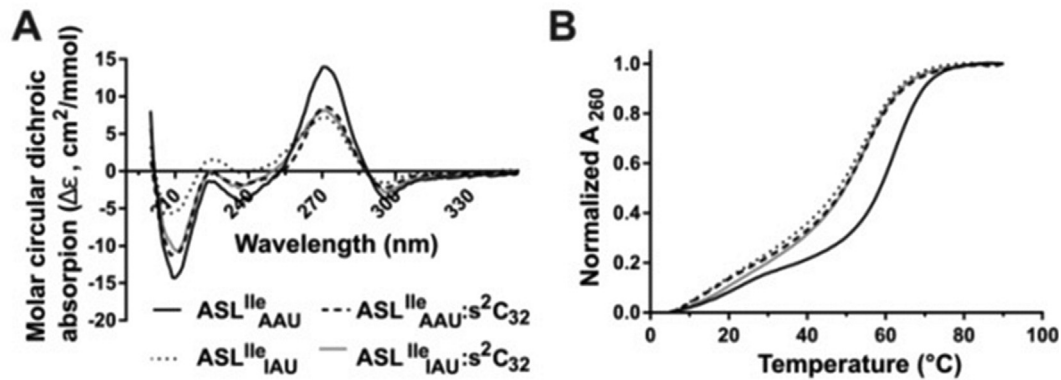


Fig. 2. Thermal stability and base stacking of the *S. cerevisiae* ASL^{Ile} constructs. (A). Circular dichroism (CD) spectra of the four ASL^{Ile} constructs. Spectra are the averages of six normalized molar circular dichroic absorption spectra with data points collected at a resolution of 1 nm. (B) UV-monitored thermal denaturation and renaturation spectra of the ASL^{Ile} constructs (1 mL; ~0.2 OD₂₆₀) collected at 260 nm. The profiles shown are the first derivative plots of the averages of four repeated denaturation/renaturation cycles (5–95 °C; 1 °C/min). ASL, anticodon stem and loop.

Table 1. Thermal stability and properties^a of the four ASL^{Ile} constructs.

ASL ^{Ile}	ΔH (kcal/mol)	ΔS (cal/K*mol)	ΔG (kcal/mol, 37 °C)	T _m (°C)	Hyperchromicity (%)
ACG	-53.7 ± 0.6	-159.7 ± 1.1	-4.18 ± 0.02	63.2 ± 0.2	35.4 ± 1.2
ACG-s ² C ₃₂	-38.0 ± 8.3	-115.3 ± 24.9	-2.22 ± 0.59	56.1 ± 0.9	27.6 ± 1.7
ICG	-38.7 ± 0.4	-117.0 ± 22.1	-2.37 ± 0.55	57.1 ± 0.8	27.4 ± 0.9
ICG-s ² C ₃₂	-32.1 ± 3.1	-97.6 ± 9.3	-1.79 ± 0.19	55.4 ± 0.3	26.8 ± 1.3

ASL, anticodon stem and loop.

^a Determined from curve-fitting analysis of thermal denaturation curves using MeltWin, v3.5. All errors are reported as standard error of the mean.

Two experimental values are often consulted to give insight into base stacking within a nucleic acid construct and to identify the changes that occur upon introduction of modified nucleosides: the change in hyperchromicity observed during thermal denaturation and the ellipticity calculated by CD spectroscopy [2,20]. The CD spectra of all four ASL constructs exhibited positive ellipticity (Δε) between 250 and 290 nm ($\lambda_{\text{max}} \approx 270$ nm), a hallmark of A-form RNA (Fig. 2A) that also decreased upon introduction of either modification singly or together. The magnitude of the change was similar for all modification states, and this decrease in ellipticity, consistent with the observed changes in hyperchromicity, also suggested a decrease in base stacking. The introduction of s²C₃₂ or I₃₄ singly or simultaneously to *S. cerevisiae* ASL^{Ile}_{AAU} caused a significant reduction of ~8% in observed hyperchromicity relative to that of the unmodified ASL, suggesting a decrease in base stacking (Fig. 2B). These results agree well with previous studies that reported that modifications to the 5' side of the ASL, such as those at or upstream of wobble position 34, tend to disorder and destabilize the structure [24,43]. They also mirror the reported effect of inosine on base stacking in the

ASL^{Arg} constructs, although the effect of s²C₃₂ is significantly more pronounced in *S. cerevisiae* ASL^{Ile}, perhaps an effect of the local chemical environment [14].

The s²C₃₂-mediated restriction of tRNA wobble decoding is a functional property observable in other tRNA species

The striking discovery that the introduction of 2-thiocytydine at position 32 in *E. coli* tRNA^{Arg1}_{ICG} acts to restrict its ability to wobble decode its noncognate CGA codon (yet still correctly reading CGC and CGU) led to the exploration, in this study, of the effect of the presence of an s²C₃₂ on the ability of other tRNAs to recognize adenine in the third codon position. *S. cerevisiae* tRNA^{Ile}_{IAU}, such as *E. coli* tRNA^{Arg2}, contains native C₃₂, I₃₄, and A₃₈ nucleosides but otherwise contains neither an endogenous s²C₃₂ modification nor any other sequence homology to tRNA^{Arg1} or tRNA^{Arg2}. To investigate the functional effect of s²C₃₂, ribosome A-site binding of *S. cerevisiae* ASL^{Ile}_{IAU} and ASL^{Ile}_{IAU}:s²C₃₂ was assessed *in vitro* to its cognate (AUC) and synonymous wobble codons (AUU and AUA) using 70S

