RluD, a highly conserved pseudouridine synthase, modifies 50S subunits more specifically and efficiently than free 23S rRNA

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ABSTRACT

Pseudouridine modifications in helix 69 (H69) of 23S ribosomal RNA are highly conserved among all organisms. H69 associates with helix 44 of 16S rRNA to form bridge B2a, which plays a vital role in bridging the two ribosomal subunits and stabilizing the ribosome. The three pseudouridines in H69 were shown earlier to play an important role in 50S subunit assembly and in its association with the 30S subunit. In *Escherichia coli*, these three modifications are made by the pseudouridine synthase, RluD. Previous work showed that RluD is required for normal ribosomal assembly and function, and that it is the only pseudouridine synthase required for normal growth in *E. coli*. Here, we show that RluD is far more efficient in modifying H69 in structured 50S subunits, compared to free or synthetic 23S rRNA. Based on this observation, we suggest that pseudouridine modifications in H69 are made late in the assembly of 23S rRNA into mature 50S subunits. This is the first reported observation of a pseudouridine synthase being able to modify a highly structured ribonucleoprotein particle, and it may be an important late step in the maturation of 50S ribosomal subunits.

Keywords: RNA modification; pseudouridine; ribosome biogenesis; RluD

INTRODUCTION

Ribosomes are complex, multisubunit, macromolecular machines responsible for all cellular protein synthesis. At least three RNA species and >50 proteins need to be assembled together to produce a functional ribosome. The sequential and orderly nature of this complex assembly process has prompted comparisons to a factory assembly line optimized for efficiency, timeliness, and quality control (Dlakić 2005; Talkington et al. 2005). While the broad outlines of the stepwise nature of ribosome biogenesis have been well documented (Culver 2003; Klein et al. 2004; Talkington et al. 2005), the role of non-ribosomal factors and post-transcriptional RNA modifications in this process have only begun to be appreciated (King et al. 2003; Nazar 2004; Dlakić 2005; Gutgsell et al. 2005; Talkington et al. 2005; Baxter-Roshek et al. 2007).

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More than 100 chemically distinct post-transcriptional RNA modifications are known (Rozenski et al. 1999), of which pseudouridines, the 5-ribosyl isomers of uridine, are the most abundant. Pseudouridines are believed to confer extra stability and rigidity to the local RNA structure surrounding the modified base (Davis et al. 1998; Cabello-Villegas and Nikonowicz 2005). Consistent with these observations, pseudouridines are found in highly structured RNA species whose tertiary structure is important for their function (including tRNA, rRNA, snRNA, and tmRNA); they are usually absent in mRNA (Charette and Gray 2000), though they have been observed in the leader sequences that are *trans*-spliced onto all mRNAs in trypanosomes (Liang et al. 2002).

The 16S and 23S ribosomal RNAs of *Escherichia coli* contain 11 pseudouridines clustered predominantly in functionally important regions of the ribosome. These pseudouridines are made by seven pseudouridine synthases in an energy- and cofactor-independent manner (Del Campo et al. 2001). The synthases are specific to one or more sites on the rRNA, but no uridine is known to be modified by more than one synthase (Ofengand et al. 2001). The synthases belong to two families: RsuA (RsuA, RluB, RluE, and RluF) and RluA (RluA, RluC, and RluD).

RluD is the Ψ synthase responsible for the formation of Ψ1911, Ψ1915, and Ψ1917 on helix 69 (H69) in domain IV of 23S rRNA. These are some of the most conserved pseudouridine sites in ribosomes (Ofengand et al. 2001). H69 itself is a critical region of 23S rRNA that is important for efficient ribosomal assembly (Gutgsell et al. 2005; Ali et al. 2006). Along with helix 44 on 16S rRNA, H69 forms bridge B2a, one of the 12 intersubunit bridges that maintain the 70S ribosome (Yusupov et al. 2001; Schuwirth et al. 2005; Korostelev et al. 2006; Selmer et al. 2006). Bridge B2a is part of the peptidyl transferase center and has been proposed to interact with the anti-codon arm of A- and P-site tRNAs (Bashan et al. 2003). Consistently, deletion of H69 is dominantly lethal in *E. coli* (Ali et al. 2006). Moreover, bases A1912 and U1917 in H69 have been shown to be absolutely essential while a U1915C mutation results in a severe growth phenotype (Liiv et al. 2005; Hirabayashi et al. 2006). Remarkably, deletion of the RluD synthase results in serious growth and ribosomal assembly defects (Raychaudhuri et al. 1998; Gutgsell et al. 2005). Taken together, these observations indicate that the three modifications on H69 are important for ribosome assembly and stability as well as for efficient protein synthesis.

A hitherto unaddressed question regarding these pseudouridylations is: When are these modifications made? In other words, at what stage during the assembly of the ribosome are these uridines modified? In this work, we show that RluD can modify H69 on 50S ribosome subunits. We also show that RluD is far more efficient on 50S subunits than on synthetic or free 23S RNA. This is the first such reported observation for a large ribosomal subunit pseudouridine synthase. We suggest that RluD is a ribosomal assembly factor that may be involved in the late stages of maturation of the large ribosomal subunit.

