YrdC exhibits properties expected of a subunit for a tRNA threonylcarbamoyl transferase

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ABSTRACT

The post-transcriptional nucleoside modifications of tRNA's anticodon domain form the loop structure and dynamics required for effective and accurate recognition of synonymous codons. The N^6-threonylcarbamoyladenosine modification at position 37 (t^6A_{37}), 3′-adjacent to the anticodon, of many tRNA species in all organisms ensures the accurate recognition of ANN codons by increasing codon affinity, enhancing ribosome binding, and maintaining the reading frame. However, biosynthesis of this complex modification is only partially understood. The synthesis requires ATP, free threonine, a single carbon source for the carbamoyl, and an enzyme yet to be identified. Recently, the universal protein family Sua5/YciO/YrdC was associated with t^6A_{37} biosynthesis. To further investigate the role of YrdC in t^6A_{37} biosynthesis, the interaction of the *Escherichia coli* YrdC with a heptadecamer anticodon stem and loop of lysine tRNA (ASL^{Lys}_{UUU}) was examined. YrdC bound the unmodified ASL^{Lys}_{UUU} with high affinity compared with the t^6A_{37}-modified ASL^{Lys}_{UUU} ($K_d = 0.27 \pm 0.20$ μM and 1.36 ± 0.39 μM, respectively). YrdC also demonstrated specificity toward the unmodified versus modified anticodon pentamer UUUUA and toward threonine and ATP. The protein did not significantly alter the ASL architecture, nor was it able to base flip A_{37}, as determined by NMR, circular dichroism, and fluorescence of 2-aminopuine at position 37. Thus, current data support the hypothesis that YrdC, with many of the properties of a putative threonylcarbamoyl transferase, most likely functions as a component of a heteromultimeric protein complex for t^6A_{37} biosynthesis.

Keywords: substrate binding; fluorescence; 2-aminopurine; enzyme mechanism; t^6A_{37} synthetase

INTRODUCTION

The high fidelity of translation is critical to the formation of properly functioning proteins. Inaccurate decoding at the ribosome can result in a myriad of diseases and afflictions. Therefore, it is crucial that transfer RNAs (tRNAs) have high affinity and precise recognition of their cognate, synonymous codons. Post-transcriptional modifications of nucleosides in the anticodon stem and loop domain (ASL) are known to play a critical role in maintaining the accuracy of translation (Björk 1995; Agris 1996, 2008). Two of the most frequently modified nucleosides in the ASL are found at the first, or wobble, position 34 of the anticodon and at the universal purine position 37, 3′-adjacent to the anticodon (Nishimura 1972; Agris 1996). Nucleosides 34 and 37 are also two of the most diversely and chemically rich modified nucleosides found in all RNAs (Agris 1996; Jühling et al. 2009). These modifications define anticodon architecture, restrict dynam-ics of the ASL, enhance codon affinity and ribosome binding, as well as reduce translational frameshifting (Urbonavicius et al. 2001; Agris 2004; Lin et al. 2010).

The highly conserved purine 37 has extensive modification chemistries, each requiring multiple enzymatic steps (Agris 1996). Of these, the universal N^6-threonylcarbamoyladenosine modification (t^6A_{37}) ensures the accurate recognition of ANN codons (Fig. 1A,C; Nishimura 1972). The t^6A_{37}-modified nucleoside has been shown to be essential for ribosome-mediated codon binding by $tRNA^{Lys}_{UUU}$, $tRNA^{Arg}_{UCU}$, and $tRNA^{Ile}_{GAU}$ and stabilizes anticodon interaction with the cognate and wobble codons on the ribosome-bound mRNA (Miller et al. 1976; Weissenbach and Grosjean 1981; Yarian et al. 2002). This modification has also been shown to be an identity determinant for recognition of the cognate tRNA by at least one aminoacyl-tRNA synthetase (Nureki et al. 1994). Structural analysis by nuclear magnetic resonance spectroscopy (NMR) and X-ray crystallography has shown that the t^6A_{37} modification of $tRNA^{Lys}_{UUU}$ is required for proper anticodon architecture to induce codon binding (Stuart et al. 2000; Murphy et al. 2004). The modification creates an open, but ordered and structured, architecture for the
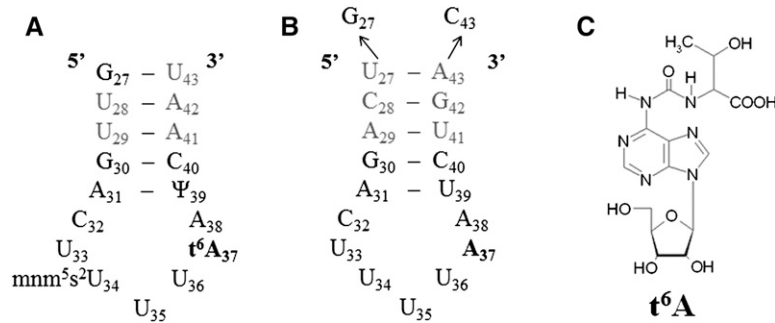
FIGURE 1. (*A*) Nucleotide sequence of the fully modified *E. coli* tRNA$^{Lys}_{UUU}$ anticodon stem and loop domain. (*B*) Sequence of the unmodified human tRNA$^{Lys}_{UUU}$ anticodon stem and loop domain, ASL$^{Lys}_{UUU}$. The terminal U$_{27}$•A$_{43}$ base pair of the natural sequence has been replaced by a G$_{27}$•C$_{43}$ base pair to increase the stability of the stem. (*C*) The N^6-threonylcarbomoyladenosine (t⁶A$_{37}$) modification found at position 37 of tRNA$^{Lys}_{UUU}$.

loop by negating intraloop base-pairing of U$_{33}$•A$_{37}$ and by enhanced base stacking on the 3′ side of the anticodon (Stuart et al. 2000).

Although this modification is required for accurate translation, the mechanism of t⁶A$_{37}$ biosynthesis is not well understood. The reaction requires ATP, free threonine, and a single carbon source (Chheda et al. 1972; Powers and Peterkofsky 1972; Elkins and Keller 1974; Körner and Söll 1974). Studies of t⁶A biosynthesis in vitro have demonstrated that *Escherichia coli* extracts contain an enzymatic component required for synthesis, but the identity of that enzyme remains unknown (Elkins and Keller 1974; Körner and Söll 1974). Recently, molecular genetic analyses have linked the Sua5/YciO/YrdC protein family with t⁶A$_{37}$ formation through mutation studies (El Yacoubi et al. 2009).

YrdC is a member of the universal Sua5/YciO/YrdC family of proteins that is present as separate proteins or domains within larger proteins, and is highly conserved from *E. coli* to humans. Because of its ubiquitous expression, structure, and homologs' function, YrdC has been thought to play a role in protein synthesis (Chen et al. 2003). *E. coli* YrdC has also been linked to ribosome biogenesis, specifically 16S rRNA maturation (Kaczanowska and Rydén-Aulin 2004, 2005). However, precise details as to its roles in these functions remain unclear.

The crystal structure of *E. coli* YrdC, solved as a dimer, revealed an α/β twisted open-sheet with a positively charged concave surface that is suggestive of a double-stranded RNA binding site (Teplova et al. 2000). tRNA binding was experimentally verified and showed YrdC selectively binds tRNA$^{Thr}_{CGU}$ lacking the t⁶A$_{37}$ modification over its modified counterpart (El Yacoubi et al. 2009). YrdC is essential in *E. coli* and *yrdC* expression is required for the formation of t⁶A$_{37}$ in *E. coli* tRNA (Baba et al. 2006; El Yacoubi et al. 2009). The *E. coli* protein has been shown to preferentially bind ATP and ADP over UTP and CTP; however, ATPase activity required for t⁶A$_{37}$ biosynthesis has yet to be observed (El Yacoubi et al. 2009).