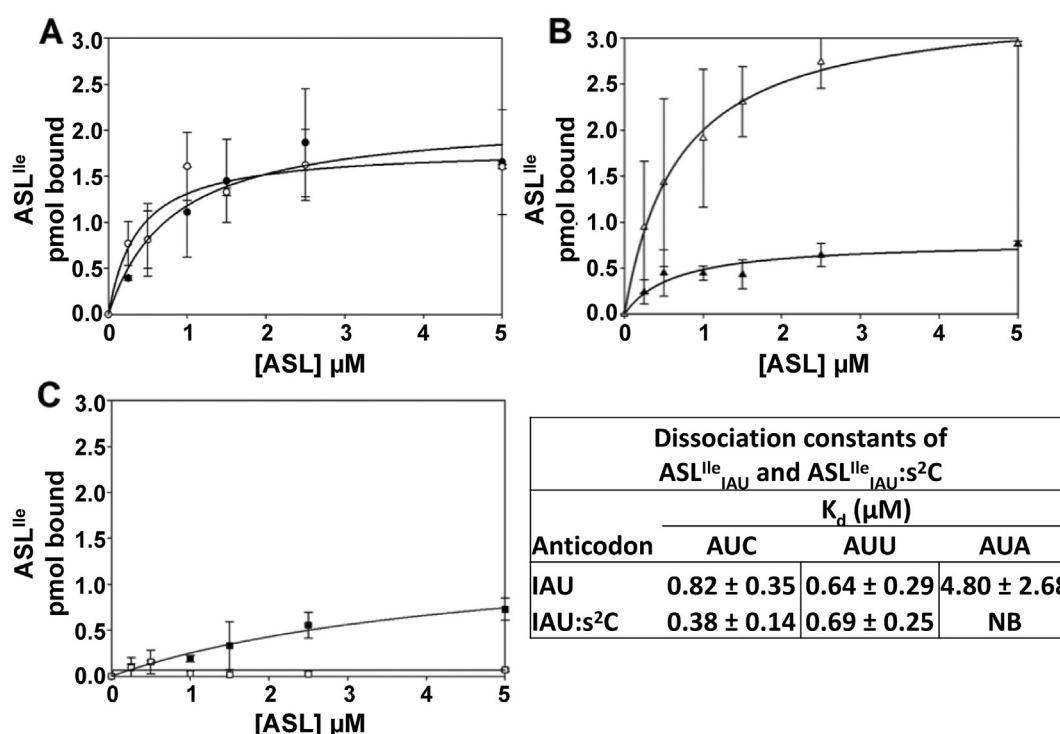


Fig. 3. Ribosomal A site codon binding by modified and unmodified *S. cerevisiae* ASL^{Ile}_{IAU}. ASL^{Ile}_{IAU} (open symbols; ○ △ □) and ASL^{Ile}_{IAU:s²C₃₂} (filled symbols; ● ▲ ■) bound in the ribosomal A site to mRNA codons (A) AUC, (B) AUU, and (C) AUA. Error bars represent standard error of the mean. Table shows dissociation constants (K_d) derived from ribosomal A site codon binding assays and calculated from a one-site saturation ligand binding model in SigmaPlot, version 13.0 (Systat Software, San Jose, CA). “NB” indicates no measurable binding above background. ASL, anticodon stem and loop.

E. coli ribosome binding assays. Consistent with the predictions of the wobble hypothesis for ASLs with inosine in position 34 [37], ASL^{Ile}_{IAU} bound to its cognate AUC codon and to AUU and AUA, with physiologically relevant binding constants (Fig. 3A–C; Fig. 3 table inset). The addition of the 2-thiocytidine modification at position 32, however, completely abrogated binding of the *S. cerevisiae* ASL^{Ile}_{IAU:s²C₃₂} to the noncognate codon AUA. This negative function for the s²C₃₂ modification, in which it restricts the expected wobble decoding of inosine of codons ending in adenine, is identical to that observed in ribosomal A site binding assays of *E. coli* ASL^{Arg1}_{ICG:s²C₃₂} to CGA [14].

X-ray crystallographic structures of modified *E. coli* ASL^{Arg1}_{ICG} and ASL^{Arg2}_{ICG} constructs bound to mRNA on the ribosome are nearly identical

The thermodynamic and ribosome binding studies of the *E. coli* ASL^{Arg1}_{ICG} and the ASL^{Arg2}_{ICG} in the context of an *S. cerevisiae* tRNA^{Ile}_{IAU} suggested that the 2-thiocytidine modification in position 32 has a demonstrable effect on the thermodynamic proper-

ties, base stacking, and wobble decoding ability of the ASL region. Recently published NMR solution structures of several ASL^{Arg1} and ASL^{Arg2} constructs bearing different combinations of the modifications s²C₃₂, I₃₄, and m²A₃₇, however, were virtually identical and gave few hints as to the structural basis for the functional effect of s²C on inosine wobbling [14]. Interestingly, all four available NMR structures of modified ASL^{Arg} domains adopted noncanonical 5'-UNCG-3' tetraloop conformations, rather than the expected U-turn motif for ribosomal A site binding, although binding studies showed all four ASLs to be competent for ribosomal interaction [14].

To determine whether a structural effect of s²C₃₂ on ASL structure and codon binding might instead become observable in the context of mRNA binding on the ribosome, where the ASL would be expected to have adopted its functional U-turn conformation, four x-ray crystal structures were determined by molecular replacement. Native *Thermus thermophilus* 30S crystals were soaked with solutions containing each of the variously modified ASL^{Arg} constructs and one of two hexameric RNA oligonucleotides (5'-CG(U/C)AAA-3') (Fig. 4 and Table S2). The four

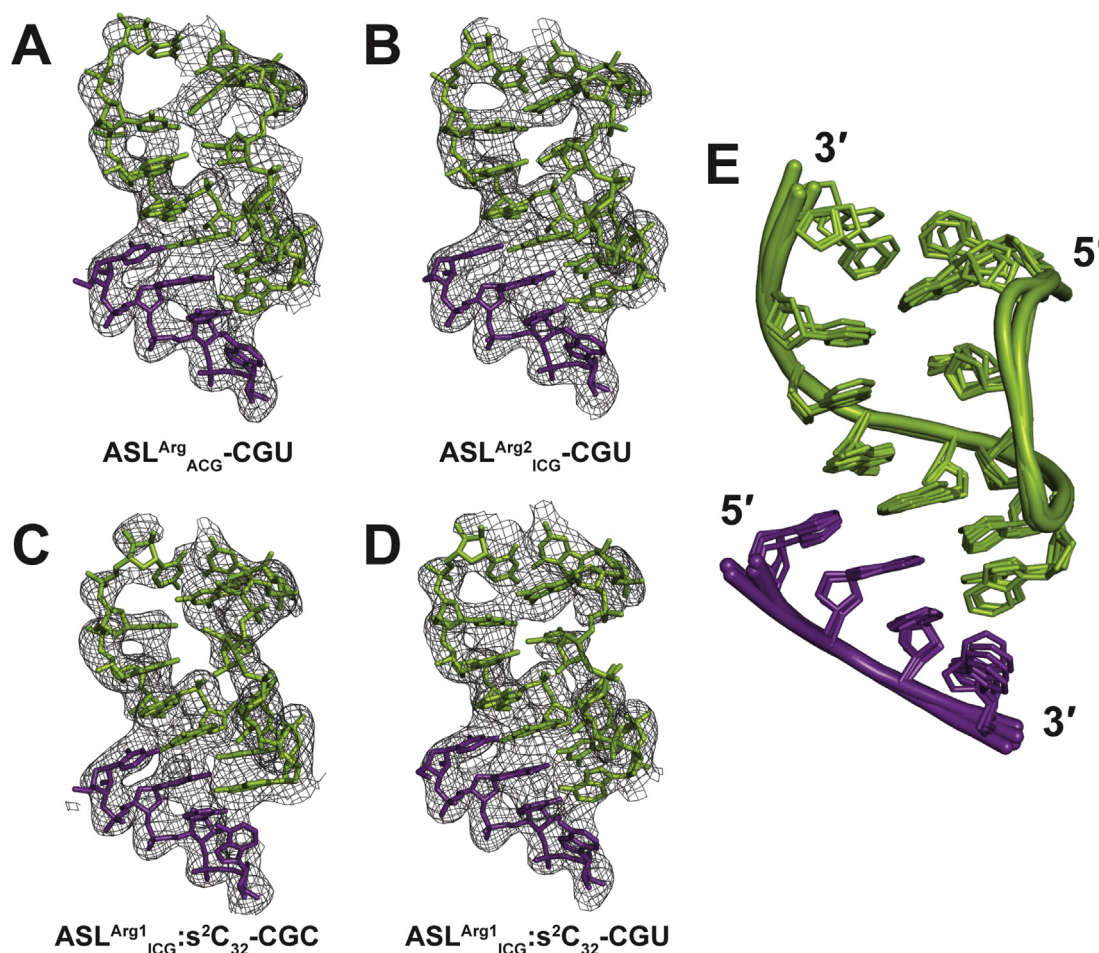


Fig. 4. Crystal structures of *E. coli* ASL^{Arg1} and ASL^{Arg2} constructs on the ribosome responding to the arginine codons CGC and CGU. *E. coli* ASL^{Arg} constructs (green with anticodons) and mRNAs (violet) were soaked into empty cryoprotected *T. thermophilus* 30S ribosome crystals. (A) ASL^{Arg}_{ACG} bound to the arginine codon CGU; (B) ASL^{Arg2}_{ICG} bound to CGU; (C) $ASL^{Arg1}_{ICG:s^2C_{32}}$ bound to CGC; and (D) $ASL^{Arg1}_{ICG:s^2C_{32}}$ bound to CGU. Structures are displayed with 2mFo-DFc maps contoured at $\sigma = 1.25$. (E) Overlay of ASL^{Arg} crystal structures. The interactions of the ASLs with the various mRNA codons for all four structures were aligned using ASL^{Arg}_{ACG} as reference. ASL, anticodon stem and loop.