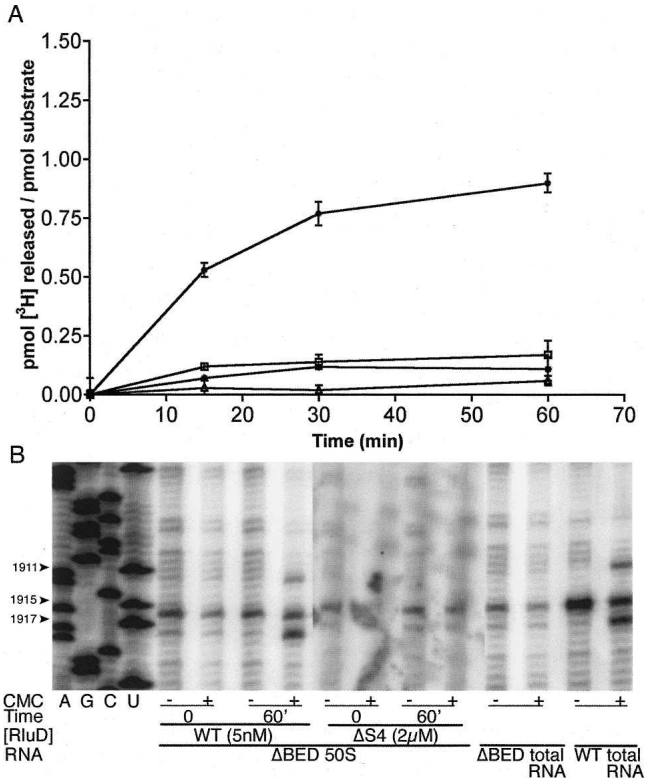

FIGURE 1. A comparison of pseudouridine modification activity of wild-type RluD (WT) and ΔS4 RluD (ΔS4) on ΔBED 50S subunits with the activity of WT RluD on full-length 23S RNA and domain IV of 23S RNA, as monitored by tritium release and Ψ sequencing analyses. (*A*) Time course of pseudouridylation activity on 50S subunits extracted from a ΔBED strain and synthetic full-length 23S RNA and domain IV of 23S RNA. Reactions were carried out as described in Materials and Methods. Reaction mixtures contained 200 nM substrate RNA and the indicated amounts of purified His-tagged protein. The amount of pseudouridine modification was monitored by the tritium release assay. (●) 5 nM WT RluD on 50S, (△) 2 μM ΔS4 RluD on 50S, (□) 20 nM WT RluD on full-length 23S RNA, (✖) 20 nM WT RluD on domain IV of 23S RNA. The average of three experiments is shown. Error bars represent standard deviation. (*B*) Ψ sequencing analysis of RNA extracted from 50S subunits from *A* at the earliest (0′) and last (60′) time points. RNA was reacted with (+) or without (−) CMC following the standard sequencing protocol. While several stops can be seen, only CMC-dependent changes in intensity are indicative of Ψ. The three RluD target uridines (1911, 1915, and 1917) are marked by black arrowheads.

RESULTS AND DISCUSSION

RluD is more efficient on 50S particles than on free 23S rRNA

RluD was initially identified and characterized based on its activity on synthetic, free 23S RNA (Huang et al. 1998; Raychaudhuri et al. 1998; Wrzesinski et al. 2000). However, as shown in Figure 1, neither synthetic 23S RNA nor domain IV (nucleotides 1658–2001) of 23S RNA are effective substrates for RluD. In fact, the rate of reaction with these substrates is so slow that equimolar or higher concentrations of enzyme are required to detect significant activity on these RNAs (Fig. 2A). Moreover, although positions 1911, 1915, and 1917 on H69 were modified when additional enzyme was added to the reaction (Fig. 2B), nonspecific modification of other nontarget uridines was also observed. For example, at the highest concentration (2 μM) of RluD examined in Figure 2, tritium release equivalent to the conversion of ~7–8 uridines per RNA molecule was seen. Considering that the target sites of RluD are only the three uridines in helix 69, these data suggest that RluD can act nonspecifically on free 23S RNA. This conclusion is further strengthened by our observation that, among the pseudouridine synthases tested, only RluD is able to produce this nonspecific tritium release, which suggests that this effect is most likely not due to contaminating enzymes in our protein preparations.

These observations prompted us to hypothesize that free RNA might not be the natural substrate of RluD. To test this, we examined the activity of RluD on 50S ribosomal subunits extracted from cells lacking the three