The yeast homolog, Sua5, contains a YrdC domain and has been suggested to function in translational regulation (Na et al. 1992). More recently, the gene product of *sua5* from *Saccharomyces cerevisiae* has been shown to play a role in t⁶A$_{37}$ biosynthesis (El Yacoubi et al. 2009). In this study *sua5* deletion mutants led to the loss of t⁶A$_{37}$. Additionally, reading frame maintenance of translation was adversely affected by loss of Sua5 and t⁶A$_{37}$ (Lin et al. 2010).

Several co-crystal structures of RNA-modifying enzymes and their substrate RNAs have been solved, providing information about their mechanisms of action (for review, see Byrne et al. 2009). For example, the MnmA protein of *E. coli* is required for the substitution of a 2-thionyl for the 2-carbonyl of the wobble position uridine-34 in tRNA$^{Gln}_{UUG}$, tRNA$^{Glu}_{UUC}$, and tRNA$^{Lys}_{UUU}$. The co-crystal structure of the MnmA and tRNA$^{Glu}_{UUC}$ reveals an interaction of the protein with the tRNA's D-stem and ASL. The U$_{34}$ is flipped out of the anticodon base stack in order for MnmA to gain access to its substrate (Numata et al. 2006). This interaction appears to be moderated by a base-specific recognition of the anticodon (Kambampati and Lauhon 2003). Another RNA modification enzyme, *E. coli* MiaA, catalyzes the transfer of the isopentyl-moiety from dimethyl-allyl pyrophosphate to A$_{37}$ of tRNAs with an A$_{36}$-A$_{37}$-A$_{38}$ sequence to form i⁶A$_{37}$ and has been co-crystalized with the *E. coli* tRNA$^{Phe}_{GAA}$ (Chimnaronk et al. 2009; Seif and Hallberg 2009). The interaction of MiaA with tRNA$^{Phe}_{GAA}$ displays a mutually induced fit in which the anticodon loop unfolds, and U$_{33}$, G$_{34}$, A$_{35}$, and A$_{37}$ are flipped outside of the canonical 3′-base stack of the loop (Seif and Hallberg 2009). This base flipping mechanism makes target nucleosides more accessible and is common to many RNA and DNA modification enzymes (for review, see Roberts and Cheng 1998).

In this study, we combined a series of biochemical and structural approaches to investigate the interactions of *E. coli* YrdC with its potential substrates. We show that YrdC selectively binds the unmodified ASL$^{Lys}_{UUU}$ and threonine. The interaction of YrdC with ASL$^{Lys}_{UUU}$ did not appear to induce a structural change in A$_{37}$, indicating that the enzymatic mechanism for modification does not include base flipping or that YrdC may function in concert with other proteins in order to constitute the enzymatic activity required for t⁶A$_{37}$ biosynthesis.

RESULTS

YrdC selectively binds unmodified ASL$^{Lys}_{UUU}$

The tRNAs responding to ANN codons and having the t⁶A$_{37}$ modification or a derivative of it such as m⁶t⁶A$_{37}$ or

$ms^2t^6A_{37}$, depending on tRNA species and organism, include tRNAs for arginine, isoleucine, lysine, and threonine. The affinity of YrdC for the full-length *E. coli* tRNA$^{Thr}_{CGU}$ lacking only the t^6A_{37} modification has been shown through determination of equilibrium dissociation constants ($K_d = 0.11 \pm 0.05$ μM) to be greater than that of its fully modified counterpart ($K_d = 0.62 \pm 0.13$ μM) (El Yacoubi et al. 2009). Because the site of threonylcarbamoylation is within the anticodon domain of the tRNA, we wanted to determine if the heptadecamer ASL (Fig. 1) was sufficient for recognition and binding by YrdC. Change in the intrinsic fluorescence of the two tryptophans of YrdC (residues W89 and W106) was utilized to measure the protein's binding and to calculate apparent binding constants for variously modified ASLs, smaller oligonucleosides, mononucleoside triphosphates, and amino acids. First, YrdC was titrated with several unmodified ASLs to determine its ability to recognize specifically the ASL of an un-
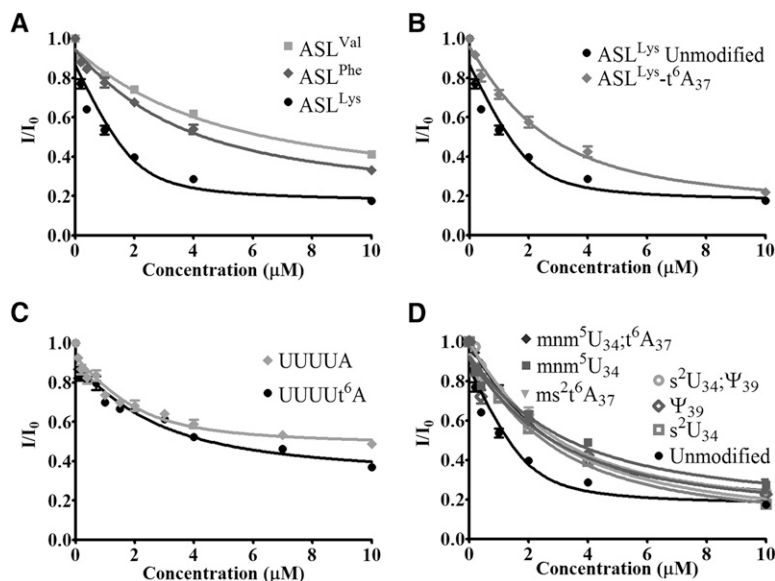

FIGURE 2. Changes in YrdC fluorescence with the addition of differentially modified ASLs. (*A*) The titration of YrdC with unmodified ASL$^{Lys}_{UUU}$, ASL$^{Val3}_{UAC}$, and yeast ASL$^{Phe}_{GAA}$. (*B*) The titration of YrdC with the unmodified ASL$^{Lys}_{UUU}$ and the t^6A_{37}-modified ASL$^{Lys}_{UUU}$-t^6A_{37}. (*C*) The titration of YrdC with nucleoside pentamers analogous to the tRNA^{Lys} anticodon region, U$_{33}$UUUA$_{37}$, and U$_{33}$UUUt^6A_{37}. (*D*) The titration of YrdC with variously modified ASL$^{Lys}_{UUU}$ with the modifications 5-methylaminomethyluridine, mnm^{5}U$_{34}$, 2-thiouridine, s^{2}U$_{34}$, pseudouridine, Ψ$_{39}$, and the 2-methylthio derivative of t^6A_{37}, ms$^2t^6A_{37}$.

modified tRNA substrate. The anticodon stem and loop sequence of the human tRNA$^{Lys}_{UUU}$ (ASL$^{Lys}_{UUU}$) (Fig. 1B) was used because the base pair content of its stem contributes considerably more stability to this small hairpin than that of the *E. coli* ASL$^{Lys}_{UUU}$. The stem of the human ASL$^{Lys}_{UUU}$ is stabilized by two internal G•C base pairs and a terminal G•C base pair substituting for an A•U in order to conduct NMR and crystallographic studies (Fig. 1B). Human ASL$^{Lys}_{UUU}$ and the *E. coli* ASL$^{Lys}_{UUU}$ have otherwise identical anticodon loop sequences and closely related modified nucleosides (Jühling et al. 2009). Previous studies have successfully used the human ASL$^{Lys}_{UUU}$ as a common context to study the structural effects of both the *E. coli* and human modifications (Durant et al. 2005).

The tryptophan fluorescence of YrdC was quenched by almost 80% with the addition of increasing concentrations of ASL$^{Lys}_{UUU}$ and at an ASL$^{Lys}_{UUU}$/protein ratio of as little as 2/1. *E. coli* ASL$^{Val3}_{UAC}$ has an A$_{37}$, but it is modified to m^{6}A$_{37}$. Yeast ASL$^{Phe}_{GAA}$ has a G$_{37}$, and it is modified to wyobutosine (yW$_{37}$). In contrast to the titration of YrdC with ASL$^{Lys}_{UUU}$, its titration with *E. coli* ASL$^{Val3}_{UAC}$ or yeast ASL$^{Phe}_{GAA}$ resulted in at most a 60% reduction in fluorescence at a ratio of 5/1. The affinity of YrdC for unmodified ASL$^{Lys}_{UUU}$, as determined from the equilibrium dissociation constant ($K_d = 0.27 \pm 0.2$ μM), was significantly greater than that of the other unmodified ASLs that had been tested (Fig. 2A; Table 1). Thus, the recognition of ASL$^{Lys}_{UUU}$ by YrdC in comparison to that for ASL$^{Val3}_{UAC}$ and ASL$^{Phe}_{GAA}$ implies some sequence-dependent specificity.