crystal structures comprise the unmodified ASL^{Arg}_{ACG} with the codon CGU, the ASL^{Arg2}_{ICG} with CGU, and the $ASL^{Arg1}_{ICG:s^2C_{32}}$ with CGC and CGU. Not unexpectedly, diffraction-quality crystals of an s^2C_{32} -modified ASL^{Arg1}_{ICG} in complex with the CGA codon were unattainable. The antibiotic paromomycin was included as an established crystallographic aid in facilitating the formation of the closed form of the 30S subunit; this is known to improve diffraction resolution and result in better ordered electron density for the tRNA moiety [54,55].

The electron density maps are not identical, but no significant differences were observed between the four crystal structures (Fig. S4). Unbiased model refinement resulted in structures having a maximum root-mean-square deviation (RMSD) between all four of the ribosome-bound inosine-containing ASLs with and without the s^2C_{32} modification of

1.09 Å (crystallographic parameters and RMSDs, Tables S2 and S3). Therefore, the small density differences that were observed are likely to be experimental variability. In all four structures, the stem of the ASL^{Arg}_{ICG} constructs exhibited the geometric features characteristic of an A-form RNA conformation with Watson-Crick base pairs [56]. The structures of the codon-anticodon base pairs are in conventional Watson-Crick or wobble geometry, as expected. The structure of the 30S ribosomal subunit is identical within experimental error to other tRNA-codon recognition ribosome structures published previously [56–58]. The 2-thiocytidine at position 32, when present, caused no discernible perturbation to any structural feature of the ASL compared with structures in which it was absent.

In 2004, Murphy and Ramakrishnan [57] published two related structures, depicting shorter *E. coli*

ASL^{Arg2}_{ICG} constructs (bearing no other modifications) in complex with cognate CGC and noncognate CGA codons on the 30S *E. coli* ribosome. They used these structures as the basis of an experimental demonstration that the wobble inosine-adenosine pair adopts an *I*_{anti}/*A*_{anti} geometry at the anticodon-codon interface. When comparing these structures to the crystal structures solved previously, which did not include a CGA-bound structure, a salient point of divergence emerged (Fig. 5A and B). The orientation of the C₃₂ nucleobase when ASL^{Arg2}_{ICG} is bound to

CGA [57] deviates from the orientation observed when the ASL^{Arg2}_{ICG} instead binds CGU (or CGC). When the backbones of the structures of ASL^{Arg2}_{ICG} bound to the CGA and CGC codons are aligned, the C₃₂ nucleobase can be observed to have rotated by 38° (Fig. 5B). The hallmark hydrogen bonds forming the stable bifurcated C₃₂-A₃₈ cross-loop interaction are abrogated under inosine-adenosine wobble binding. The amine group on A₃₈ (N6) is within hydrogen bonding distance of N3 on C₃₂ when the codon is CGC (or CGU). When the ASL binds to the CGA codon, the distance between A₃₈ (N6) and C₃₂ (N3) is close to 5 Å, which prevents any direct cross-loop interaction.

It was not possible to obtain a crystal structure of the most interesting ribosome-bound complex, containing an s²C₃₂-modified ASL^{Arg1}_{ICG} interacting with CGA. This is an anticipated absence, given that no binding was detected between those species experimentally. Having observed, however, that the unmodified C₃₂ of ASL^{Arg2}_{ICG} exhibits a conformational distortion when bound to CGA, it was interesting to speculate that the replacement of an oxygen atom with a sulfur atom at that position might compound the effect observed. Because a complex between ASL^{Arg1}_{ICG}-s²C₃₂ and a CGA codon does not form on the ribosome and an x-ray crystal structure could not be obtained, *in silico* MD methods were used to study the effect of 2-thiocytydine on inosine-adenosine binding at the anticodon-codon interface.

Force field parameters developed for the modified nucleoside 2-thiocytydine agree with experimental properties

Force field parameters for MD are readily available for inosine, a common modified nucleoside, but it was necessary to develop and validate a set of novel force field parameters for 2-thiocytydine to simulate s²C-containing RNA molecules accurately. Briefly, AMBER(Assisted Model Building with Energy Refinement) type parameters were developed as described in detail in the [Supplementary Materials](#). Predictions from simulation about the distributions of the *syn-anti* glycosidic and sugar pucker angles of 2-thiocytydine in solution were compared to experimental results obtained by NMR (Figs. S1 and S2) and found to be in good agreement. The difference in the free energy of folding between the ASL^{Arg2}_{ICG} and ASL^{Arg1}_{ICG}:s²C₃₂ hairpins determined experimentally by UV-monitored thermal denaturation studies ($\Delta\Delta G_{37} = 0.5 \pm 0.10$ kcal/mol) [57] was also in good agreement with that obtained from simulation using the new s²C force field parameters ($\Delta\Delta G_{37} = 0.37 \pm 0.12$ kcal/mol) (Fig. S3). These results suggest that the force field parameter set for 2-thiocytydine correctly captured the *in vitro* behavior