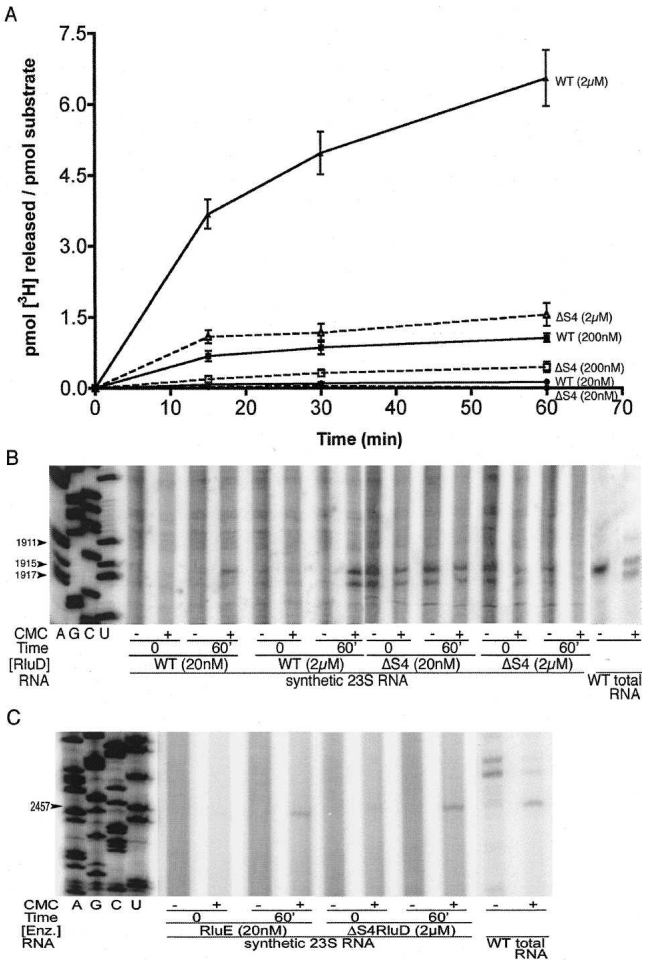

FIGURE 2. Pseudouridine modification activity of wild-type RluD, ΔS4 RluD, and wild-type RluE on synthetic 23S RNA, as monitored by tritium release and/or Ψ sequencing analyses. (*A*) Time course of pseudouridylation activity on synthetic RNA at different enzyme concentrations. Reactions were carried out as described in Materials and Methods. Reaction mixtures contained 200 nM RNA and the indicated amounts of purified His-tagged protein. The extent of pseudouridine modification was monitored using the tritium release assay. (——) WT RluD, (– – –) ΔS4 RluD. (●) 20 nM WT RluD, (■) 200 nM WT RluD, (▲) 2 μM WT RluD. (○) 20 nM ΔS4 RluD, (□) 200 nM ΔS4 RluD, (△) 2 μM ΔS4 RluD. The average of three experiments is shown. Error bars represent standard deviation. (*B*) Ψ sequencing analysis of RNA extracted from reactions in *A* at lowest (20 nM) and highest (2 μM) concentrations of wild-type RluD (WT) and ΔS4 RluD (ΔS4) at the earliest (0′) and last (60′) time points. RNA was reacted with (+) or without (−) CMC following the standard sequencing protocol. The three RluD target uridines (1911, 1915, and 1917) are marked by black arrowheads. No change in intensity is evident between (+) and (−) CMC lanes for ΔS4, signifying the lack of any modification at the three sites. (*C*) Ψ sequencing analysis of synthetic 23S RNA extracted from pseudouridylation reactions at lowest (20 nM) and highest (2 μM) concentrations of wild-type RluE and ΔS4 RluD at the earliest (0′) and last (60′) time points. RNA was reacted with (+) or without (−) CMC following the standard sequencing protocol. The RluE target uridine (2457) is marked by a black arrowhead.

pseudouridine modifications on helix 69. For this purpose, a mutant strain was constructed in which *rluD* as well as two other pseudouridine synthase genes (*rluB* and *rluE*) were deleted (ΔBED). Deletion of *rluB* and *rluE* genes (singly or in combination) has no effect on growth or ribosome profiles on sucrose gradients (data not shown). Ribosomes from the triple deletion strain were employed as substrates so that the activity of multiple synthases could be tested on the same ribosome preparation.

Although Δ*rluD* strains normally grow very poorly on agar plates and in liquid culture, rapidly growing suppressors soon predominate. These strains still lack the three pseudouridines on H69 but exhibit near normal colony sizes, growth rates, and ribosome profiles on sucrose gradients (Gutgsell et al. 2005). Ribosomes from one such suppressor of the ΔBED strain were analyzed at low and high Mg^{2+} and were found to lack any aberrant sized particles (data not shown). Moreover, the sedimentation rates of the 30S, 50S, and 70S particles were identical to those of the wild-type MG1655 strain.

50S subunits were purified from this suppressor strain for use as the substrate in in vitro assays. As shown in Figure 1A, even at sub-stoichiometric amounts of enzyme (5 nM), RluD can efficiently pseudouridylate H69 in the context of these 50S ribosomal subunits. Primer extension analysis showed that this activity is specific to the three target sites on H69 (Fig. 1B). In contrast, even at a higher amount of enzyme (20 nM), the activity on free 23S RNA is at least fivefold lower (Fig. 1A). Even this poor activity is likely nonspecific, since primer extension analysis shows no modification in H69 (cf. Fig. 1B and Fig. 2B). This suggests that the physiological substrate for RluD is likely to be a structured ribonucleoprotein particle.

Interestingly, although primer extension analysis shows that at least two uridines on H69 are modified by RluD (pseudouridylation of the third uridine, U1915, is more difficult to discern, since U1915 is also methylated, which produces a stop in primer extension assays), only about 1 equivalent of tritium is released in the tritium release assay (Fig. 1A). This could be due to two reasons. Our protocol for the isolation of ribosome subunits may result in preparations that are only partially competent to act as RluD substrates. Alternatively, RluD may pseudouridylate only one of the three sites per RNA molecule. In other words, not all 23S RNA molecules may have all three pseudouridines on H69. We are in the process of trying to distinguish between these two possibilities.

