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 N⁶-threonylcarbamoyladenine modification at position 37 (t⁶A₃₇), 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 t⁶A₃₇ biosynthesis. To further investigate the role of YrdC in t⁶A₃₇ 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 t⁶A₃₇-modified ASL^{Lys}_{UUU} ($K_d = 0.27 \pm 0.20 \mu\text{M}$ and $1.36 \pm 0.39 \mu\text{M}$, 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 A₃₇, as determined by NMR, circular dichroism, and fluorescence of 2-aminopurine 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 t⁶A₃₇ biosynthesis.

Keywords: substrate binding; fluorescence; 2-aminopurine; enzyme mechanism; t⁶A₃₇ 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 N⁶-threonylcarbamoyladenine modification (t⁶A₃₇) ensures the accurate recognition of ANN codons (Fig. 1A,C; Nishimura 1972). The t⁶A₃₇-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 t⁶A₃₇ 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

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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 U₂₇•A₄₃ base pair of the natural sequence has been replaced by a G₂₇•C₄₃ base pair to increase the stability of the stem. (C) The N⁶-threonylcarbamoyladenine (t⁶A₃₇) modification found at position 37 of tRNA^{Lys}_{UUU}.

loop by negating intraloop base-pairing of U₃₃•A₃₇ 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 t⁶A₃₇ 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 t⁶A₃₇ 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 t⁶A₃₇ modification over its modified counterpart (El Yacoubi et al. 2009). YrdC is essential in *E. coli* and *yrnC* expression is required for the formation of t⁶A₃₇ 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 t⁶A₃₇ 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 t⁶A₃₇ biosynthesis (El Yacoubi et al. 2009). In this study *sua5* deletion mutants led to the loss of t⁶A₃₇. Additionally, reading frame maintenance of translation was adversely affected by loss of Sua5 and t⁶A₃₇ (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 U₃₄ 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 dimethylallyl pyrophosphate to A₃₇ of tRNAs with an A₃₆-A₃₇-A₃₈ sequence to form i⁶A₃₇ and has been co-crystallized with the *E. coli* tRNA^{Phe}_{GAA} (Chimnarong 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 U₃₃, G₃₄, A₃₅, and A₃₇ 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 A₃₇, 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 t⁶A₃₇ biosynthesis.

RESULTS

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

The tRNAs responding to ANN codons and having the t⁶A₃₇ modification or a derivative of it such as m⁶t⁶A₃₇ 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 ($K_d = 0.11 \pm 0.05 \mu M$) to be greater than that of its fully modified counterpart ($K_d = 0.62 \pm 0.13 \mu M$) (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 unmodified 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^6A_{37} . Yeast ASL^{Phe}_{GAA} has a G_{37} , and it is modified to wybutosine (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 ($K_d = 0.27 \pm 0.2 \mu M$), 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.

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, U₃₃UUUA₃₇, and U₃₃UUUt⁶A₃₇. (D) The titration of YrdC with variously modified ASL^{Lys}_{UUU} with the modifications 5-methylaminomethyluridine, mnm⁵U₃₄, 2-thiouridine, s²U₃₄, pseudouridine, Ψ₃₉, and the 2-methylthio derivative of t^6A_{37} , ms² t^6A_{37} .

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, $K_d = 1.36 \pm 0.39 \mu M$ (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 U₃₃UUUA₃₇ and U₃₃UUUt⁶A₃₇ are composed of the invariant U₃₃ 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 (μ M)	$\pm$
ASL ^{Lys} _{UUU} unmodified	0.27	0.20
ASL ^{Lys} _{UUU-t⁶A₃₇}	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 ⁶ A ₃₇	1.31	0.59
ASL ^{Lys} _{UUU-mnm⁵U₃₄t⁶A₃₇}	1.20	0.20
ASL ^{Lys} _{UUU-mnm⁵U₃₄}	1.87	0.73
ASL ^{Lys} _{UUU-s²U₃₄Ψ₃₉}	1.54	0.27
ASL ^{Lys} _{UUU-s²U₃₄}	1.64	0.29
ASL ^{Lys} _{UUU-ms²t⁶A₃₇}	1.92	0.64
ASL ^{Lys} _{UUU-Ψ₃₉}	1.82	0.88
ASL ^{Lys} _{UUU-2AP₃₇}	0.17	0.21

nucleoside A₃₇. 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 t⁶A-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 A₃₇ and possible product t⁶A₃₇.