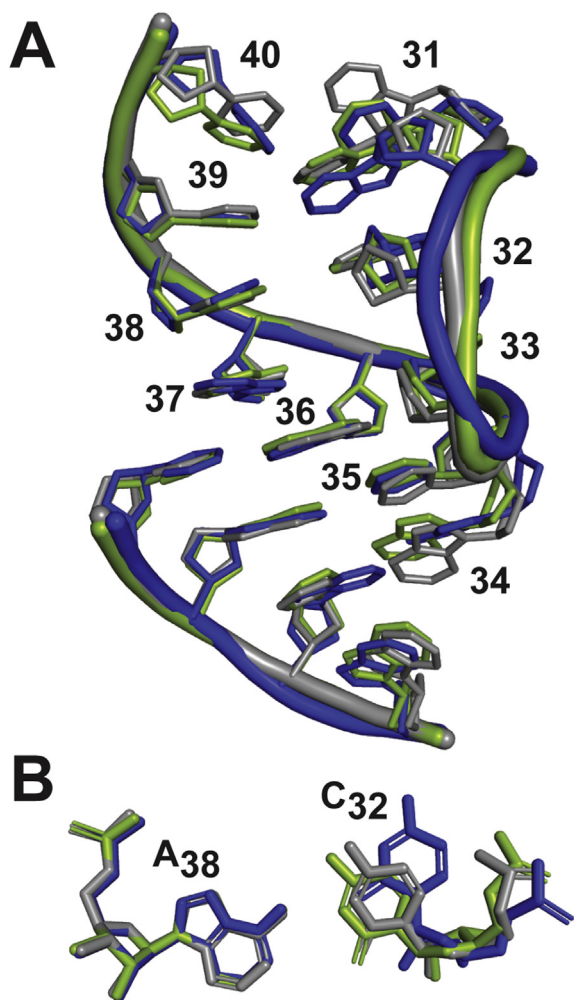


Fig. 5. Comparison of ASL^{Arg} crystal structures with previously solved structures. (A) The ASL^{Arg2}_{ICG} (gray) interaction with the mRNA codon CGU is used as a representative of the structural set and superimposed onto the previously solved structures of the truncated ASL^{Arg2}_{ICG} binding the CGC codon (green) and the CGA codon (blue) [57]. (B) A comparison of the C₃₂-A₃₈ base pair with the ASL^{Arg2}_{ICG} construct reveals a deviation in the structure when bound to the rare codon CGA; same coloration as in (A). The structure results from the altered geometry of the I₃₄:A₃ anticodon-codon base pair. ASL, anticodon stem and loop.

of the nucleoside, and it was used in the remainder of the studies reported here.

MD simulations of ASL^{Arg}_{ICG} constructs bound to mRNA on the ribosome indicate that the purine:purine I₃₄-A₃ interaction destabilizes the canonical U-turn motif

To investigate the effects of nucleoside modifications on the structure of tRNA^{Arg} bound to mRNA on the ribosome, simulations were performed for ASL^{Arg}_{ICG} binding to each synonymous arginine codon (CGC, CGU and CGA) with and without the s²C modification at position 32. Initially, a 10-ns simulation of each of the structures with the entire ribosome intact demonstrated that the system does not exhibit any structural rearrangements, in either the presence or absence of the modification. Expected interaction between the ribosomal subunit and its tRNA and mRNA substrates (specifically between tRNA and mRNA residues and ribosome nucleosides G530, A1492, A1493) are maintained throughout the simulations. Therefore, to simplify the system and permit longer simulations, the system of interest was thereafter defined as composed of the tRNA and mRNA moieties, an intact stable fragment of the ribosomal RNA which includes all residues making direct contacts with the tRNA and/or mRNA, and associated Mg²⁺ ions. All simulations were run for 50 ns.

As predicted by numerous previous structural studies, when ASL^{Arg1}_{ICG} or ASL^{Arg2}_{ICG} binds CGC or CGU, the inosine at position 34 of the ASL interacts in standard wobble geometry with the smaller pyrimidine ring (U₃/C₃) of the third nucleoside in the mRNA codon [54,57,59]. The standard C1'-C1' distance between a purine-pyrimidine base pair is in the range of 10.2–10.8 Å [37], a characteristic that can be observed in these MD simulations as well when the mRNA codon is either CGC or CGU. In this conformation the canonical U-turn interaction, in which U₃₃ interacts with the phosphate group of G₃₆, is also maintained, as previously observed in simulations and experimentally obtained structures [60–62].

In the case in which the ASL^{Arg}_{ICG} binds the CGA codon, however, I₃₄ must form a wobble base pair with the larger purine ring of adenosine (A₃), an interaction which occupies a broader spatial extent [38,57]. To accommodate the larger adenosine in position 3 of the CGA codon, the C1'-C1' distance between I₃₄ and A₃ becomes 2.5 Å greater than the standard purine-pyrimidine distance, occupying nearly 13 Å total [37]. Previous reports based on x-ray crystallography suggested that when ASL^{Arg2}_{ICG} binds CGA on the ribosome, the ASL absorbs this structural perturbation chiefly through modest changes in torsion angles between the C1 and P atoms of the inosine [57]. Examination of these

simulation results confirmed that those torsion angle alterations occur but also demonstrated that the purine-purine displacement at the wobble position propagates further than I₃₄. This is reflected in a significantly distorted U-turn in which the U₃₃, instead of interacting with the phosphate group of G₃₆ as in a canonical U-turn [60–62], now interacts with C₃₅ through the O2' hydroxyl group (Fig. 6A and B). This altered hydrogen bonding brings about two important changes: (a) it introduces a kink in the ASL domain that results in a rotation of the C₃₂ nucleobase away from the center of the loop domain, as also observed experimentally by x-ray crystallography [60–62] and (b) U₃₃ rotates toward the outside, opening the loop cavity for hydration water molecules. In this conformation, the classic bifurcated cross-loop hydrogen bond between C₃₂ and A₃₈ is disrupted (Fig. 6C and D) and is instead replaced by dynamic water-mediated interactions involving the N6 and O2 of the tRNA's A₃₈ and C₃₂ bases, respectively, with one or two bridging water molecules. This behavior is in contrast to the binding of ASL^{Arg2}_{ICG} to CGC or CGU, in which the wobble position inosine forms a standard Watson-Crick bond with a pyrimidine nucleoside, and N6 of A₃₈ and N3 of C₃₂ participate in the classic bifurcated hydrogen bond. In this confirmation, the oxygen on the second position (O2) of C₃₂ faces away from the interior of the loop domain. When C₃₂ is unmodified and the codon is CGA, it appears clear that the O2 directly participates in maintaining the loop hydrated with at least one dynamic H₂O molecule (Fig. 7 & Supplementary video). The absence of these water molecules in the published crystal structure [57] is not surprising because of their highly dynamic nature (Supplementary video).

Supplementary video related to this article can be found at <https://doi.org/10.1016/j.jmb.2019.12.016>.

A canonical interaction and structure are obtained when ASL^{Arg2} containing only I₃₄ binds CGU or CGC but CGA binding results in a distortion of the U-turn that propagates as far as the C₃₂-A₃₈ cross-loop interaction. It therefore seemed probable that the 2-thiocytydine modification at position 32 of ASL^{Arg1}_{ICG}:s²C₃₂ would compound the structural instability obtained when a purine-purine interaction occupies the wobble position. Accordingly, the same simulations were performed on the system in which s²C₃₂ replaced C₃₂ *in silico*. A stable interaction does not form naturally between the mRNA CGA codon on the ribosome and the s²C₃₂-modified ASL^{Arg1}_{ICG}; thus, the simulation included an extra set of constraints on the ASL itself, holding it fixed in its initial distorted U-turn structure such that the binding interface between codon and anticodon could not be destabilized. In this way, we were able to account for the failure of ASL^{Arg1}_{ICG}:s²C₃₂ to form a complex with CGA under biological conditions.