The requirement for a structured particle for the activity of RNA modification enzymes is not without precedent. It has been shown that RsuA, the small subunit pseudouridine synthase responsible for generating Ψ516 on 16S RNA, is maximally active on a partially assembled particle consisting of the first 678 nucleotides (nt) and a subset of 30S proteins (Wrzesinski et al. 1995). In addition, neither 16S RNA nor reconstituted 30S subunits could be modified

by this enzyme. These observations suggest that RsuA may modify U516 during an intermediate stage in the assembly of the 30S subunit. Other RNA modification enzymes such as methyltransferases are known to require partial or fully assembled ribosomal subunits for maximal activity (Tscherne et al. 1999; Bügl et al. 2000; Andersen and Douthwaite 2006; Basturea and Deutscher 2007). It is likely that all of these enzymes recognize an RNA structure or fold that is formed only in the context of the assembled subunit, and not in the context of free RNA. Moreover, one cannot discount the possibility that the modification enzymes may interact with ribosomal proteins or other assembly factors that may be part of the subunit during a particular stage of its assembly.

Substrate specificity of RluD

In vivo, RNA modification enzymes, including RluD, are generally exquisitely specific for their target sites. However, as shown above, in vitro, RluD can act nonspecifically on free 23S RNA. Consequently, it was of interest to examine whether other RNAs and structured particles might also function as substrates. For this purpose, the activity of increasing amounts of RluD on 16S and 23S RNAs, either free or in assembled ribosomal particles, was determined. As is evident from Figure 3, at low amounts of enzyme (Fig. 3, open bars), RluD was most active on 50S subunits lacking any pseudouridines on helix 69, and this activity changed little as the amount of enzyme was increased. In contrast, RluD was essentially inactive on mutant 30S particles at all enzyme amounts tested. Moreover, 23S and 16S RNA purified from ΔBED Ψ synthase mutant ribosomes were also poor substrates at low enzyme levels. However, activity on these RNAs increased dramatically with increasing amounts of enzyme, providing evidence that RluD loses its specificity on free RNA at high levels of enzyme, in agreement with that observed with synthetic 23S rRNA (Fig. 2). Inasmuch as there was no significant change in RluD activity on 50S subunits even when additional enzyme was added, it appears that nonspecific base modifications do not occur when the RNA is in the context of the 50S subunit. Thus, the structure of the ribosomal subunit appears to modulate the enzyme's specificity and accessibility to its target sites.

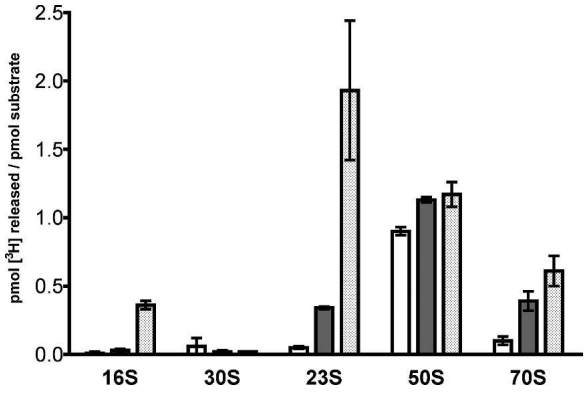

FIGURE 3. RluD is specific to the 50S subunit. The pseudouridylation activity of increasing amounts (20 nM [open], 200 nM [gray], and 2 μM [stippled]) of purified, His-tagged, full-length RluD was compared on different RNA species extracted from ΔBED mutant cells. The substrates were maintained at a constant concentration of 200 nM of RNA. The substrates included 16S RNA (16S), 30S subunits (30S), 23S RNA (23S), 50S subunits (50S), and 70S ribosomes (70S). The 16S and 23S RNA substrates were extracted from their respective subunits as described in Materials and Methods. The reactions were carried out for an hour as described in Materials and Methods. An average of two experiments is shown. Error bars represent standard deviation.

In all cases, a low level of activity was seen with mutant 70S ribosomes. This may be due to a decreased stability of the ribosomes and an increased propensity to dissociate into component subunits during the course of the reaction. As expected, the enzyme was inactive on similarly isolated WT ribosome subunits, and very poorly active on RNAs extracted from the ribosomes at high enzyme amounts (data not shown).

To understand these observations at the structural level, we docked the recently reported structure of full-length RluD (PDB ID: 2IST; Hur et al. 2006) on helix 69 in the structure of the *E. coli* 50S ribosomal subunit (PDB ID: 2AWB; Schuwirth et al. 2005). RluD was docked in an orientation that allows U1915 to enter its active site pocket (Fig. 4). When positioned in this manner, RluD fits nicely on the solvent-exposed and extended H69 in the 50S subunit, with very few clashes with other regions of ribosomal RNA or protein. Moreover, this docking positions the S4-like domain of RluD very close to the base of the H69 stem–loop, suggesting that this region (which is a junction of three helices—helix 68, 69, and 70) may adopt a conformation in the 50S subunit that is recognized by RluD.

