Different sensitivity of H69 modification enzymes RluD and RlmH to mutations in *Escherichia coli* 23S rRNA

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1. Introduction

Post-transcriptional modification of nucleosides is a conserved feature of ribosomal RNA (rRNA). Pseudouridines (Ψ) and ribose methylations are the most common modifications, followed by addition of methyl groups to various positions of bases. Modified nucleosides are mostly found at conserved positions in or near the functionally important regions of the ribosome [1–4].

Regardless of their localization and conservation, the function of the modified nucleosides remains largely enigmatic. Altogether, hydrophilic pseudouridines and hydrophobic methylations are proposed to conspire to ensure the formation and the stability of the rRNA conformational changes needed for optimal ribosome activity and for binding of ligands. Another function of the modified nucleosides could be to act as structural checkpoints in the ribosome assembly process [5–7].

One of the heavily modified regions of the bacterial ribosome is the stem-loop 69 (H69) of the 23S rRNA. The importance of this rRNA region is underscored by the following: i) H69 is the large subunit component of the evolutionally highly conserved inter-
modifications like m^3Cm and m^3Ψ whose synthesis involves two specific enzymes. As a rule, most rRNA modification enzymes modify a single nucleotide that is present at a defined position. However, exceptions to this rule are: KsgA that methylates two neighboring adenosines in 16S rRNA; RluC and RluD enzymes that both introduce 3 pseudouridines to specific sites in 23S rRNA; and RluA that introduces one pseudouridine both into 23S rRNA and some specific tRNA species [41–43]. Modification enzymes recognize their rRNA substrates at various stages of the ribosome subunit assembly [44]. In vitro studies have revealed that some modifications are added to the naked rRNA, while others require the presence of ribosomal proteins, or alternatively, rRNA structural motifs that only form with the aid of certain ribosomal proteins [1,6]. In the case of tRNA, some modification enzymes seem to have a strict requirement for the sequence neighboring its target nucleoside, whereas others do not, recognizing rather structural motifs beyond the vicinity of the target nucleoside [45]. The specificity determinants of the bacterial rRNA modification enzymes have not been studied in detail.

As mentioned, there are two pseudouridine synthases in E. coli that can isomerize three uridines each. RluC isomerizes U955, U2504, and U2580 that are located in various regions of the large subunit and do not appear to share any common traits either on the primary or on the secondary structure level [41,46]. RluD, on the other hand, isomerizes U1911, U1915, and U1917, all of which are located in close proximity of one another in the H69 of the 23S rRNA [42,47]. Unlike the pseudouridines synthesized by RluC, the ones in H69 are highly conserved [46]. Such multi-site activities make it intriguing to define the specificity determinants of the corresponding enzymes.

It has been shown that RluD exhibits a remarkably unspecific activity on the in vitro transcribed protein-free 23S rRNA [42,43,48]. However, when 50S subunits isolated from the RluD deficient strain were used as a substrate, RluD exhibited a fast and specific activity toward the H69 of 23S rRNA [48]. By introducing a set of point mutations into the H69, we have previously shown that RluD is in vivo highly specific to positions 1911 and 1917, while its specificity toward the position 1915 could not be determined due to experimental limitations caused by the ambiguous nature of the methylation of the base (U or Ψ) at this position [49]. In the aforementioned study, uridines introduced into positions 1912, 1914, 1916 and 1919 were not isomerized into pseudouridine [49]. This finding contradicts the assumption by Ofengand and coworkers [46] that RluD modifies all uridines in the loop region of the H69 irrespective of their exact position. Only the A1916U and A1916G mutations had a noticeable negative effect on the RluD activity in vivo, inhibiting formation of H69 pseudouridines, suggesting that A1916 serves as an important specificity determinant for RluD [49]. We have also established that the pseudouridines in the H69 are formed independently of each other and that their formation does not seem to follow any specific order [49,50]. It has been shown that RluD acts during the late stages of the ribosome large subunit assembly in vivo, unlike the majority of the large subunit specific modification enzymes [44,48,49]. More recently, we have determined the kinetic parameters of the RluD protein verifying its fast kinetics when the 50S subunit is used as the substrate [50]. Despite the numerous findings mentioned above, it is still unclear what structural elements of the 50S subunit are recognized by the RluD protein and are mediating its intriguing substrate specificity.