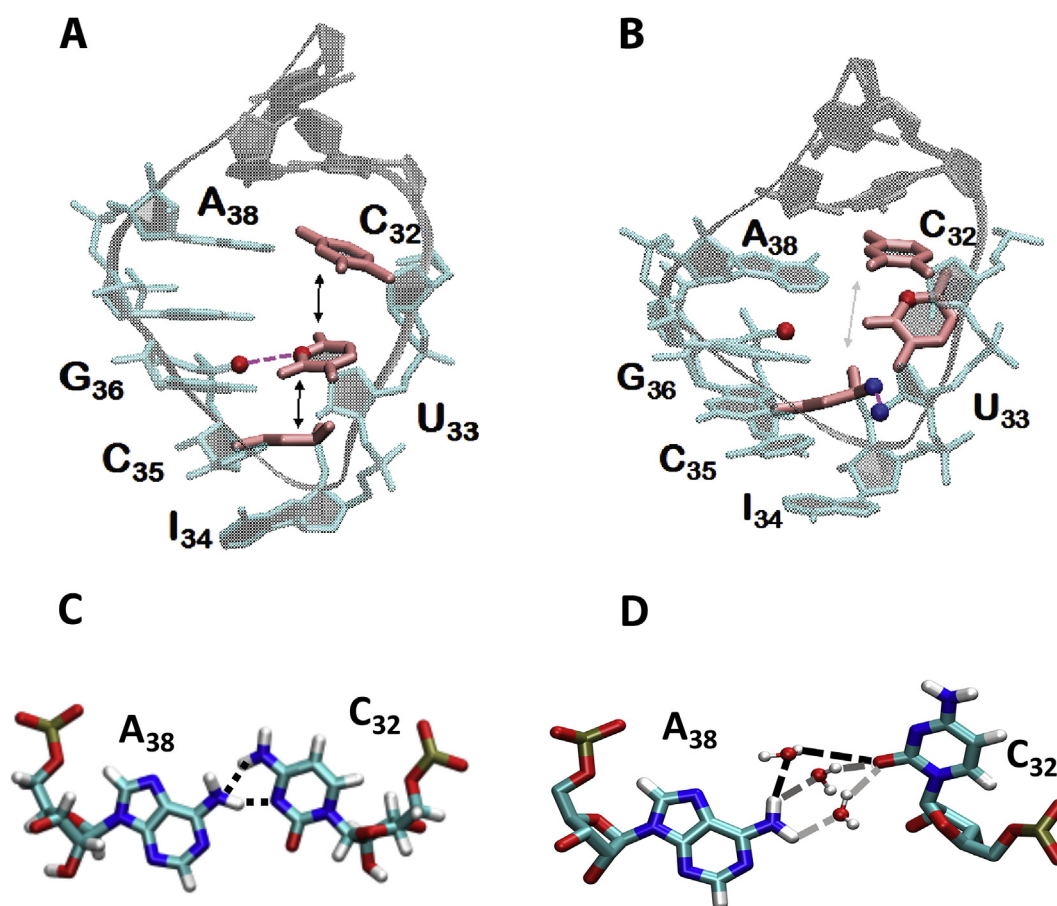


Fig. 6. Molecular dynamics (MD)-simulated comparison of the U-turns and A₃₈-C₃₂ interactions in ASL^{Arg1}_{ICG} binding the codons CGU (PDB ID: 6MKN) and CGA (PDB ID: 1XNQ). (A) Canonical U-turn with stacking and U₃₃-G₃₆ interaction (ASL^{Arg2}_{ICG} bound to codon CGU). (B) Noncanonical U-turn with U₃₃-C₃₅ interaction. The stacking interaction and U₃₃-G₃₆ interaction are absent (ASL^{Arg2}_{ICG} bound to codon CGA). (C) A₃₈-C₃₂ interaction via hydrogen bonds (ASL^{Arg1}_{ICG} bound to codon CGU). (D) Water-mediated interaction between A₃₈-C₃₂ (ASL^{Arg1}_{ICG} bound to codon CGA). ASL, anticodon stem and loop.

The addition of s²C₃₂ to ASL^{Arg1}_{ICG} had no discernible effect on the structure of the mRNA-bound ASL on the ribosome when the bound codon is CGC or CGU and a purine-pyrimidine pair occupies the wobble position. In contrast, when the codon is CGA and the codon-anticodon interaction is enforced, the MD simulations demonstrated that the larger and less electronegative sulfur atom replacing O2 in the s²C₃₂-modified ASL^{Arg1}_{ICG} is not able to coordinate water and stabilize the water-mediated C₃₂-A₃₈ interaction as efficiently as the oxygen (Supplementary video). The S2-for-O2 substitution is expected to have both enthalpic and entropic penalties. The larger sulfur atom and the longer C=S bond result in a reduction of available space for water molecules inside the loop of the ASL^{Arg1}_{ICG}:s²C₃₂ construct when in complex with CGA, as observed in simulation (Fig. 7). In the presence of s²C₃₂, the water molecules are confined to three distinct clusters, while in the unmodified case the water molecules are relatively dispersed in the

accessible space. The confined, more ordered distribution of water in the presence of the modified s²C₃₂ implies that the water-mediated interaction occupies a smaller number of possible configurations, resulting in an entropic penalty. The reduced enthalpy arising from the weakened water-mediated interaction and the entropic penalty as a result of the fewer accessible configurations for the water-mediated hydrogen bond between the A₃₈ and C₃₂ both contribute to destabilization of the loop structure.

Furthermore, in the absence of constraints on the ASL^{Arg1}_{ICG}:s²C₃₂ in complex with CGA, the U-turn-stabilizing U₃₃ (O2') - C₃₅ (OP) hydrogen bond is observed to break, in favor of a U₃₃ (O2') - I₃₄ (OP) hydrogen bond, further destabilizing the positioning of U₃₃ in the loop. While the simulations did not sample the timescales necessary to observe spontaneous unbinding of the ASL from the CGA codon in response to the sulfur modification, they do provide valuable molecular insights into the interactions that

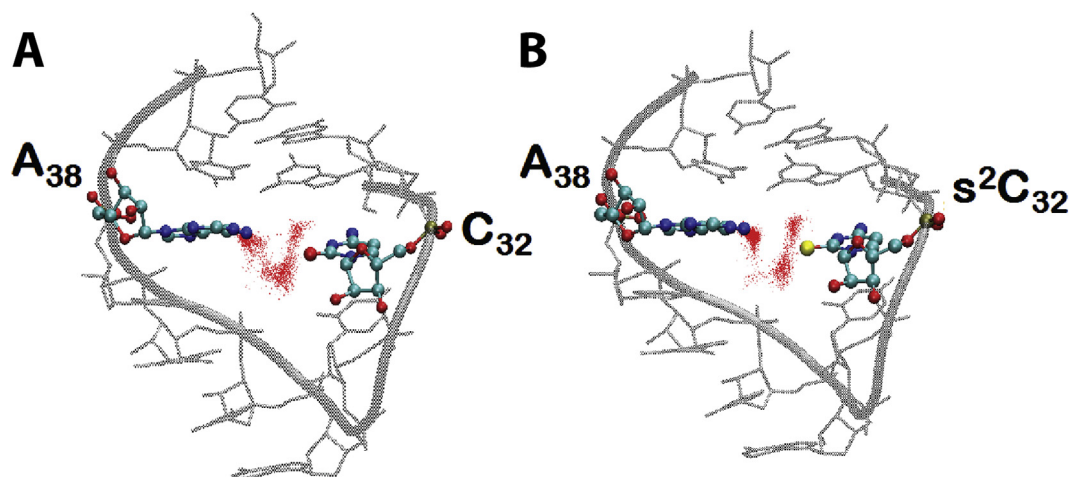


Fig. 7. The MD simulation of the distribution of water inside the loop of the modified ASL^{Arg1}_{ICG}:s²C₃₂ and the ASL^{Arg2}_{ICG} in binding to the codon. The MD simulation was conducted in a water box with 4.3×10^4 water molecules. Each dot represents the oxygen of a water molecule which is within 3.5 Å of the O₂/S₂ of C₃₂ and the amine nitrogen, N₆, of A₃₈, where the water molecule can establish hydrogen bonds with both these groups. Snapshots were recorded every ps. (Supplementary video). ASL, anticodon stem and loop; MD, molecular dynamics.

weaken and finally break. Consequently, they provide insight to the substantial decrease of the experimentally observed binding affinity between tRNA^{Arg1}_{ICG}:s²C₃₂ and its cognate codon CGA.