Should YrdC be the threonylcarbamoyltransferase or a subunit of the enzyme, it would be expected to have a lower affinity for the product of the reaction, the modified ASL$^{Lys}_{UUU}$-t^6A_{37}, than for the substrate, the unmodified ASL$^{Lys}_{UUU}$. We assessed the affinity of YrdC for the ASL$^{Lys}_{UUU}$-t^6A_{37} and found that YrdC bound the ASL$^{Lys}_{UUU}$-t^6A_{37} with a significantly lower affinity, $K_d = 1.36 \pm 0.39$ μM (Fig. 2B; Table 1). This preferred recognition of the unmodified ASL substrate, taken together with the specificity for the ASL$^{Lys}_{UUU}$, suggests that YrdC may very well be involved in t^6A_{37} synthesis through its direct interaction with the ASL, specifically A$_{37}$. These results are consistent with the previously reported binding of YrdC to full-length tRNA$^{Thr}_{CGU}$ where the only modification missing from the tRNA$^{Thr}_{CGU}$ was the t^6A_{37}. The difference in binding affinity of YrdC to the full-length, fully modified tRNA$^{Thr}_{CGU}$ versus the tRNA$^{Thr}_{CGU}$ lacking only the t^6A modification was approximately fivefold (El Yacoubi et al. 2009), comparable to the differences in affinity we have observed for ASL$^{Lys}_{UUU}$-t^6A_{37} and unmodified ASL$^{Lys}_{UUU}$.

To determine if YrdC would recognize a tRNA$^{Lys}_{UUU}$ fragment as small as a pentamer and to further test its specificity for unmodified A$_{37}$, the protein's ability to bind and discriminate between two anticodon region oligonucleotides was examined (Fig. 2C). The oligomers U$_{33}$UUUA$_{37}$ and U$_{33}$UUUt^6A_{37} are composed of the invariant U$_{33}$ responsible for tRNA's canonical "U-turn" just prior to the anticodon, the anticodon UUU, and the substrate

TABLE 1. Apparent dissociation constants (K_d) of YrdC and RNA species

RNA	K_d (μM)	±
ASL^{Lys}_{UUU} unmodified	0.27	0.20
ASL^{Lys}_{UUU}-t^6A_{37}	1.36	0.39
ASL^{Val3}_{UAC} unmodified	3.67	1.03
ASL^{Phe}_{GAA} unmodified	2.52	0.77
$U_{33}UUU$-A_{37}	0.68	0.40
$U_{33}UUU$-t^6A_{37}	1.31	0.59
ASL^{Lys}_{UUU}-mnm^5U_{34};t^6A_{37}	1.20	0.20
ASL^{Lys}_{UUU}-mnm^5U_{34}	1.87	0.73
ASL^{Lys}_{UUU}-s^2U_{34};Ψ_{39}	1.54	0.27
ASL^{Lys}_{UUU}-s^2U_{34}	1.64	0.29
ASL^{Lys}_{UUU}-$ms^2t^6A_{37}$	1.92	0.64
ASL^{Lys}_{UUU}-Ψ_{39}	1.82	0.88
ASL^{Lys}_{UUU}-$2AP_{37}$	0.17	0.21

nucleoside A_{37}. As evidenced by the dissociation constants derived from the change in tryptophan fluorescence, YrdC exhibited a considerable affinity for the unmodified pentamer in comparison to that for the t^6A-modified pentamer (Table 1). The pentamers were not recognized as well as the heptadecamer ASL. The affinity of YrdC for the unmodified pentamer was only a third that for the hairpin ASL^{Lys}_{UUU}. It appears that YrdC is able to differentially recognize the anticodon sequence of ASL^{Lys}_{UUU} in the absence of the hairpin, stem, and loop structure and to discern between the putative substrate A_{37} and possible product t^6A_{37}.

The affinity of YrdC for differentially modified ASL^{Lys}_{UUU} constructs was tested by fluorescence quenching of YrdC to observe the effect that modifications, in general, may have on its recognition of the ASL (Fig. 2D). The completely modified, naturally occurring *E. coli* tRNA$^{Lys}_{UUU}$ contains 5-methylaminomethyl-2-thiouridine-34, $mnm^5s^2U_{34}$, t^6A_{37} and pseudo-uridine 39, Ψ_{39} (Fig. 1A). The affinity of YrdC for the unmodified ASL^{Lys}_{UUU} was at least fourfold greater than that for the modified ASL^{Lys}_{UUU} constructs (Table 1). The ASL^{Lys}_{UUU}-mnm^5U_{34};t^6A_{37} and the ASL^{Lys}_{UUU}-$ms^2t^6A_{37}$ with the mammalian 2-methylthio-t^6A_{37} would be expected to bind with an affinity comparable to that of ASL^{Lys}_{UUU}-t^6A_{37} because the A_{37} in each is modified with an N^6-threonyl-carbamoyl-moiety. Indeed, these two modified ASL^{Lys}_{UUU} bound to YrdC with apparent dissociation constants more reflective of the t^6A_{37}-modified ASL ($K_d = 1.20 \pm 0.20$ and 1.92 ± 0.64, respectively) than that of the unmodified ASL^{Lys}_{UUU} (Table 1). The modified ASLs lacking t^6A_{37}, ASL^{Lys}_{UUU}-s^2U_{34}, ASL^{Lys}_{UUU}-s^2U_{34};Ψ_{39}, ASL^{Lys}_{UUU}-Ψ_{39}, and ASL^{Lys}_{UUU}-mnm^5U_{34} would be expected to be recognized by YrdC with K_d values similar to the unmodified ASL^{Lys}_{UUU}, because they are much closer to the putative natural substrate of t^6A_{37} biosynthesis. Surprisingly, the dissociation constants for the ASLs with s^2U_{34}, mnm^5U_{34}, and Ψ_{39} were similar to those for ASLs with the threonyl-carbamoyl modification at A_{37}. s^2U_{34}, mnm^5U_{34}, and

$mnm^5s^2U_{34}$ are known to contribute order to the ASL^{Lys}_{UUU} structure (Stuart et al. 2000; Sundaram et al. 2000; Murphy et al. 2004; Durant et al. 2005). Ψ_{39}, located two nucleosides 3′ to A_{37}, is known to stabilize the 3′ side of the anticodon loop (Durant and Davis 1999; Yarian et al. 1999) Therefore, the contributions of these modifications to the conformation and dynamics of the ASL could impart a structure to the anticodon loop less favorable for YrdC recognition. The chronology of events leading to the fully modified anticodon domain of the tRNA is not known, so it is possible that these post-transcriptional modifications are sequential and that formation of t^6A_{37} is the primary event.

Saturation transfer difference NMR (STD-NMR) was conducted as an additional independent method to verify the unmodified ASL^{Lys}_{UUU} binding by YrdC. STD-NMR is able to detect the presence of binding equilibria and provides information to identify protons of ligands in direct contact with the protein (Mayer and Meyer 1999). In the STD spectrum, only the protons representing molecules interacting with the protein will be observable. The reference spectrum depicts the ribose (6–4.5 ppm) and the aromatic (8.5–6.5 ppm) regions for unmodified ASL^{Lys}_{UUU} (Fig. 3A) and unmodified ASL^{Val3}_{UAC} (Fig. 3B). In the lower STD spectrum of the unmodified ASL^{Lys}_{UUU}, there is considerable recovery of many ribose peaks. There is also a significant recovery of specific resonances in the aromatic region of the spectrum, indicating that YrdC is making contacts with what appears to be a select set of the bases. In contrast, the recovery of the ribose protons of unmodified ASL^{Val3}_{UAC} is less and the aromatic protons show no observable signal. This recognition of the backbone is not unexpected considering the K_d of YrdC for ASL^{Val3}_{UAC} (Table 1). Given these results with the fluorescence quenching data, it is very likely that YrdC recognizes the RNA substrate in a sequence-dependent manner by directly interacting with certain bases.