In many bacterial species, including E. coli, the pseudouridine at position 1915 of the H69 is methylated at its N3 position by the SPOUT family methyltransferase RlmH [33,51,52]. The RlmH enzyme has a unique specificity in two aspects. First, it is the only RNA methyltransferase in bacteria that methylates pseudouridine and, furthermore, the only RNA methyltransferase described in any organism that methylates pseudouridine at its N3 position (Modomics http://modomics.genesilico.pl/; RNA modification database http://rna-mdb.cas.albany.edu/RNAmods/; as of November, 2011). In eukaryotes, the small subunit specific pseudouridine N1-methyltransferase Nep1 has been found to be associated with the Bowen-Conradi syndrome [53,54]. The second peculiarity of the RlmH enzyme is its specificity for the 70S ribosome, not displayed by any other bacterial rRNA modification enzyme, assigning it to the final steps of ribosome maturation process [50]. Modeling of the crystal structure of the RlmH protein into the crystal structure of the 70S ribosome suggests that RlmH makes extensive contacts with the small subunit and interacts with the large subunit only in the H69 region [52]. As with RluD, the exact mechanism and the determinants in H69 involved in target site recognition by RlmH remain unanswered.

Our goal in the present study was to shed light on the substrate recognition mechanism of rRNA modification enzymes, by analyzing the effects of the mutations introduced into the 23S rRNA H69 on the modification activities of the RluD and RlmH enzymes in the cell-free system.

2. Experimental procedures

2.1. Strains and plasmids

Generation of the ArlmH and ArluD/ArmlmH strains, single and double gene knockouts (rlmH and rluD genes replaced with kanamycin and chloramphenicol resistance cassettes, respectively) of E. coli MG1655 strain, is described in [51]. E. coli MG1655 strain [55] was used as the control.

rRNA gene variants with mutations corresponding to A1912U, C1914U, A1916U, A1916G, A1919U, A1919G, or A1960G substitutions in 23S rRNA were inserted into the ptBB-Tag expression vector in which the rrnB operon is under the control of the inducible tac promoter [29]. For this study we constructed 23S rRNA variant H69 + GC, H69 + AU and H69A1908/1922. PCR was used for site directed mutagenesis to delete bases C1908 and C1922 in case of H69A1908/1922 and to insert G/A between bases C1908, C1909 and C/U between bases C1921, C1922 of 23S rRNA in case of H69 + GC and H69 + AT. All the expressed 23S rRNA variants also contain a streptavidin-binding aptamer in helix 25 for affinity purification of the mutant ribosomes [56].
2.2. Preparation of ribosomes

*E. coli* MG1655 wild-type, ΔrlmH knockout, and ΔrluD/ΔrlmH double knockout strains were grown in 2.4 L of 2xYT media containing 25 μg/ml of kanamycin in the case of ΔrlmH strain and 25 μg/ml of kanamycin and 20 μg/ml of chloramphenicol in the case of ΔrluD/ΔrlmH strain. Ribosomes were isolated from the exponentially growing cells at OD_{600} ~ 0.8. Bacteria were collected by low-speed centrifugation at 4 °C and re-suspended in lysis buffer (20 mM Tris/HCl pH 7.5, 100 mM NH₄Cl, 10 mM Mg-acetate, 0.5 mM EDTA, 6 mM β-mercaptoethanol, 20 U/ml DNase I, and 2 mg/ml lysozyme). French press was used to disrupt the cells. Cell lysate was cleared by centrifugation at 12 000 rpm for 30 min at 4 °C and diluted two-fold with TKNM-10 buffer (10 mM Tris/HCl pH 8.0, 60 mM NH₄Cl, 60 mM KCl, 10 mM Mg-acetate, and 6 mM β-mercaptoethanol). A total of 3500 A_{260} units of cell lysate was layered onto a 10%–35% (w/w) sucrose density gradient in TKNM-10 buffer and centrifuged at 25,000 rpm for 18.5 h (ω^2t = 4.6 × 10^11) at 4 °C in a Beckman Ti-45 rotor. Sucrose gradients were analyzed with a Beckman Ti-14 rotor. Sucrose gradients were analyzed with a Beckman Ti-14 rotor. The 70S ribosome pellet was suspended in 20 mM Mg-acetate, 60 mM KCl, 60 mM NH₄Cl, 10 mM Tris/HCl pH 8.0, 6 mM β-mercaptoethanol at 37 °C for 10 min mRNA extraction was carried out as described in [49].