The catalytic and RNA binding (S4-like) domains of RluD are quite distinct, and their relative orientations may vary. Indeed, in the initial structures of RluD (Del Campo et al. 2004; Mizutani et al. 2004; Sivaraman et al. 2004), the S4-like domain was disordered. In the recent Hur et al. (2006) structure, the S4-like domain is visible, although three residues in the linker domain cannot be seen (PDB ID: 2IST). Given this variability (as also seen in the variable positioning of RNA binding domains in other Ψ synthases; Del Campo et al. 2004; Mizutani et al. 2004), the arrangement shown in Figure 4 should be considered tentative. However, the spacing between the two domains and the general orientation of the S4-like domain with respect to the catalytic domain strongly suggest the involvement of the region around helices 68, 69, and 70 of 23S RNA in RluD specificity. S4 domains typically recognize helical junctions (Powers and Noller 1995).

In the 70S ribosome, when 30S and 50S subunits associate, helix 69 comes into close contact with helix 44

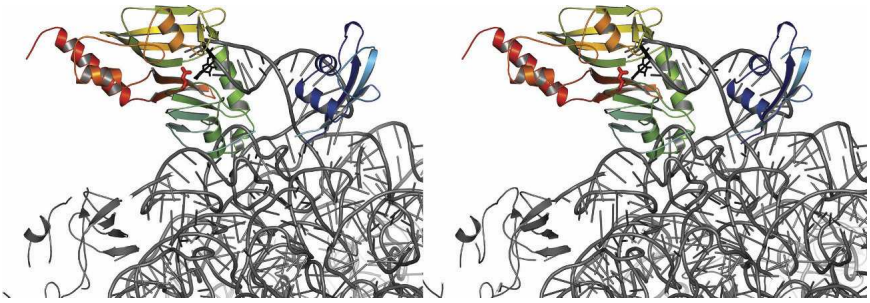

FIGURE 4. Model of RluD docked on the H69 stem–loop in the 50S subunit, poised to modify U1915. A stereo view shown focuses on H69 in the 50S (gray cartoon) with RluD represented by a colored ribbon. Full-length RluD (PDB ID: 2IST) was manually docked on to the H69 stem–loop of the *E. coli* 50S subunit (PDB ID: 2AWB) as described in Materials and Methods. The catalytic Asp (D139) of RluD and U1915 of 23S RNA are depicted as red and black stick models, respectively. Three residues (73–75) connecting the S4-like domain to the catalytic domain of RluD are not shown, since they are missing in the 2IST structure.

on 16S RNA to form bridge B2a. This prevents access to the stem–loop and could explain why 70S ribosomes are poor substrates for this enzyme. This conclusion is also supported by the observation that RsmE, which methylates U1498 on helix 44 of 30S subunits, cannot methylate that site on intact 70S ribosomes (Basturea and Deutscher 2007).

Interestingly, RluD catalyzed fewer nonspecific modifications on 23S RNA from mutant ribosomes than on synthetic RNA (cf. Fig. 2A and Fig. 3). This could be due to two reasons. First, RNA purified from ribosomes is likely to be in a more "native-like" structure than synthetic, in vitro transcribed RNA. Thus, activity on this RNA may be lower due to reduced access to nontarget uridines, many of which may be buried within the folded RNA structure. Second, synthetic RNA is completely devoid of rRNA modifications. Perhaps in vitro, RluD can modify some nontarget uridines, which in vivo normally are modified by other Ψ synthases. We favor the second possibility because the amount of modification of mutant and wild-type 23S RNA was not altered even after these RNAs had been denatured and quick cooled on ice to remove "native" structure (data not shown).

Role of the S4-like domain of RluD

Five of the seven ribosomal Ψ synthases possess N-terminal S4-like domains. Named because of homology with the S4 ribosomal protein, these regions are believed to be RNA binding domains. The S4-like domain is required for the specificity and activity of RluD (Figs. 1, 2). At first glance, ΔS4 RluD seems to be weakly active on synthetic 23S RNA as shown by the tritium release assay (Fig. 2A). However, primer extension analysis of this RNA across the H69 region showed that the truncated enzyme is not specific for the three H69 uridines (Fig. 2B; there is no change in band intensity in the absence or presence of CMC in the ΔS4 RluD lanes). Two other rRNA synthases, RluA and RluE, lack peripheral domains, including an S4-like domain. We hypothesized that, without the S4-like domain, RluD may be able to modify uridines usually modified by RluA or RluE (Ψ746 and Ψ2457, respectively). Since U746 is in the middle of a highly modified region of 23S RNA, we chose to examine the region around U2457. Primer extension analysis confirmed that ΔS4 RluD could indeed modify this RluE site (Fig. 2C). This observation suggests that a core synthase domain may be inherently able to recognize uridines in several contexts and that the role of the peripheral domains is to direct this intrinsic specificity of each synthase toward particular sites.