YrdC recognizes threonine and ATP

The biosynthesis of t^6A_{37} utilizes free threonine from the cell as the threonyl-moiety for production of the threonylcarbamoyl (Powers and Peterkofsky 1972). If YrdC is involved in the enzymatic pathway, it could potentially bind threonine, ATP, and/or the carboxylphosphate. The binding of threonine by YrdC was determined by monitoring the protein's tryptophan fluorescence while titrating with the amino acid (Fig. 4A). YrdC bound threonine with a $K_d = 0.04 \pm 0.10$ μM. This very tight binding indicates the likelihood of a specific binding site on YrdC for threonine. The same experiment was conducted with serine. The binding affinity of YrdC for threonine was threefold higher than that for serine ($K_d = 0.12 \pm 0.16$ μM).

STD-NMR was also conducted to identify the binding epitope of threonine. In the STD spectrum, only the peaks representing bound protons will be seen. A STD signal was
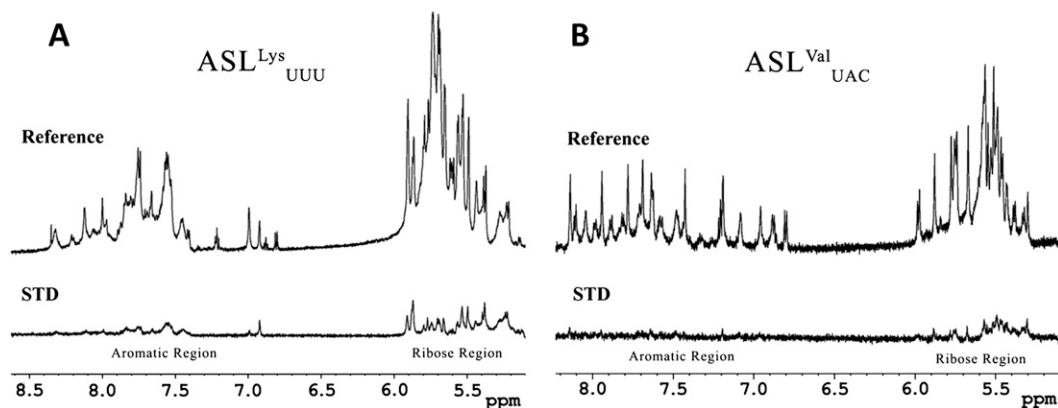
FIGURE 3. Saturation transfer difference (STD)-NMR of YrdC with unmodified ASL$^{Lys}_{UUU}$ (*A*) and unmodified ASL$^{Val3}_{UAC}$ (*B*). For each set of experiments, there is a one-dimensional NMR reference spectrum (*top*) and a STD spectrum of recovered signals (*bottom*). The spectral region shown displays the characteristic RNA chemical shifts for the aromatic protons (8.5 to 6.5 ppm) and the ribose protons (6.0 to 5.0 ppm). The STD-NMR spectrum records the proton signals from those residues of the ASL that have been bound by YrdC. The standard one-dimensional ^{1}H-NMR recapitulate previously published data for the unmodified ASL$^{Lys}_{UUU}$ (Stuart et al. 2000) and unmodified ASL$^{Val3}_{UAC}$ (Vendeix et al. 2008).

observed with 1 mM threonine, and all of the peaks were recovered (Fig. 4B). It appears from the differential changes in the threonine resonances that YrdC binds threonine with a preference for the Hβ. An additional STD-NMR experiment was performed to observe the selectivity of YrdC with several amino acids. Because of their similarities, serine, valine, and aspartic acid were added together with threonine to compete for binding to YrdC. It appears that YrdC can in fact preferentially bind threonine over the other amino acids present (Fig. 4C). YrdC also recognized serine, but to a lesser extent than threonine, as determined by integration of the peaks. The peak at 4.1 ppm represents the Hβ of threonine and is recovered by 48%. The Hα threonine peak is 14% recovered, and the serine peaks were only 10% and 8% recovered, respectively. This is most likely due to the recognition of serine's and threonine's common elements. Since valine is not bound, we believe YrdC must be distinguishing the OH that serine and threonine have in common, and not the methyl group.

YrdC's preferential binding of ATP and ADP had been reported previously; however, the protein's affinity for the nucleotides had not been determined (El Yacoubi et al. 2009). Using trypto-phan fluorescence quenching, YrdC was titrated with ATP and GTP (Fig. 5). The protein bound ATP and GTP with estimated K_d values of 1.04 ± 0.67 μM and 1.31 ± 0.91 μM, respectively.

YrdC does not induce a significant change in ASL$^{Lys}_{UUU}$ structure, particularly at A$_{37}$

Modification of A$_{37}$ to t^{6}A$_{37}$ may involve a conformational perturbation of the anticodon stem and loop or, minimally, a conformational change in A$_{37}$. Evidently, a conformational change in the tRNA is not required for all modification recognition mechanisms. The protein MnmA,
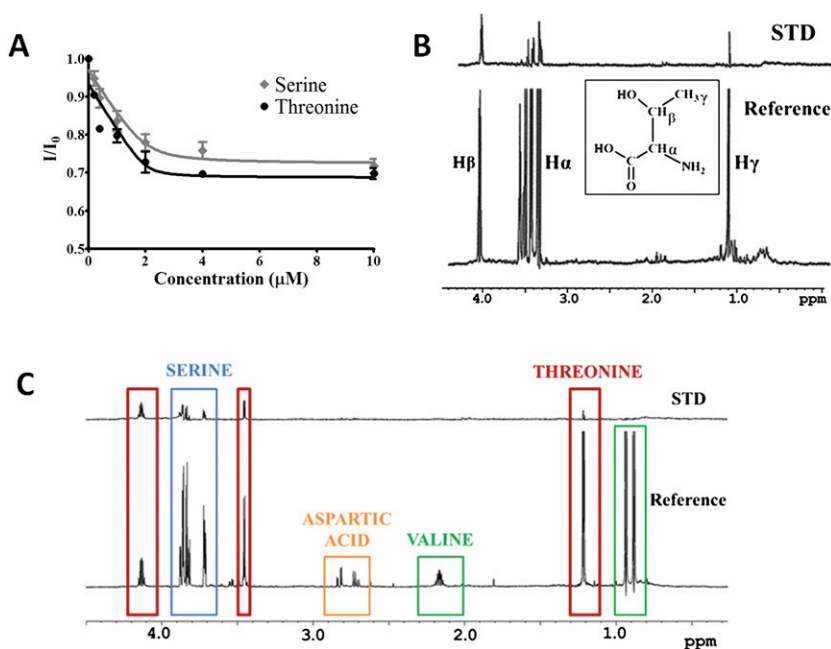

FIGURE 4. Changes in fluorescence of YrdC with additions of the amino acids threonine and serine (*A*) and STD-NMR of threonine in the presence of YrdC (*B*). The one-dimensional reference spectrum with 1 mM threonine (*bottom*) and the STD spectrum (*top*). (*C*) Competition of amino acids in binding to YrdC. The STD-NMR experiment was conducted with YrdC and equal molar concentrations of serine (blue), threonine (red), aspartic acid (orange), and valine (green).
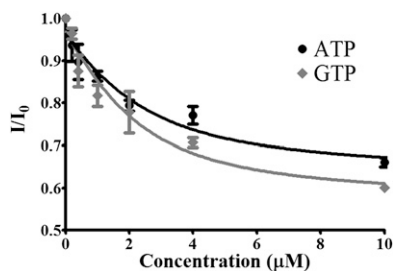
FIGURE 5. YrdC binding of ATP and GTP. YrdC was titrated with ATP and GTP and binding of the nucleotide-5′-triphosphates was measured by observing changes in tryptophan fluorescence.