Discussion

The effect of modified nucleosides on the ability of tRNA to recognize codons has been discussed ever since 1966, when Crick published the wobble hypothesis and proposed that an inosine occupying position 34 of the anticodon can bind A, C, or U [37]. Other modifications, particularly those at positions 34 and 37, affect the ASL in a variety of ways, including restricting codon recognition, enabling translocation, prestructuring of the ASL to bind the ribosomal A site in addition to expanding, and restricting wobble recognition [22,58,63]. In fact, the ASL of the two tRNA^{Arg} isoacceptors (tRNA^{Arg1}, tRNA^{Arg2}) discussed here contain another modification at position 37, 2-methyladenosine (m²A₃₇) [1,34]. Aliphatic modifications at position 37 of tRNAs are commonly shown to stabilize the codon-anticodon pairs by additional stacking interactions and thus enhance binding affinity of the tRNA-mRNA complex [1,34]. However, in the case of ASL^{Arg1}_{ICG} and ASL^{Arg2}_{ICG}, ribosomal binding studies show that the m²A₃₇ modification also reduces or negates binding of the ASL to its cognate and near cognate codons [14]. A complete structural and dynamic explanation for this counterintuitive result is being pursued using MD simulations and is a part of our ongoing investigative efforts.

Other than s²C₃₂, modifications at position 32 are not uncommon. Modified nucleosides that have been

found at this position include N3-methylcytidine (m³C), 2'-O-methyl-(cytidine, uridine and adenosine) (Cm, Um and Am) [64,65] and pseudouridine (Ψ). The m³C₃₂ modification has been found to impart resistance to oxidative stress in yeast and higher eukaryotes when present in tRNA^{Ser} and tRNA^{Thr} and in tRNA^{Arg}, respectively [66]. When absent, the lack of Cm at position 32 on tRNA^{Phe} negatively affects translational efficiency of the tRNA [67].

Among the several modifications that occur at position 32; however, the s²C₃₂ modification is rare. The nucleoside s²C was first reported in an *E. coli* tRNA^{Ser} [68]. An *in vitro* investigation of the synthesis of the T4 phage coat protein in *E. coli* showed that the triply modified tRNA^{Arg1}_{ICG}:s²C₃₂,m²A₃₇ was required to maintain the translational reading frame; substitution of C₃₂ for s²C₃₂ increased the incidence of frameshift mutations during translation [47]. A *Salmonella enterica* mutant unable to synthesize 2-thiocytidine exhibited no overall growth disadvantage, but the reintroduction of the modification was observed to decrease the rate of peptide translation for the rare CGA codon and not the common CGU [69]. This finding was in broad agreement with the recent discovery that, while *E. coli* ASL^{Arg1}_{ICG} (identical to ASL^{Arg2}_{ICG}) carrying an unmodified C₃₂ binds all three wobble hypothesis–predicted mRNA codons, the s²C₃₂-modified ASL^{Arg1}_{ICG} was completely unable to bind to CGA, although it bound CGC and CGU with physiological binding constants [14]. The negative influence of s²C₃₂ on I₃₄ decoding of A3 was clearly replicated with a yeast tRNA^{Ile} ASL construct. Yet, after determining three NMR structures of variously modified ASL constructs of tRNA^{Arg} [14] and four crystal structures of ASL^{Arg1}_{ICG} with and

ASL^{Arg2}_{ICG} without s²C₃₂ on the ribosome in response to various codons, the mechanism by which s²C₃₂ was modulating the ability of I₃₄ to read A3 was not clear. A comparison of crystal structures containing the CGU/CGC codon with one containing the CGA codon revealed that in the case of I₃₄-A₃, the hallmark interactions of the canonical U-turn are modified to accommodate the larger purine-purine base pair. This evident structural difference was, however, not itself sufficient to explain the role of s²C₃₂ in negating binding of the ASL^{Arg1}_{ICG}:s²C₃₂ to the CGA codon. Taken together, the aforementioned results do suggest both a global role for s²C₃₂ in preventing frameshift mutations during protein translation and a specific role in restricting the ability of tRNA^{Arg1}_{ICG} to decode the rare arginine codon CGA.

MD simulation of the I₃₄-A3 interaction in explicit water appears to have revealed a possible mechanism based on dynamics and the ordering of water by s²C₃₂ between the ASL nucleosides C₃₂ and A₃₈. The interaction between the nucleosides Y₃₂-R₃₈ at the base of the loop domain of the ASL is typically characterized by a single or bifurcated hydrogen bond [36]. The strength of this interaction appears to be carefully modulated. While a stronger interaction, similar to that of a canonical base pair, could lead to a loss in flexibility of the loop domain at this position, a weaker interaction could conversely result in loss of stacking between Y₃₂ and U₃₃ and result in a disruption of the functional U-turn conformation of the ASL. Indeed, the identity of the bases occupying these positions and the nature of their interaction correlates strongly to the affinity of the tRNA for the ribosomal A site for cognate and near cognate codons [51]. The nature of the C₃₂-A₃₈ interaction is attributed to energetic penalties and structural changes that affect the codon-anticodon helix in cognate and near cognate recognition [51]. Thus, we hypothesized that tRNAs having s²C₃₂ have an altered anticodon loop conformation and enthalpy penalties whereas interaction of C₃₂ with A₃₈ could significantly affect wobble of I₃₄ to A3.

In the crystal structure of the ASL^{Arg2}_{ICG} bound to the CGA codon at the ribosomal A site, the direct cross-loop interaction between C₃₂ to A₃₈ is disrupted in the presence of the I₃₄-A3 interaction [57]. In our MD simulations, we observed that the subtle distortion reported at the codon-anticodon mini-helix of ASL^{Arg2}_{ICG} due to the I₃₄-A3 purine-purine base pair results in a substantially distorted, noncanonical U-turn upstream. This, in turn opens up the loop domain for hydration waters and brings about a water-mediated interaction between C₃₂ to A₃₈ involving the O2 atom of C₃₂. It is well established that pseudouridine at position 32 uses a water-mediated base-backbone interaction to stabilize the 32–38 interaction pair [36]. Replacing C₃₂ with s²C₃₂ in the ASL^{Arg1}_{ICG} construct weakens the water-mediated interaction, due to the larger size and weaker

electronegativity of the sulfur atom. We hypothesize that this diminished interaction makes formation of the required noncanonical U-turn less favorable, which destabilizes the interactions within the loop and with the mRNA and leads to the inability of s²C₃₂-modified ASL^{Arg1}_{ICG} to bind to the CGA codon.

It is interesting to note that the rare modification s²C₃₂ affects the decoding capacity of the ASL^{Arg1}_{ICG} only in the case of the similarly rare codon CGA. It does not significantly affect the structure or the ribosomal binding capacity of the ASL^{Arg1}_{ICG} to the more common CGU and CGC codons of arginine, nor does its absence affect the growth rate or physiology, under laboratory conditions, of bacteria in which it is native [69]. This observation permits speculation that the incorporation of the s²C₃₂ modification in tRNA^{Arg1}_{ICG} may have originally been an evolutionary accident but that its negative effect on the already inefficient decoding of the rare codon CGA conferred an advantage in some environments.