2.4. Determination of pseudouridines by CMCT/alkali treatment and primer extension analysis

The CMCT/alkali treatment of the 23S rRNA followed by the primer extension analysis was used to determine the presence of the pseudouridines in the H69. The CMCT/alkali treatment was performed as described by Ofengand and coworkers [57]. 15 μg of rRNA was dissolved in 20 μl of deionized water. 80 μl of BEU buffer (7 M urea, 4 mM EDTA, 50 mM Bicine/NaOH pH 8.5) and 20 μl of CMCT/BEU (1 M CMCT in BEU buffer) were added to the rRNA solution. Alternatively, 100 μl of BEU buffer was added to the rRNA solution, serving as the negative control. Both samples were incubated at 37 °C for 10 min for the CMC modification of the U, G, and Ψ residues. rRNA was precipitated with addition of 38 μl of 4 M Na-acetate and 600 μl of cold 96% ethanol, and by incubation at −20 °C for 10 min rRNA precipitate was collected by centrifugation at 13 000 rpm for 10 min at 4 °C. The supernatant was carefully removed and rRNA was washed twice with 600 μl of 70% ethanol. RNA precipitate was air-dried at 37 °C for 10 min rRNA was dissolved in 50 μl of NPK buffer (20 mM NaHCO₃, 30 mM Na₂CO₃, and 2 mM EDTA) and incubated at 37 °C for 4 h to allow for the removal of the CMC group from the U and G residues. rRNA was precipitated and washed as described above. rRNA precipitate was dissolved in 20 μl of deionized water and stored at −20 °C.

Pseudouridine sequencing of rRNA was carried out by the primer extension analysis using primer U1 (CAG CCT GCC CAT CAT TAC GCC) complementary to the positions 1972–1992 of *E. coli* 23S rRNA, [α-^32^P]dCTP (Amersham Biosciences), and AMV reverse transcriptase (Fermentas Life Sciences) according to manufacturer's protocol. The resulting DNA fragments were precipitated with ethanol and separated on a 7% poly-acrylamide/8 M urea denaturing gel. The gel was transferred to Whatman 3 MM paper and vacuum-dried. Radioactivity was visualized by a Typhoon Phosphorimager (GE Healthcare).

2.5. In vitro *RlmH* treatment

Methyltransferase activity of *RlmH* protein on ribosomes with single point mutations in 23S rRNA was tested in vitro. Non-tagged *RlmH* protein was purified as described in [50]. Reaction mixture (25 μl) containing 10 pmol of affinity purified 50S subunits and 10-fold molar excess of *RluD* protein was pre-incubated for 10 min at 37 °C in modification buffer (50 mM Tris/HCl pH 8.0, 100 mM NH₄Cl, 20 mM MgCl₂, and 1 mM DTT). Likewise, 20 pmol of 30S subunits, increasing concentrations of purified *RlmH* protein (final concentration 0.25/0.41/0.6/1.2 pmol/μl), and 100 μM [α-H]-S-adenosyl-L-methionine 1500 dpm/μmol (PerkinElmer) were pre-incubated for 10 min at 37 °C in the same modification buffer (reaction volume 25 μl). Pre-incubation reactions were mixed together and incubate at 37 °C for 1 min.

Reaction products were precipitated with 2 ml of ice-cold 5% TCA. Samples were incubated for 30 min on ice prior to collection on glass fiber filters (Whatman). Filters were washed with 10 ml of 5% TCA, followed by 2 ml of 70% ethanol, and allowed to air dry. Radioactivity was determined by scintillation counting using Optiphase HiSafe III scintillator (PerkinElmer).

2.6. In vitro association of 70S ribosomes

One A_{260} unit of 50S subunits and 10-fold molar excess of *RluD* were pre-incubated for 10 min at 37 °C in the modification buffer
(50 mM Tris/HCl pH 8.0, 100 mM NH₄Cl, 20 mM MgCl₂, and 1 mM DTT). Likewise, one A₂₅₀ unit of 30S subunits was incubated with two-fold molar excess of RlmH and 100 µM S-adenosyl-L-methionine for 10 min at 37 °C in the same buffer (50 µl reaction volume was used for both pre-incubation mixtures). Pre-incubation reactions were mixed together and incubate at 37 °C for 1 min. The samples were subsequently diluted with 900 µl of modification buffer and loaded onto a 10%–25% (w/w) sucrose gradient in the same buffer. 70S ribosomes, and 50S and 30S subunits were fractioned by centrifugation at ω̇t = 2.58 × 10⁻⁶ m/s in a Beckman SW-28 rotor at 4 °C.

3. Results

3.1. Expression and purification of the variant ribosomes

In scope of the current study, a number of single base alterations (A1912U, C1914U, A1916U, A1916C, A1919G, and A1919U, outlined in Fig. 1) were introduced into the H69 region of 23S rRNA. The A1912U, A1919G, and A1919U mutations have previously been shown to severely impair ribosomal functioning in vitro [29,30]. Mutation A1960G, also analyzed in this study, is located in the 23S rRNA stem-loop H71 that forms in part of the inter-subunit bridge B3 [9]. In addition, 23S rRNA containing a GC base pair insertion in the H69 stem region (H69 + GC) (Fig. 1) were analyzed.

We were not able to analyze ribosomes containing 23S rRNA variants A1916G, A1919U, ΔC1908(C/G1922), and H69 + AU (See Experimental procedures) probably due to an assembly defect or instability of the mutant ribosomes. Interestingly, 23S rRNA variant A1916G can be assembled into 50S subunits in E. coli wild-type strain [39]. Absence of RluD protein has been shown to cause ribosome assembly defects in cells [46]. Thus, it is possible that the assembly of specific 23S rRNA variants into ribosomes depends on the interaction of the pre-50S particles with RluD protein.