responsible for thiolation of U_{34} recognizes tRNA in a predominantly rigid body manner by flipping out U_{34} without affecting the rest of the tRNA structure (Numata et al. 2006). However, all other co-crystals of RNA-modifying enzymes with their tRNA substrates to date have depicted structural changes in the tRNA and/or the enzyme (for reviews, see Ishitani et al. 2008; Byrne et al. 2009). For that reason, we employed circular dichroism (CD) spectroscopy to determine if YrdC induces a conformational change in the unmodified ASL^{Lys}_{UUU}. CD spectra are sensitive to structural changes in base stacking within an RNA and in response to the binding of a protein, changes in temperature, pH, and metal binding (Chen et al. 1993; Agris et al. 1999). Changes in base stacking are monitored by the positive ellipticity found between 250 and 290 nm. This spectral window is particularly advantageous for observing the RNA in presence of YrdC because proteins have a spectral null in this exact wavelength range. ASL^{Lys}_{UUU} was titrated with increasing concentrations of YrdC to observe the effect the protein had on the RNA structure (Fig. 6). Each spectrum representing a different concentration of YrdC was within experimental error of the spectrum in the absence of the protein and therefore, reported no global change in base stacking. Even at twice the concentration of the RNA, YrdC did not induce an observable change in the ASL.

An alteration of only the A_{37} nucleoside conformation may not have been observable by CD because the nucleoside was one of 17 residues being averaged in the spectroscopy. Therefore, to investigate any possible perturbation of the A_{37} with the binding of YrdC, ASL^{Lys}_{UUU} was synthesized with a fluorescent analog of adenosine, 2-aminopurine ribonucleoside, at position 37 ($2AP_{37}$) (Fig. 7A). Though not capable of a Watson-Crick base-pairing, 2AP in loops is commonly used to probe nucleic acid structure because it rarely affects structure adversely, and its fluorescence is typically enhanced when its environment is disturbed as a result of decreased stacking interactions (Rachofsky et al. 2001). Therefore, if a conformational change of A_{37} occurs such as flipping outside of the anticodon loop base stack, an increase in the fluorescence would be expected. To ensure that YrdC recognition of the ASL^{Lys}_{UUU}-$2AP_{37}$ was comparable to

that of the unmodified ASL^{Lys}_{UUU}, the affinity was assessed by monitoring tryptophan fluorescence as described above (Fig. 7B). YrdC recognized ASL^{Lys}_{UUU}-$2AP_{37}$ with a $K_d = 0.17 \pm 0.21$ μM, comparable to that for ASL^{Lys}_{UUU}. The 2AP fluorescence of ASL^{Lys}_{UUU}-$2AP_{37}$ ($\lambda_{ex} = 310$nm, $\lambda_{em} = 375$ nm) was monitored while titrating the ASL^{Lys}_{UUU} with YrdC. With the addition of YrdC, the 2AP fluorescence is quenched (Fig. 7C). This suggests that upon protein binding, A_{37} is stacking with either adjacent bases or aromatic amino acids and is not being flipped to a position exposed to solvent. Since $2AP_{37}$ is within the flexible loop region, it is likely that binding of YrdC creates a more structured local environment for A_{37}, possibly in the binding pocket.

Two physicochemical approaches, CD and 2AP fluorescence, indicated that the structure and conformational dynamics of the ASL^{Lys}_{UUU}, and those of the target A_{37}, were not significantly altered when bound by YrdC. In order to determine at the level of atomic resolution whether or not the ASL or the nucleoside were subjected to a structural change, we employed NMR spectroscopy. The nucleoside resonances for ASL^{Lys}_{UUU} had been assigned previously (Durant and Davis 1999; Stuart et al. 2000), and the chemical shifts of many of the aromatic resonances of the RNA, including cross-peaks for A_{37}, were clearly observable in the presence of the protein (Fig. 8). After collecting a Nuclear Overhauser Effect spectrum (NOESY) of the ASL^{Lys}_{UUU} alone, it was titrated with YrdC to a final stoichiometry of 1:1 and another spectrum collected. No significant changes occurred in the resonances of the RNA
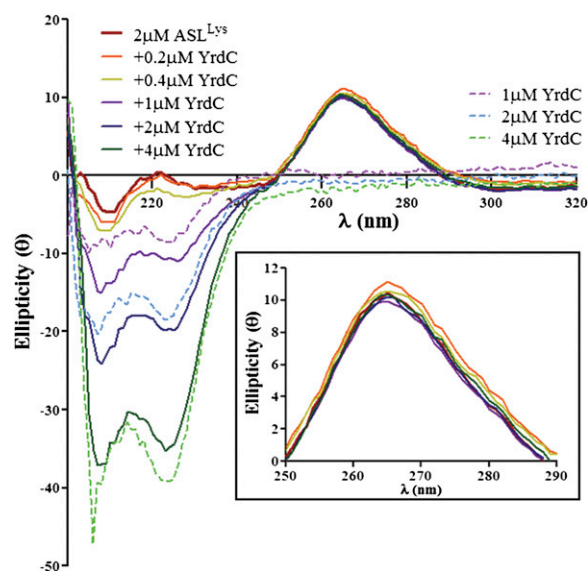

FIGURE 6. Circular dichroism (CD) spectra of the unmodified ASL^{Lys}_{UUU} titrated with YrdC. The unmodified ASL^{Lys}_{UUU} (2 μM) was titrated with YrdC (0.2–4.0 μM), and the resulting CD spectra were collected. In addition, the spectra of YrdC alone were collected at the same concentrations. A magnification of the 250- to 290-nm spectral region is shown to examine changes in the RNA base stacking with addition of YrdC (*inset*).
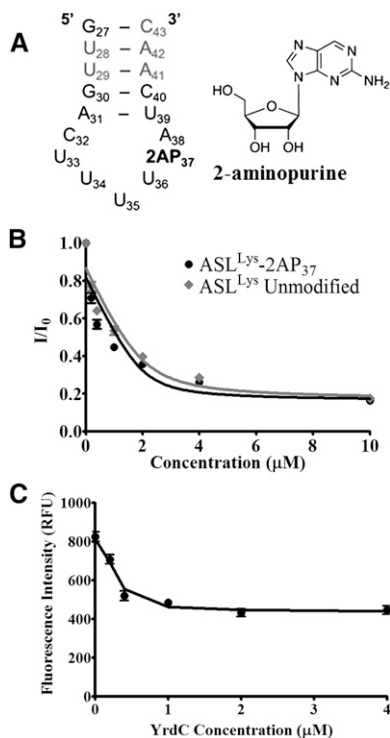
FIGURE 7. Nucleoside A_{37} monitored for base flipping. (*A*) The nucleoside sequence of the 2-aminopurine ribonucleoside ($2AP_{37}$) construct. (*B*) Fluorescence changes in the tryptophans of YrdC with addition of the unmodified ASL^{Lys}_{UUU} and the $2AP_{37}$-modified ASL^{Lys}_{UUU}-$2AP_{37}$. (*C*) Changes in fluorescence of $2AP_{37}$ ($\lambda_{ex} = 310$ nm; $\lambda_{em} = 375$ nm) with addition of increasing concentrations of YrdC.

when observing the nucleobase aromatic protons. However, the other substrates required for the biosynthesis of t^6A_{37}, ATP, threonine, and what is suspected to be an activated bicarbonate such as carboxylphosphate, were missing from the experiment. Therefore, ATP and then threonine were added to the YrdC-ASL^{Lys}_{UUU} sample in two steps. Again, the NMR spectra depicted no significant peak movement or broadening in the ASL amino region. All four of the spectra overlay completely with minor shifts in some resonances for A_{37} and A_{38} and slight shifts in the loop uridines. With the addition of threonine, line broadening was observed and resulted in the loss of several peaks (data not shown). This could be due to a number of reasons including the YrdC binding of the RNA or the high concentration of molecules in the sample decreasing tumbling.