Considering the five ribosomal Ψ synthases that contain S4-like domains, one may then ask, what determines whether the synthases remain specific for their respective sites and not overlap with each other? The simplest mechanism to account for this is that their S4-like domains differ. Minor variations in their structures could impart certain specificities to individual synthases. Indeed, structural superposition of the S4-like domain of RluD (PDB ID: 2IST; Hur et al. 2006) on the S4-like domain of RsuA (PDB ID: 1KSK; Sivaraman et al. 2002) shows small, but significant, differences that could account for their distinct specificities. However, this by itself may not be enough to explain the precise targeting of these enzymes. Another mechanism may take advantage of the assembly process for the ribosomal subunits. Ψ synthases could follow a temporal order of modification, which is governed by the process of the assembly of the 23S RNA into the 50S subunit. As the 23S RNA matures and the various ribosomal proteins are added, different sites could be presented in unique structural contexts that are recognized only by their respective enzymes.

Unraveling the temporal sequence of rRNA modification

Understanding the temporal order of rRNA modification would be very informative from the standpoint of ribosomal biogenesis. It would allow placement of modification events on an assembly map of the ribosome, and may provide clues to the functions of the modified residues. In this regard, a close look at the pseudouridine modifications on ribosomal RNA and the substrate specificities of their corresponding Ψ synthases reveals some interesting points. For example, the lone 16S RNA Ψ synthase is able to modify a ribonucleoprotein particle that consists of the first

678 bases and an unknown assortment of proteins (Wrzesinski et al. 1995). From the 30S assembly map (Talkington et al. 2005) and the crystal structure of the *E. coli* ribosome (PDB ID: 2AW7), it can be deduced that RsuA modifies 16S RNA once the 5′ domain primary binding proteins have bound the RNA.

In contrast, the substrate specificities of 23S RNA Ψ synthases are less well understood. The recently reported crystal structure of RluA in complex with the anticodon stem–loop (ASL) region of tRNA (Hoang et al. 2006) demonstrated how this dual-specificity synthase could bind and modify its rRNA and tRNA substrates. In the pseudouridylation of U746 on helix 35 of 23S RNA, RluA efficiently modifies free RNA (Raychaudhuri et al. 1999). This uridine is part of a highly modified stem–loop region and is adjacent to both m^1G745 and m^5U747 (Raychaudhuri et al. 1999). Interestingly, $RlmA^I$, the methyltransferase responsible for modifying G745, also prefers free RNA as a substrate (Hansen et al. 2001). Although the substrate specificity of RlmC, the methyltransferase responsible for U747, has not yet been studied in detail (Madsen et al. 2003), it is reasonable to expect that this enzyme will also be active only on free RNA. This is because helix 35, encompassing this region, is buried within the structure of the 50S subunit and is inaccessible in mature 50S subunits. Thus, these three modifications can be placed early in the 23S maturation pathway.

In this work, we have shown that RluD is able to modify 50S subunits much more efficiently than free 23S rRNA. This suggests that RluD may modify H69 at a late step in the maturation of the 23S RNA into the 50S subunit. Among the other pseudouridine synthases, we have observed that RluE (Ψ2457) also modifies free RNA efficiently and specifically (P.P. Vaidyanathan and A. Malhotra, unpubl.). Since in the assembled structure of the *E. coli* 50S subunit, 5S RNA and ribosomal protein L16 normally would block access to U2457, it is likely that RluE modifies domain V of 23S RNA at an early stage in 50S assembly.

Modification enzymes as quality control inspectors

The U2552 methyltransferase, RlmE, is the only other 23S RNA modification enzyme that is able to modify fully mature 50S subunits (Bügl et al. 2000). As was found with deletion of RluD, removal of RlmE also results in severe ribosomal assembly defects (Bügl et al. 2000; Gutgsell et al. 2005). These enzymes join a growing list of prokaryotic ribosomal assembly factors that promote proper ribosomal assembly and folding. Other 23S RNA pseudouridine synthases, including RluB and RluC, also have been termed ribosomal assembly factors based on their association with, and enrichment on, immature pre-50S particles (Jiang et al. 2006).

These indications that some Ψ synthases serve as ribosomal assembly factors prompt us to further speculate about possible secondary roles of these enzymes. Helix 69 and its three pseudouridines are important for ribosome assembly and stability in vivo (Gutgsell et al. 2005; Ali et al. 2006), and ribosomes in which the helix 69 stem–loop is deleted produce a dominant lethal phenotype. Surprisingly, ribosomes lacking the helix 69 stem–loop display near normal accuracy, fidelity, and translocation rates in in vitro assays (Ali et al. 2006). This indicates that, even though these mutant subunits have trouble maturing into ribosomes in vivo, they are functionally competent. It is conceivable that RNA modification enzymes play a role in this process as quality control inspectors, monitoring the progress of ribosome assembly. As individual ribosomes reach particular folded states, appropriate ''check marks'' are placed on them in the form of modifications, allowing them to continue to the next stage. An absence of the modification, because a ribosome is not in the right conformation, may mark that ribosome as ''defective'' and increase the chances of it being degraded (Song and Nazar 2002; LaRiviere et al. 2006). Presumably, the reduction of functional ribosomes is what sickens cells in Δ*rluD* and Δ*rlmE* strains. Further studies will be required to probe this connection between RNA modifications and ribonucleases.