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, mnm⁵s²U₃₄, t⁶A₃₇ and pseudo-uridine 39, Ψ₃₉ (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-mnm⁵U₃₄t⁶A₃₇} and the ASL^{Lys}_{UUU-ms²t⁶A₃₇} with the mammalian 2-methylthio-t⁶A₃₇ would be expected to bind with an affinity comparable to that of ASL^{Lys}_{UUU-t⁶A₃₇} because the A₃₇ in each is modified with an N⁶-threonyl-carbamoyl-moiety. Indeed, these two modified ASL^{Lys}_{UUU} bound to YrdC with apparent dissociation constants more reflective of the t⁶A₃₇-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 t⁶A₃₇, ASL^{Lys}_{UUU-s²U₃₄}, ASL^{Lys}_{UUU-s²U₃₄Ψ₃₉}, ASL^{Lys}_{UUU-Ψ₃₉}, and ASL^{Lys}_{UUU-mnm⁵U₃₄} 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 t⁶A₃₇ biosynthesis. Surprisingly, the dissociation constants for the ASLs with s²U₃₄, mnm⁵U₃₄, and Ψ₃₉ were similar to those for ASLs with the threonyl-carbamoyl modification at A₃₇. s²U₃₄, mnm⁵U₃₄, and

mnm⁵s²U₃₄ 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). Ψ₃₉, located two nucleosides 3' to A₃₇, 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 t⁶A₃₇ 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 t⁶A₃₇ utilizes free threonine from the cell as the threonyl-moiety for production of the threonyl-carbamoyl (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 $K_d = 0.04 \pm 0.10$ μ M. 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 ($K_d = 0.12 \pm 0.16$ μ M).

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^{Val}_{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 ¹H-NMR recapitulate previously published data for the unmodified ASL^{Lys}_{UUU} (Stuart et al. 2000) and unmodified ASL^{Val}_{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 H β . 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 H β of threonine and is recovered by 48%. The H α 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 tryptophan 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 μ M and 1.31 ± 0.91 μ M, respectively.

YrdC does not induce a significant change in ASL^{Lys}_{UUU} structure, particularly at A₃₇

Modification of A₃₇ to t⁶A₃₇ may involve a conformational perturbation of the anticodon stem and loop or, minimally, a conformational change in A₃₇. 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 U₃₄ recognizes tRNA in a predominantly rigid body manner by flipping out U₃₄ 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 A₃₇ 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 A₃₇ with the binding of YrdC, ASL^{Lys}_{UUU} was synthesized with a fluorescent analog of adenosine, 2-aminopurine ribonucleoside, at position 37 (2AP₃₇) (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 A₃₇ 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}-2AP₃₇ 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}-2AP₃₇ with a $K_d = 0.17 \pm 0.21 \mu\text{M}$, comparable to that for ASL^{Lys}_{UUU}. The 2AP fluorescence of ASL^{Lys}_{UUU}-2AP₃₇ ($\lambda_{\text{ex}} = 310\text{nm}$, $\lambda_{\text{em}} = 375\text{nm}$) 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, A₃₇ is stacking with either adjacent bases or aromatic amino acids and is not being flipped to a position exposed to solvent. Since 2AP₃₇ is within the flexible loop region, it is likely that binding of YrdC creates a more structured local environment for A₃₇, 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 A₃₇, 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 A₃₇, 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 $_{37}$ (λ_{ex} = 310 nm; λ_{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 ± 0.20 μ M) 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 ± 0.05 μ M) (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. 1H -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 $\times$ 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 A₃₇ 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 t⁶A₃₇. 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 2AP₃₇ 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 A₃₇. It is possible that the NMR solution conditions may not have been optimal for observation of a conformational change, as in vitro t⁶A₃₇ biosynthesis has been shown to require magnesium and bicarbonate (Elkins and Keller 1974).

The proposed t⁶A₃₇ biosynthesis requires two ATP-dependent steps: formation of carboxylphosphate from bicarbonate and ATP and its transfer to the amino group of adenosine to form N⁶-carbamoyl-adenosine, and then activation of the carbamoyl moiety of N⁶-carbamoyl-adenosine 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 t⁶A₃₇ 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 A₃₇ 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 t⁶A₃₇ 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}-t⁶A₃₇ was chemically synthesized with the 2',5'-protected 3'-phosphoramidite of t⁶A₃₇ by the Nucleic Acid Facility of North Carolina State University. The oligonucleoside pentamers UUUUA and UUUUt⁶A were chemically synthesized in solution using the phosphotriester chemistry (Agris et al. 1997).

His₆-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 μ L of 2 μ M YrdC (25 mM potassium phosphate, 100 mM NaCl at pH 6.8) to final concentrations of 0.2–10 μ M 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}-2AP₃₇ 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 A₂₆₀ 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 overlaid. 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% D₂O (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% D₂O (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, ¹³C, and ¹⁵N 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% D₂O). 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% D₂O.

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