DISCUSSION

In 1974, an enzymatic activity capable of the synthesis of t^6A in a t^6A-deficient tRNA was found in a crude extract from an *E. coli* culture (Elkins and Keller 1974; Körner and Söll 1974). Over 30 years later, there is little information about the enzyme(s) responsible for this reaction. Molecular genetic analyses point to the Sua5/YciO/YrdC protein family

as having a role in t^6A_{37} formation. The knockdown of the *yrdC* gene results in a loss of t^6A_{37} in *E. coli* tRNAs, and the knockout of the *sua5* gene in *S. cerevisiae* causes inadequate t^6A_{37} production (El Yacoubi et al. 2009; Lin et al. 2010). Because of its double-stranded RNA-binding ability and suspected ATPase activity, YrdC has emerged as the most likely candidate for the role of N^6-adenosine threonyl-carbamoyltransferase in *E. coli*.

YrdC is able to recognize small hairpin RNAs, more specifically the ASL of $tRNA^{Lys}_{UUU}$, as we have shown by tryptophan fluorescence quenching, STD-NMR, and novel Raman spectroscopy techniques (data not shown). It binds with sequence specificity and preferentially binds unmodified ASL^{Lys}_{UUU} compared with the modified counterpart, ASL^{Lys}_{UUU}-t^6A_{37}, and the unmodified ASL^{Val3}_{UAC} and yeast ASL^{Phe}_{GAA}. In fact, YrdC binds the unmodified ASL^{Lys}_{UUU} with an affinity ($K_d = 0.27 \pm 0.20$ μM) very comparable to its reported recognition of the full-length, native *E. coli* $tRNA^{Thr}_{CGU}$, lacking only the t^6A_{37} ($K_d = 0.11 \pm 0.05$ μM) (El Yacoubi et al. 2009). YrdC binding of the ASL^{Lys}_{UUU}-t^6A_{37} exhibited a fivefold increase in K_d compared with that for the unmodified ASL^{Lys}_{UUU}. This result is also analogous to the previously reported full-length, fully modified $tRNA^{Thr}_{CGU}$ versus $tRNA^{Thr}_{CGU}$ lacking only t^6A_{37} (El Yacoubi et al. 2009). YrdC exhibited an affinity for ASL^{Lys}_{UUU} similar to that for the full-length $tRNA^{Thr}_{CGU}$ lacking t^6A_{37}, indicating that most of the important binding determinants are within the ASL region of the tRNA and not within other domains. The twofold difference in K_d between the respective pairs (fully modified $tRNA^{Thr}_{CGU}$ versus the ASL^{Lys}_{UUU}-t^6A, and the $tRNA^{Thr}_{CGU}$ lacking only t^6A_{37} versus the unmodified ASL^{Lys}_{UUU}) could be the result of YrdC interactions with more than just the anticodon domain or could reflect differences in recognition of the two natural substrate ASL sequences ($tRNA^{Thr}_{CGU}$ vs. $tRNA^{Lys}_{UUU}$). At this time, our results do not distinguish between these two explanations for the difference in equilibrium binding
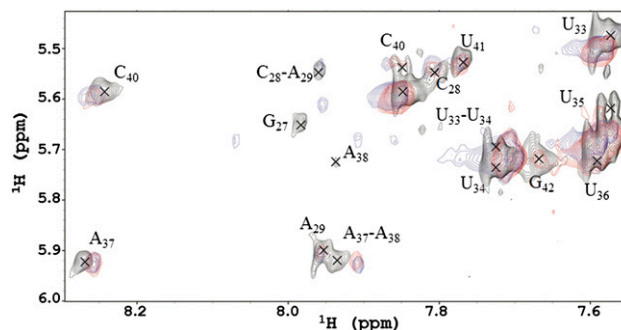

FIGURE 8. ^{1}H-NMR NOESY spectra of unmodified ASL^{Lys}_{UUU} with additions of YrdC and ATP. The spectra were collected in 10% D_2O at 25°C. A region of the ASL^{Lys}_{UUU} spectrum (7.4–8.4 ppm × 5.4–6.0 ppm) is practically free of overlapping resonances from the protein. The connectivities between the ASL^{Lys}_{UUU} aromatic protons and the H1′ of the ribose are annotated: black, unmodified ASL^{Lys}_{UUU} alone; blue, addition of YrdC; and red, addition of ATP to YrdC and the ASL.

constant. The previously reported lack of binding to a fully unmodified tRNA$^{Thr}_{CGU}$ transcript (El Yacoubi et al. 2009) could be due to the importance of other modifications outside of the anticodon domain or to the tertiary folding of the tRNA (Helm 2006) and not due to a requirement for a direct interaction with YrdC.

Many modification enzymes display a mechanism of action that introduces a structural change in the tRNA to create a more accessible nucleoside for modification (Ishitani et al. 2008; Byrne et al. 2009). Typically the nucleobase to be modified is flipped, removing it from an internal stacking interaction and perhaps hydrogen bonding, and exposes it to the solvent and the protein active site. Unmodified A$_{37}$ is located within the anticodon loop of ASL$^{Lys}_{UUU}$ (Durant and Davis 1999; Stuart et al. 2000). Therefore, we hypothesized that a base flip would be required for the enzymatic addition of the threonylcarbamoyl-group to form t^{6}A$_{37}$. However, our present studies do not indicate a base flipping mechanism. Results from titrating of the unmodified ASL$^{Lys}_{UUU}$ with YrdC and monitoring either the CD spectra or the fluorescence of 2AP$_{37}$ did not indicate that a change in structure of the ASL had taken place. This may be due to the absence of other protein components required for catalysis, a requirement for the hydrolysis of ATP, or the limitations of these two methods in exhibiting a change. NMR spectra of the unmodified ASL$^{Lys}_{UUU}$ in the presence of YrdC followed by additions of ATP and threonine also did not show a noticeable structural change to the ASL or movement of A$_{37}$. It is possible that the NMR solution conditions may not have been optimal for observation of a conformational change, as in vitro t^{6}A$_{37}$ biosynthesis has been shown to require magnesium and bicarbonate (Elkins and Keller 1974).

The proposed t^{6}A$_{37}$ biosynthesis requires two ATP-dependent steps: formation of carboxylphosphate from bicarbonate and ATP and its transfer to the amino group of adenosine to form N^6-carbamoyladenosine, and then activation of the carbamoyl moiety of N^6-carbamoyladenosine with a second ATP followed by addition of threonine via attack by its α-amine (Garcia and Goodenough-Lasua 1998). It is possible that YrdC is involved in the latter step of the pathway by positioning threonine and the tRNA substrate into the active site of the protein responsible for catalytic activity. Further research is necessary to elucidate this enzymatic biosynthesis. However, the data presented here along with prior research support the hypothesis that YrdC is a subunit of a complex that constitutes the enzymatic properties for t^{6}A$_{37}$ biosynthesis where YrdC selectively recognizes and binds the tRNA substrates, ATP and threonine, and the holoenzyme functions as the threonylcarbamoyl transferase. Another possible scenario, based on the binding affinities alone and not on cell concentrations of the ligands, has YrdC binding tRNA and threonine, but not ATP. The observed ATP binding may be nonspecific due to recognition of ATP in the A$_{37}$ binding pocket on the protein. An additional subunit of the enzyme complex may have the ATPase activity. Recently, the KEOPS/EKC complex in yeast has been shown to be required for t^{6}A$_{37}$ biosynthesis (Srinivasan et al. 2011). A member of this complex, Kae1, is an ATPase and is highly conserved. Its bacterial ortholog, YgjD, was also shown to be required for modification in *E. coli*. Further examination of the structural and biochemical interactions between YrdC, its substrates, and the other possible subunits of the threonylcarbamoyl transferase should offer more information about this essential enzymatic pathway.

MATERIALS AND METHODS

Sample preparation and purification

All ASL samples except one were chemically synthesized by Dharmacon (Thermo Fisher) using "ACE" chemistry (Scaringe et al. 1998). The ASLs were de-protected per manufacturer's instructions and dialyzed against 25 mM potassium phosphate buffer (pH 6.8), and 100 mM NaCl using a 3500-Da cutoff. The ASL$^{Lys}_{UUU}$-t^{6}A$_{37}$ was chemically synthesized with the 2′,5′-protected 3′-phosphoramidite of t^{6}A$_{37}$ by the Nucleic Acid Facility of North Carolina State University. The oligonucleoside pentamers UUUUA and UUUUt^{6}A were chemically synthesized in solution using the phosphotriester chemistry (Agris et al. 1997).