It is increasingly acknowledged that tRNA modification profiles can be reprogrammed, under various stresses and during environmental changes, to better read specific codons over their synonymous counterparts [70–72]. The reprogramming of tRNA wobble modifications has already been shown to lead to enhanced translation of codon-biased mRNAs (known as modification-tunable transcripts, or MoTTs) with roles in survival and stress response [72,73]. It is not difficult to imagine a similar tunable role for s²C₃₂ in regulating the translation of MoTTs containing disproportionate stretches of rare CGA codons, which have been shown in both *E. coli* and *S. cerevisiae* to promote ribosomal stalling and affect translation negatively under ordinary cellular conditions [70,74,75]. An investigation of proteins consisting of a generous percentage of arginines coded by CGA codons and their functions in the bacteria would be useful in understanding the physiological impact of this modification [76].

Material and Methods

Oligonucleotide preparation

The modified nucleoside 2-thiocytidine (s²C) was synthesized chemically (Trilink Biotechnologies, San Diego, CA) and then derivatized to the 5'-O-BzH-2'-O-ACE-protected 2-thiouridine-3'-(methyl-N,N-diisopropyl) phosphoramidite (Dharmacon Products; Thermo Fisher, Lafayette, CO). The chemically synthesized heptadecameric *E. coli* ASL^{Arg1} [4] and *S. cerevisiae* ASL^{Ile} constructs [4,77] were also produced by ACE chemistry [52] to contain A₃₄ or I₃₄ with or without s²C₃₂ (Dharmacon Products; Thermo Fisher). After deprotection via standard protocol, the RNA was desalted and dialyzed against water

using a 3500 Da cutoff membrane (Pierce) followed by resuspension in 20 mM sodium phosphate buffer, pH 6.8.

UV-monitored thermal denaturation and CD

ASL^{Ile}_{IAU} RNA samples (Fig. 1C) were prepared as aforementioned at concentrations adjusted to ~0.3 absorbance units at 260 nm. Thermal denaturations and renaturations were monitored by UV absorbance at 260 nm with a Cary 100 UV-visible spectrophotometer (Agilent) using Thermal software. Five successive denaturations and renaturations were conducted over a temperature range of 5–95 °C using 1-cm pathlength cuvettes. The temperature ramp rate was 1.0 °C/min with a data sampling interval of 1 min. Thermodynamic parameters were obtained by fitting absorbance versus temperature profiles using the curve-fitting program Meltwin (version 3.5). After normalization, the first derivative was determined and smoothed by cubic splines. CD spectra on each sample were collected immediately after thermal denaturation studies as a series of six scans on a Jasco 600 spectropolarimeter (Jasco, Inc.) at 20 °C in a 1-cm pathlength quartz cuvette. All data were baseline corrected using a control containing buffer only. Data were analyzed by normalizing the spectra to molar circular dichroic absorbance using simultaneously collected absorbance data to calculate the concentrations of each sample ($\Delta\epsilon = \theta/32980 \cdot C \cdot L \cdot N$) [78].

Codon-specific ribosomal binding assays

E. coli 70S ribosomes were purified from *E. coli* MRE 600 cultures and used in codon-specific ribosomal A site binding assays as described previously [14]. Together with ASL^{Ile}_{IAU} constructs, the following mRNA sequences based on the T4 gp32 message and *S. cerevisiae* tRNA^{Ile}_{IAU} were used. (A-site codon sequences are bold and underlined.):

- (1) GGCAAGGAGGUAAAAAUG**AUC**GCACGU
- (2) GGCAAGGAGGUAAAAAUG**AUUG**GCACGU
- (3) GGCAAGGAGGUAAAAAUG**AUAG**GCACGU

Heptadecamer ASLs were 5'-³²P-radiolabeled and purified using preparative denaturing 15% polyacrylamide gel electrophoresis. mRNA-programmed ribosomes (250 nM ribosome and 2.5 μM mRNA) were P-site saturated with tRNA^{fMet} and incubated for 1 h at 37 °C with varying concentrations (0, 0.25, 0.5, 1.0, 1.5, 2.5, 5 μM) of unlabeled ASL spiked with up to 2 kCPM radiolabeled ASL in ribosome binding buffer (50 mM HEPES, pH 7; 30 mM KCl; 70 mM NH₄Cl; 1 mM DTT; 100 μM EDTA; 20 mM MgCl₂ adjusted to pH 7 with 2 M NaOH). Binding reactions were then filtered through a 0.22 μM nitrocellulose filter (Whatman) using a modified micro-sample filtration manifold (Whatman Schleicher & Schuell Minifold) 96-well dot blot apparatus [79,80]. A standard curve was generated by spotting known amounts of radiolabeled ASL onto a small strip of nitrocellulose filter. Filters were imaged using a Typhoon phosphorimager (GE Healthcare), and radioactive spot density was

calculated using ImageQuant TL software (Amersham). Binding constants were calculated using the one-site-specific binding function in Prism v3 (Graphpad). An internal positive control using unmodified yeast ASL^{Phe} binding to polyuridylic acid and nonspecific negative controls using a control mRNA with GCG in the A-site position were performed in tandem with every experiment. All binding curves were performed in triplicate in each of three independent experiments. Determination of ribosome activity and usability of mRNAs was accomplished using filter binding assays consisting of only ribosomes and 5'-³²P-radiolabeled mRNAs. This approach to assessing the binding of tRNA to ribosomes by treating it in the manner of a standard ligand-binding experiment has historical precedents as far back as 1966 [81] and also appears in a large number of more modern studies of ribosome/tRNA interaction [82–84].

Crystallization

Chemically synthesized hexamer mRNA oligonucleotides (5'-CG(U/C)AAA-3') were purified by preparative polyacrylamide electrophoresis (Thermo Fisher Scientific). The 30S ribosome subunits were purified from *T. thermophilus* followed by crystallization and cryoprotection in 26% (v/v) MPD, 100 mM K-MES (pH 6.5), 75 mM NH₄Cl, 200 mM KCl, and 15 mM MgCl₂ as described [85]. Empty, cryoprotected 30S ribosome crystals were then soaked in cryoprotection solution containing 80 μM paromomycin, 300 μM ASL, and 300 μM mRNA [54,55]. After soaking for at least 48 h, crystals were plunged in liquid nitrogen and stored until data collection. Paromomycin, an antibiotic, induces a closed conformation of the 30S ribosomal subunit without affecting the conformation of the A-site tRNA [55], resulting in improved density and resolution of the ASL [54].