In conclusion, we have found that RluD prefers a structured ribosomal subunit for its activity. Inefficient and nonspecific activity on free RNA suggests that the RNA needs to be properly folded to be accurately and efficiently recognized as a substrate. This observation can be rationalized using structural docking models between the enzyme and helix 69 on the ribosome. Helix 69 is present on the side of the 50S subunit that interfaces with the 30S subunit. This loop is solvent-exposed in the crystal structures of the 50S subunits and, therefore, is easily accessible for modification (Fig. 4). Our results present strong evidence for modification of this stem–loop at a late stage in the assembly of the 50S subunit. This is consistent with accumulating evidence that points toward a growing list of RNA modification enzymes that also function as ribosomal assembly factors. While the role of RluD and similar modification factors is not completely clear, an interesting possibility is that these factors may be involved in ribosome quality control.

MATERIALS AND METHODS

Strains

The triple pseudouridine synthase (*rluB*, *rluE*, and *rluD*) knockout strain was generated from WT MG1655 cells. *rluB* and *rluE* were sequentially removed by the gene disruption method of Datsenko and Wanner (2000), to create VA19. *rluD* was inactivated in this strain by P1 mediated transduction of *rluD*::*Kan* (Gutgsell et al. 2005) to generate the triple knockout strain, PVP028. The RNase I^- mutation was introduced into this strain by P1 mediated

transduction of an *rna*::*Tet* allele from strain, CAN20–12E (Deutscher et al. 1984), resulting in PVP029. This strain (referred to as ΔBED) was used for all mutant ribosome extractions. An MG1655 strain from which the *rna* gene was similarly knocked out served as wild type for the purposes of this study.

Materials

[5-^{3}H]-Uridine and [5-^{3}H]-UTP were purchased from GE Heathcare, Inc. [α-^{32}P] dATP was obtained from Perkin-Elmer. All restriction enzymes were purchased from New England Biolabs. T7 RNA Polymerase, RNase-free DNase I, Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT), Taq DNA polymerase, and RNasin ribonuclease inhibitor were from Promega. KOD high fidelity DNA polymerase was obtained from Stratagene. Zero blunt end pTOPO vector cloning kit was from Invitrogen.

In vitro synthesis of RNA

23S RNA and domain IV of 23S RNA were synthesized in vitro by runoff transcription with T7 RNA polymerase from plasmids containing the cloned genes. Plasmid pCW1 containing the 23S rRNA gene, *rrnB*, has been previously described (Weitzmann et al. 1990). The domain IV region of *rrnB* (bases 1658–2001) was amplified from pCW1 by PCR using KOD DNA Polymerase. The N-terminal primer was 5′-GAAGCT**GGTACC***TAATACGACT CACTATAG*GGTGAAGGAACTAGGC-3′. This primer contained a KpnI site (indicated in bold) and a T7 promoter sequence (indicated in italics). The C-terminal primer, in reverse orientation, was 5′-GAAGTA**CTGCAGCTTAAG**GGGTGGAGACAGCC TGG-3′ and introduced PstI (CTGCAG) and AflII (CTTAAG) restriction sites (indicated in bold).

The amplified fragment was cloned into the pTOPO vector following manufacturer's protocols and the sequence was confirmed by DNA sequencing. The plasmids were linearized with AflII and used as templates without further purification. Typical transcription reactions were carried out in 40 mM Tris-Cl, pH 8.0, 10 mM DTT, 0.1% Triton-X, 1 mM spermidine, 40 mM $MgCl_2$, 400 U/mL RNasin, 5 mM rNTPs, 100 ug/mL plasmid template, 1 U/mL inorganic pyrophosphatase, and 1.2 U/μL T7 RNA polymerase for 4 h at 37°C. The reaction was then treated with 40 U/mL of RNase-free DNase for 20 min at 37°C. RNA was precipitated by the addition of 1/3 volume of 3 M NaOAc, pH 5.2, and 5/3 volume of sterile DEPC-treated water. Isopropanol was added to a final concentration of ~38%. The RNA was pelleted by centrifugation at 14,000 rpm for 10 min. The pellet was air-dried and dissolved in 250 μL 0.1 M NaOAc. The RNA was then phenol:chloroform-treated and chloroform:isoamylalcohol-extracted twice, ethanol-precipitated, washed with 70% ethanol, air-dried, and dissolved in a suitable volume of DEPC-treated water. Tritium labeling was carried out by addition of 200 μCi of [5-^{3}H]-UTP (GE Healthcare, Inc.) to the reaction mix.

Protein purification

His-tagged RluD was purified by sequential column chromatography, as previously described (Del Campo et al. 2003). The protein was diluted and stored at −20°C in buffer E (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 2 mM EDTA, 0.5 mM DTT, and 45% glycerol). N-terminal his-tagged RluE and ΔS4 mutant of RluD were expressed using the pET28a expression system (Novagen, Inc.) and purified by cobalt metal-affinity chromatography using Talon resin following manufacturer's instructions (Clontech).

Buffers

Cell Resuspension Buffer (CRB) contained 50 mM HEPES-KOH, pH 7.5, 100 mM NH_4Cl, 6 mM β-mercaptoethanol, and 10 mM $Mg(OAc)_2$. High-salt CRB was the same as CRB, but contained 2 M NH_4Cl. Ribosome Dissociation Buffer (RDB) was the same as CRB, but with 2 mM $Mg(OAc)_2$.