His$_6$-tagged *E. coli* YrdC protein was overexpressed in *E. coli* as described previously and purified by Ni-NTA affinity chromatography to a purity of >95% by SDS-PAGE (El Yacoubi et al. 2009). Protein integrity was verified by crystallography.

Fluorescence quenching

The inherent fluorescence of the two tryptophans present in YrdC was observed at room temperature using a SpectraMax Gemini XS microplate reader (Molecular Devices). Data were collected using accompanying SoftMax Pro software (Molecular Devices). For fluorescence quenching of YrdC, the tryptophans were excited at 295 nm and their emission collected at 345 nm, similar to the method described by Teplova et al. (2000). Triplicate samples of the RNAs, NTPs, and amino acids were lyophilized to dryness in amounts corresponding to the required final concentrations. The titration was performed by resuspending lyophilized samples in 350 μL of 2 μM YrdC (25 mM potassium phosphate, 100 mM NaCl at pH 6.8) to final concentrations of 0.2–10 μM in 96-well microplates. Data were averaged and fit to a single site nonlinear regression,

$$\frac{I}{I_0} = 1 + (Y_{min} - 1)\left(\frac{(K_d + p + x) - \sqrt{(K_d + p + x)^2 - 4px}}{2p}\right),$$

where p represents the protein concentration and x equals the ligand concentration. Apparent dissociation constants were calculated with standard errors using this equation according to method described by Kurganov et al. (1972) (GraphPad Prism 5). Error bars represent the standard error of the mean.

The fluorescence of the 2-aminopurine ribonucleoside at position 37 of the ASL$^{Lys}_{UUU}$ was excited at 310 nm and the emission collected at 375 nm. YrdC samples were lyophilized to

dryness and resuspended to final concentrations of 0.2–10 μM in 350 μL of 2 μM ASL$^{Lys}_{UUU}$-2AP$_{37}$ sequentially.

CD spectroscopy

CD spectra (200–320 nm) were collected using a Jasco J600 spectropolarimeter at 25°C with a quartz cuvette having 1-cm path length. ASL sample concentrations were adjusted to ~0.27 A_{260} U/mL (2 μM). ASL (2 μM in 25 mM potassium phosphate, 100 mM NaCl at pH 6.8) was added to lyophilized YrdC to constitute final protein concentrations of 0.2–10 μM in 1 mL. Each experiment was performed in triplicate. The spectra of YrdC alone at the same concentrations were collected and overlayed. Data were plotted using Excel 2007 (Microsoft) and GraphPad Prism 5.

NMR spectroscopy

STD-NMR data were acquired on a Bruker Avance II 700 MHz spectrometer at UAlbany equipped with an ultra-sensitive triple resonance cryoprobe capable of applying pulsed field gradients along the z-axis. All experiments were conducted at 25°C. For the experiments with RNA, 3 μM YrdC and 300 μM ASL$^{Lys}_{UUU}$ or 200 μM ASL$^{Val3}_{UAC}$ was prepared in 300 μL of 99% D_2O (10 mM potassium phosphate, 100 mM NaCl at pH 6.8). The STD pulse was applied according the method described by Mayer and Meyer (1999), except the on-resonance irradiation was centered at 0 ppm in order to selectively saturate only protein. A total of 1028 scans with 16 dummy scans was collected. Amino acid samples were prepared in 600 μL of 99% D_2O (10 mM potassium phosphate, 100 mM NaCl at pH 6.8). For each experiment, the concentrations were 20 μM YrdC and 1 mM of each amino acid. The STD pulse sequence was applied according the method described by Mayer and Meyer (1999). A total of 256 scans with 16 dummy scans were collected for each.

NOESY spectra were collected on a DMX Bruker 500 MHz instrument equipped with triple-resonance ^1H, ^{13}C, and ^{15}N probe and three-axis pulsed field gradient capabilities at NCSU. All NMR data were processed using NMRPipe (Delaglio et al. 1995). Spectra were analyzed using SPARKY software (Goddard and Kneller 2008). Each 2D NOESY experiment was recorded at 25°C with a 200-msec mixing time using the WATERGATE suppression sequence. A spectrum was collected of ASL$^{Lys}_{UUU}$ sample alone (~1 mM; 25 mM potassium phosphate, 100 mM NaCl at pH 6.8 in 10% D_2O). For the additions of 0.4 mM YrdC, 1 mM ATP, and 1 mM threonine, the ASL$^{Lys}_{UUU}$ concentration was 0.4 mM in the same buffer conditions and 10% D_2O.

REFERENCES

Agris PF. 1996. The importance of being modified: roles of modified nucleosides and Mg2+ in RNA structure and function. *Prog Nucleic Acid Res Mol Biol* **53:** 79–129.

Agris PF. 2004. Decoding the genome: a modified view. *Nucleic Acids Res* **32:** 223–238.

Agris PF. 2008. Bringing order to translation: the contributions of transfer RNA anticodon-domain modifications. *EMBO Rep* **9:** 629–635.

Agris PF, Guenther R, Ingram PC, Basti MM, Stuart JW, Sochacka E, Malkiewicz A. 1997. Unconventional structure of tRNA$^{Lys}_{SUU}$ anticodon explains tRNA's role in bacterial and mammalian ribosomal frameshifting and primer selection by HIV-1. *RNA* **3:** 420–428.

Agris PF, Marchbank MT, Newman W, Guenther R, Ingram P, Swallow J, Mucha P, Szyk A, Rekowski P, Peletskaya E, et al. 1999. Experimental models of protein-RNA interaction: isolation and analyses of tRNA(Phe) and U1 snRNA-binding peptides from bacteriophage display libraries. *J Neurosci Methods* **18:** 425–435.

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomito M, Wanner BL, Mori H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2:** 2006.0008. doi: 10.1038/msb4100050.

Björk G. 1995. Biosynthesis and function of modified nucleosides. In *tRNA: Structure, biosynthesis, and function* (ed. D. Söll and U. RajBhandary), pp. 165–205. ASM Press, Washington.

Byrne R, Waterman D, Antson A. 2009. Enzyme-RNA substrate recognition in RNA-modifying enzymes. In *DNA and RNA modification enzymes: Structure, mechanisms, function, and evolution* (ed. Henri Grosjean), pp. 304–327. Landes Bioscience, Austin.

Chen Y, Sierzputowska-Gracz H, Guenther R, Everett K, Agris PF. 1993. 5-Methylcytidine is required for cooperative binding of Mg^{2+} and a conformational transition at the anticodon stem-loop of yeast phenylalanine tRNA. *Biochemistry* **32:** 10249–10253.

Chen J, Ji C, Gu S, Zhao E, Dai J, Huang L, Qian J, Ying K, Xie Y, Mao Y. 2003. Isolation and identification of a novel cDNA that encodes human yrdC protein. *J Hum Genet* **48:** 164–169.

Chheda GB, Hong CI, Piskorz CF, Harmon GA. 1972. Biosynthesis of N-(purin-6-ylcarbamoyl)-L-threonine riboside. Incorporation of L-threonine in vivo into modified nucleoside of transfer ribonucleic acid. *Biochem J* **127:** 515–519.

Chimnaronk S, Forouhar F, Sakai J, Yao M, Tron CM, Atta M, Fontecave M, Hunt JF, Tanaka I. 2009. Snapshots of dynamics in synthesizing N^6-isopentenyladenosine at the tRNA anticodon. *Biochemistry* **48:** 5057–5065.

Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. 1995. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biol NMR* **6:** 277–293.

Durant PC, Davis DR. 1999. Stabilization of the anticodon stem-loop of tRNA^{Lys,3} by an A+-C base-pair and by pseudouridine. *J Mol Biol* **285:** 115–131.