Data collection and refinement

Native RNA-bound ribosome diffraction data were collected at NE-CAT beamlines 24-ID-C and 24-ID-E of the advanced photon source and processed using X-ray Detector Software, XDS [86]. Phases were obtained by molecular replacement using PHENIX with PDB ID: 1XNR a search model [87]. The model was refined in PHENIX, followed by additional refinement, visualization, and model building in Coot [87,88]. The molecular visualization system, PyMOL was used for figure production [89]. The eLBOW module within PHENIX was used to generate geometry restraints and dictionary files for the nonstandard residues (paromomycin, inosine, 2-methyladenosine, and 2-thiocytidine) using the semiempirical quantum mechanical AM1 method [90]. Data sets were not collected at the same time; differences in resolution of the structures were, therefore, likely caused by variation in the X-ray beam rather than in the crystals themselves. A summary of data and refinement statistics is given in Table S1. The atomic coordinates for the ASL^{Arg1} and ASL^{Arg2} constructs bound to mRNA on the 30S ribosome were deposited in the Protein Data Bank under the accession numbers 6DTI (unmodified ASL^{Arg}_{ACG} binding codon CGU), 6MKN (ASL^{Arg2}_{ICG} binding codon CGU), 6MPF (ASL^{Arg1}_{ICG}:s²C₃₂ binding CGC), and 6MPI (ASL^{Arg1}_{ICG}:s²C₃₂ binding CGU).

Simulation Methods

Force field parameterization and validation

AMBER-type force field parameters for the modified nucleoside 2-thiocyridine (s^2C) were obtained and validated as described in detail in the [Supplementary Materials](#). Briefly, published values were used for the partial charges of the s^2C atoms, while AMBER-99 force field parameters and AMBER-99 parameters with the Chen-Garcia correction were used for bonded and Lennard-Jones (LJ) interactions, respectively [91,92]. To validate these force field parameters, two sets of MD simulations were performed: (i) single nucleoside simulations of cytidine and 2-thiocyridine and (ii) simulations in solution of ASL^{Arg1}_{ICG} with the 2-thiocyridine modification at position 32 and of ASL^{Arg2}_{ICG} without the s^2C_{32} . The simulated distributions of the *syn-anti* glycosidic and sugar pucker angles for s^2C in solution were then compared with experimental results obtained from NMR $^1H-^1H$ nuclear overhauser enhancement spectroscopy-heteronuclear single quantum coherence, and correlation spectroscopy-double quantum filtered (NOESY-HSQC and DQF-COSY)spectra (Figs. S1A and B), and the folding free energy difference between the ASL^{Arg2}_{ICG} and ASL^{Arg1}_{ICG}: s^2C_{32} RNA hairpins (Fig. S2; Table S1) was compared with that determined experimentally by UV-monitored thermal denaturation studies [14]. The simulation results were in good agreement with experiment, suggesting that the force field parameter set for 2-thiocyridine correctly captured the *in vitro* behavior of the nucleoside.

MD simulations

The crystal structures of the 16S ribosomal subunit of *T. thermophilus* containing the ASL domains of tRNA^{Arg1}_{ICG} and tRNA^{Arg2}_{ICG} and the mRNA codon fragment in the decoding center were obtained from the Protein Data Bank: ASL^{Arg2}_{ICG} bound to CGC (1XNR) and CGA (1XNQ) [57] or acquired crystallographically as aforementioned: unmodified ASL^{Arg}_{ACG} bound to CGU (deposited as 6DTI), ASL^{Arg2}_{ICG} bound to CGU (deposited as 6MKN), ASL^{Arg1}_{ICG}: s^2C_{32} bound to CGC (deposited as 6MPF), and ASL^{Arg1}_{ICG}: s^2C_{32} bound to CGU (deposited as 6MPI). To model the binding of ASL^{Arg1}_{ICG}: s^2C_{32} to the CGA codon, the cytosine at position 32 in the ASL^{Arg2}_{ICG}-CGA crystal structure (1XNQ) was modified to s^2C_{32} *in silico*. Missing atoms and ions in the ribosome-associated proteins were added using MOE [93].

A 10-ns all-atom simulation of each of the structures with the entire ribosome intact was used to demonstrate that the system does not exhibit any changes in the interaction between the canonical tRNA-mRNA pair and the ribosomal subunit. Thereafter, the simulation system included the tRNA and mRNA moieties, an intact stable fragment of the ribosomal RNA comprising those residues making direct contacts with the tRNA and/or mRNA, and associated Mg²⁺ ions in a solution of 1 M KCl in a 3D periodic box. The box size was 11.25 × 11.25 × 11.25 nm³ containing ~997 K⁺ ions, ~884 Cl⁻ ions and 4.3 × 10⁴ water molecules.

MD simulations were performed using Gromacs-4.6.3 and Gromacs-5.0.6 packages [94]. The MD simulations incorporated a leap-frog algorithm with a 2-fs timestep to integrate the equations of motion. The system was maintained at 300 K, using the velocity rescaling thermostat [95]. The pressure was maintained at 1 atm using the Berendsen barostat for equilibration [96,97]. The long-range electrostatic interactions were calculated using particle mesh Ewald algorithm with a real space cut-off of 1.0 nm [98]. Lennard-Jones interactions were also truncated at 1.0 nm. The TIP3P model was used represent water molecules, and the LINCS algorithm was used to constrain the motion of hydrogen atoms bonded to heavy atom [99]. The system was subjected to energy minimization to prevent any overlap of atoms, followed by 0.5 ns of equilibration in constant pressure-constant temperature ensemble ensemble and a 50-ns constant volume-constant temperature ensemble production run. During simulations, the ribosomal subunit was held in place using position restraints on its heavy atoms with a force constant of 1000 N/nm in each spatial dimension for the equilibration run and then was frozen for the rest of the simulation. When modeling the binding of ASL^{Arg1}_{ICG}: s^2C_{32} to the CGA codon, the atoms comprising the ASL were held fixed in the conformation adopted during the simulation of the binding of ASL^{Arg2}_{ICG} without s^2C_{32} to enforce the binding interface between the anticodon and the codon. Coordinates of the RNA components (rRNA, tRNA, and mRNA) of the system were stored every 1 ps for further analysis.

Accession Numbers

The final model coordinates and the corresponding structure factors were submitted to Protein Data Bank with the accession numbers PDB ID: 6DTI (unmodified ASL^{Arg}_{ACG} bound to CGU); PDB ID: 6MKN (ASL^{Arg2}_{ICG} bound to CGU); PDB ID 6MPF (ASL^{Arg1}_{ICG}: s^2C_{32} bound to CGC); and 6MPI (ASL^{Arg1}_{ICG}: s^2C_{32} bound to CGU).

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Author contributions

S.V., W.A.C., J.L.S., H.D., F.V.M., S.V.R., and K.L.S. contributed to conducting of the empirical and *in silico* experiments, analysis of data, and feedback on the manuscript; W.A.C. and K.L.S. contributed to writing; and P.F.A. contributed to concept of study, aid in preparation of figures, editing the manuscript and supplementary figures.

Conflict of interest statement

No conflict of interest exists with any of the authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jmb.2019.12.016>.

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Abbreviations used:

tRNA, transfer RNA; mRNA, messenger RNA; ASL, anticodon stem and loop; s²C, 2-thiocytidine; m²A, 2-methyladenosine; I, inosine; s²U, 2-thiouridine; MD, molecular dynamics; NMR, nuclear magnetic resonance; MoTTs, modification-tunable transcripts; ACE, 5'-silyl-2'-acetoxyethyl orthoester; CD, circular dichroism; NOESY-HSQC, nuclear overhauser enhancement spectroscopy-heteronuclear single quantum coherence; COSY-DQF, correlation spectroscopy-double quantum filtered; NPT, constant pressure-constant temperature ensemble; NVT,

constant volume-constant temperature ensemble; PME, particle mesh Ewald.

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