Ribosome labeling and extraction

An aliquot of the appropriate strain cultured in rich media until the stationary phase was diluted 100-fold in M9 medium supplemented with 0.2% glucose, the appropriate antibiotics, and 10 μCi/mL of [5-^{3}H]-Uridine (GE Healthcare Inc.). Cultures were allowed to grow at 37°C with constant shaking to an A_{600} of 0.6–0.8. Cells were pelleted by centrifugation at 5000 rpm for 10 min. Cell pellets were resuspended in 2–3 mL of CRB. Cells were then lysed by three passes through a French press at 18,000 psi. The lysates were centrifuged at 16,000 rpm for 30 min in an SS-34 rotor (Sorvall) to pellet cellular debris. The supernatant fraction was diluted twofold with high-salt CRB and centrifuged at 44,000 rpm for 4 h in the 60Ti rotor (Beckman) to pellet the ribosomes. The ribosomes were dissociated into subunits by dissolution into RDB, either by gentle rocking at 4°C for 16–20 h or by stirring at very low speed at 4°C for at least 1 h. The dissociated subunits were layered onto a 14%–32% sucrose gradient in RDB and centrifuged at 21,000 rpm for 19 h in an SW 28 rotor (Beckman). The gradients were collected using the Gilson Fraction Recovery System. Fractions corresponding to the 50S and 30S peaks were pooled, the Mg^{2+} concentration increased to 10–20 mM, and samples were concentrated using Millipore Amicon Ultra spin filters. 70S ribosomes were purified in essentially the same way except for the following buffer modifications: The ribosomal pellets were dissolved in CRB and layered onto a 14%–32% sucrose gradient also in CRB.

Tritium release assay

The tritium release assay (TRA), which measures the release of radiolabeled hydrogen from the 5-carbon position of uridine when it is converted to pseudouridine, was performed essentially as described (Wrzesinski et al. 1995). Typical reactions were performed at 37°C in 50 mM HEPES-KOH, pH 7.5, 100 mM NH_4Cl, 10 mM $Mg(OAc)_2$ (20 mM for 70S particles), 400 U/mL RNasin, and 6 mM β-mercaptoethanol. Reactions with 70S particles also included 2 mM spermidine. The reaction mixtures containing 200 nM substrate and indicated amounts of enzyme were incubated for the indicated periods of time. Reactions were stopped by adding 1 mL of 12% NoritA-activated charcoal in 0.1 N HCl. The solution was mixed and allowed to incubate at room temperature for at least 5 min. The charcoal was pelleted by centrifugation at 14,000 rpm for 5 min. The supernatant fraction was then filtered using 0.2 μ PTFE syringe filters (Nalgene). Five hundred microliters were removed for scintillation counting. This value was multiplied by two to arrive at the total radioactivity released per reaction.

Primer extension assay

The primer extension assay was performed essentially as described (Ofengand et al. 2001). Briefly, RNA (either synthetic or extracted from ribosomal particles) was purified from the reaction by two rounds of phenol:chloroform treatment and chloroform:isoamylalcohol extraction followed by ethanol precipitation. Total RNA was extracted as described (Ofengand et al. 2001). These RNA species were modified with CMC in a solution containing 7 M Urea, 50 mM Bicine, and 4 mM EDTA and incubated at 37°C for 20 min. RNA was ethanol-precipitated and dissolved in 100 μL of 50 mM Na_2CO_3 (pH 10.4) and incubated at 37°C for 4 h. RNA was then ethanol-precipitated and dissolved in 10 μL water. Four microliters of this RNA were annealed to a primer complementary to the region of the 23S rRNA under analysis. The primer was extended by AMV-RT according to the manufacturer's instructions. pCW1 plasmid DNA was used to generate the sequencing lanes. DNA sequencing was carried out using Sequenase (USB) according to the manufacturer's instructions. Primer extension products were resolved on an 8% polyacrylamide gel containing 7 M urea and detected by autoradiography with Kodak Biomax HR film or with a PhosphoImager (Molecular Imaging).

Molecular modeling and docking

The model of RluD docked onto the helix 69 stem–loop was guided by the co-crystal structure of TruB, a related pseudouridine synthase, with a 22-nt RNA stem–loop (PDB ID: 1K8W; Hoang and Ferré-D'Amaré 2001). An initial model was constructed by a two-step structural superposition of RluD and H69 on the TruB–RNA complex as follows: The coordinates of the H69 stem–loop (bases 1906–1924) were extracted from the structure of the *E. coli* 50S (PDB ID: 2AWB; Schuwirth et al. 2005) and superimposed onto the RNA ligand from the TruB–RNA complex using the FASTFIT option of LSQMAN (Kleywegt et al. 2001). RluD (PDB ID: 2IST; Hur et al. 2006) was superimposed onto TruB by the SSM superposition (Krissinel and Henrick 2004) package in COOT (Emsley and Cowtan 2004). The rotated coordinates of H69 and RluD were merged into the same file. Potential clashes were analyzed using the MolProbity package (Davis et al. 2004). The coordinates in this file were then appropriately rotated and translated to superimpose H69 onto the same region in the 50S structure using the EXPLICIT option of LSQMAN. Figure 4 was generated using PyMol (DeLano 2002).

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