Durant PC, Bajji AC, Sundaram M, Kumar RK, Davis DR. 2005. Structural effects of hypermodified nucleosides in the Escherichia coli and human tRNA^{Lys} anticodon loop: the effect of nucleosides s^{2}U, mcm^{5}U, mcm^{5}s^{2}U, mnm^{5}s^{2}U, t^{6}A, and ms^{2}t^{6}A. *Biochemistry* **44:** 8078–8089.

Elkins BN, Keller EB. 1974. The enzymatic synthesis of N-(Purin-6-ylcarbamoyl)threonine, an anticodon-adjacent base in transfer ribonucleic acid. *Biochemistry* **13:** 4622–4628.

El Yacoubi B, Lyons B, Cruz Y, Reddy R, Nordin B, Agnelli F, Williamson JR, Schimmel P, Swairjo MA, de Crécy-Lagard V. 2009. The universal Sua5/YciO/YrdC family is required for the formation of threonylcarbamoyladenosine in tRNA. *Nucleic Acids Res* **37:** 2894–2909.

Garcia G, Goodenough-Lasua D. 1998. Mechanisms of RNA-modifying and -editing enzymes. In *Modification and editing of RNA* (ed. R. Benne and H. Grosjean), pp. 135–168. ASM Press, Washington, DC.

Goddard TD, Kneller DG. 2008. SPARKY 3, University of California, San Francisco.

Helm M. 2006. Post-transcriptional nucleotide modification and alternate folding of tRNA. *Nucleic Acids Res* **34:** 721–733.

Ishitani R, Yokoyama S, Nureki O. 2008. Structure, dynamics, and function of RNA modification enzymes. *Curr Opin Struct Biol* **18:** 330–339.

Jühling F, Mörl M, Hartmann RK, Sprinzl M, Stadler PF, Pütz J. 2009. tRNAdb 2009: compilation of tRNA sequences and tRNA genes. *Nucleic Acids Res* **37:** D159–D162.

Kaczanowska M, Rydén-Aulin M. 2004. Temperature sensitivity caused by mutant release factor 1 is suppressed by mutations that affect 16S rRNA maturation. *J Bacteriol* **186:** 3046–3055.

Kaczanowska M, Rydén-Aulin M. 2005. The YrdC protein—a putative ribosome maturation factor. *Biochim Biophys Acta* **1727:** 87–96.

Kambampati R, Lauhon CT. 2003. MnmA and IscS are required for in vitro 2-thiouridine biosynthesis in *Escherichia coli*. *Biochemistry* **42:** 1109–1117.

Körner A, Söll D. 1974. N-(Purin-6-ylcarbamoyl) threonine: Biosynthesis in vitro in transfer RNA by an enzyme purified from *Escherichia coli*. *FEBS Lett* **39:** 301–306.

Kurganov B, Sugrobov N, Yakovlev V. 1972. Estimation of dissociation constant of enzyme-ligand complex from fluorometric data by "difference" method. *FEBS Lett* **19:** 308–310.

Lin CA, Ellis SR, True HL. 2010. The Sua5 protein is essential for normal translational regulation in yeast. *Mol Cell Biol* **30:** 354–363.

Mayer M, Meyer B. 1999. Characterization of ligand binding by saturation transfer difference NMR spectroscopy. *Angew Chem Int Ed Engl* **38:** 1784–1788.

Miller JP, Hussain Z, Schweizer MP. 1976. The involvement of the anticodon adjacent modified nucleoside N-[9-(β-D-ribofuranosyl) purine-6-ylcarbamoyl]-threonine in the biological function of *E. coli* tRNA-Ile. *Nucleic Acids Res* **3:** 1185–1201.

Murphy FV, Ramakrishnan V, Malkiewicz A, Agris PF. 2004. The role of modifications in codon discrimination by tRNA$^{Lys}_{UUU}$. *Nat Struct Mol Biol* **11:** 1186–1191.

Na JG, Pinto I, Hampsey M. 1992. Isolation and characterization of SUA5, a novel gene required for normal growth in *Saccharomyces cerevisiae*. *Genetics* **131:** 791–801.

Nishimura S. 1972. Minor components of transfer RNA: their characterization, localization, and function. *Prog Nucleic Acid Res Mol Biol* **12:** 49–85.

Numata T, Ikeuchi Y, Fukai S, Suzuki T, Nureki O. 2006. Snapshots of tRNA sulphuration via an adenylated intermediate. *Nature* **442:** 419–424.

Nureki O, Niimi T, Muramatsu T, Kanno H, Kohno T, Florentz C, Giegé R, Yokoyama S. 1994. Molecular recognition of the identity-determinant set of isoleucine transfer RNA from *Escherichia coli*. *J Mol Biol* **236:** 710–724.

Powers DM, Peterkofsky A. 1972. Biosynthesis and specific labeling of N-(purin-6-ylcarbamoyl) threonine of *Escherichia coli* transfer RNA. *Biochem Biophys Res Commun* **46:** 831–838.

Rachofsky EL, Osman R, Ross JB. 2001. Probing structure and dynamics of DNA with 2-aminopurine: effects of local environment on fluorescence. *Biochemistry* **40:** 946–956.

Roberts RJ, Cheng X. 1998. Base flipping. *Annu Rev Biochem* **67:** 181–198.

Scaringe SA, Wincott FE, Caruthers MH. 1998. Novel RNA synthesis method using 5′-O-Silyl-2′-O-orthoester protecting groups. *J Am Chem Soc* **120:** 11820–11821.

Seif E, Hallberg BM. 2009. RNA-protein mutually induced fit: structure of *Escherichia coli* isopentenyl-tRNA transferase in complex with tRNA(Phe). *J Biol Chem* **284:** 6600–6604.

Srinivasan M, Mehta P, Yu Y, Prugar E, Koonin EV, Karzai AW, Sternglanz R. 2011. The highly conserved KEOPS/EKC complex I essential for a universal tRNA modification, t6A. *EMBO J* **30:** 873–881.

Stuart JW, Gdaniec Z, Guenther R, Marszalek M, Sochacka E, Malkiewicz A, Agris PF. 2000. Functional anticodon architecture of human tRNA Lys3 includes disruption of intraloop hydrogen bonding by the naturally occurring amino acid modification, t6A. *Biochemistry* **39:** 13396–13404.

Sundaram M, Durant PC, Davis DR. 2000. Hypermodified nucleosides in the anticodon of tRNA^{Lys} stabilize a canonical U-turn structure. *Biochemistry* **39:** 12575–12584.

Teplova M, Tereshko V, Sanishvili R, Joachimiak A, Bushueva T, Anderson WF, Egli M. 2000. The structure of the yrdC gene product from *Escherichia coli* reveals a new fold and suggests a role in RNA binding. *Protein Sci* **9:** 2557–2566.

Urbonavicius J, Qian Q, Durand JM, Hagervall TG, Björk GR. 2001. Improvement of reading frame maintenance is a common function for several tRNA modifications. *EMBO J* **20:** 4863–4873.

Vendeix FAB, Graham WD, Dziergowska A, Gustilo E, Sproat B, Malkiewicz A, Agris PF. 2008. Anticodon domain modifications contribute order to tRNA for ribosome-mediated codon binding. *Biochemistry* **47:** 6117–6129.

Weissenbach J, Grosjean H. 1981. Effect of threonylcarbamoyl modification (t6A) in yeast tRNA Arg III on codon-anticodon and anticodon-anticodon interactions. A thermodynamic and kinetic evaluation. *Eur J Biochem* **116:** 207–213.

Yarian CS, Cain R, Basti MM, Ansari G, Guenther RH, Sochacka E, Malkiewicz A, Agris PF. 1999. Structural and functional roles of the N1- and N3-protons of Ψ at tRNA's position 39. *Nucleic Acids Res* **27:** 3543–3549.

Yarian C, Townsend H, Czestkowski W, Sochacka E, Malkiewicz AJ, Guenther R, Miskiewicz A, Agris PF. 2002. Accurate translation of the genetic code depends on tRNA modified nucleosides. *J Biol Chem* **277:** 16391–16395.