The 23S rRNA variants containing the aforementioned mutations and a streptavidin-binding aptamer in helix 25 were expressed from an IPTG-inducible plasmid in E. coli double knockout strain ΔrluD/ΔrlmH lacking both the RluD and RlmH enzymatic activities and hence also the corresponding post-transcriptional modifications in H69. It should be noted that the growth rate of ΔrluD/ΔrlmH strain is comparable to E. coli MG1655 strain and it does not display significant ribosome assembly defects (data not shown). To avoid the possible effects due to the incomplete assembly, the variant 50S subunits were isolated from the 70S particles using Streptavidin Sepharose affinity chromatography [56]. The 50S subunits containing 23S rRNA with streptavidin aptamer in helix 25, but otherwise no alterations, were purified identically and are referred to as wild-type (WT) in the present work.

3.2. Effect of the mutations on the specificity of RluD

CMCT/alkali treatment of rRNA extracted from 50S subunits followed by primer extension analysis and separation of cDNAs using poly-acrylamide gel electrophoresis was used to determine the pseudouridines in the H69 region of 23S rRNA [37].

First, the pseudouridine detection range of the CMCT/alkali treatment method was determined by mixing the 23S rRNAs isolated from the ΔrluD/ΔrlmH strain (no modifications in the H69) and from the ΔrlmH strain (pseudouridines present in the H69 but no methylation at position 1915) 50S subunits at different ratios (Fig. 2A). The presence of the reverse transcriptase stop signal on the CMCT/alkali modified RNA lane (+) and the absence of the stop signal on the unmodified RNA lane (−) indicates the occurrence of pseudouridylation at the corresponding position. As the N3 methylation of pseudouridine at position 1915 causes a reverse transcriptase stop signal irrespective of the CMCT/alkali treatment, 23S rRNA of ΔrlmH strain was used. Pseudouridine specific stop signals in H69 can be observed when ΔrlmH 23S rRNA constituted approximately 20% of the total rRNA population (Fig. 2A). Therefore, the detection limit of the CMCT/alkali-reverse transcriptase method is about 20% level of the pseudouridine formation and this method can be considered to be roughly quantitative.

Effect of mutations in 23S rRNA on the activity of RluD in vitro. The presence of pseudouridines in the H69 region was determined by CMCT/alkali treatment of 23S rRNA followed by primer extension analysis and cDNA separation on Urea/PAGE. "+" and "−" lanes correspond to CMCT/alkali treated and "−" lanes to untreated 23S rRNAs. A stronger stop signal on "+" lane as compared to "−" lane indicates the presence of a pseudouridine at a given position. Positions 1911, 1915, and 1917 of E. coli 23S rRNA are shown by arrows. A. 23S ribosomal RNAs extracted from ΔrluD/ΔrlmH lacks RluD-dependent pseudouridines and RlmH-dependent methylation in the H69 and ΔrlmH (RluD-dependent pseudouridines present but lacks the RlmH-dependent methylation in the H69) strains were mixed at different ratios (0%–100% ΔrlmH 23S rRNA and hence 0%–100% ΔrlmH) and analyzed for the occurrence of a pseudouridine specific polymerase stop signal. B. 23S rRNAs harboring the A1912U, C1914U, A1916U, A1916C, A1919G, A1919U, or A1960G mutations were purified from the 50S subunits dissociated from the ΔrluD/ΔrlmH 70S ribosomes and treated with two-fold molar excess of purified RluD protein in vitro. 23S rRNA purified from E. coli wild-type MG1655 strain ribosomes was used as a reference. C. 23S rRNA containing extra GC base pair in H69 helix region (see experimental procedures) was affinity purified from 70S ribosomes and treated 3 h with two times molar excess of RluD protein in vitro. 23S rRNA purified from E. coli MG1655 strain ribosomes was used as a control. Note that the bands corresponding to positions 1911 and 1917 of mutant 23S rRNA are shifted in respect of wt 23S rRNA because of the insertion in H69 helix region.
determined as described above (Fig. 2B). It should be noted that the RluD-directed isomerization of uridines in 50S subunits with the wild-type H69 sequence occurs at significantly lower RluD concentrations within 1 min (kcat 2 min⁻¹) [50]. Therefore, this experiment is designed to detect major effects of the mutations in the H69 on the ability of RluD to make pseudouridines in H69.

Replacement of A1912, C1914, or A1919 with uridine did not lead to a detectable difference in the RluD-directed isomerization of uridines at the native positions 1911, 1915, and 1917 as compared to ribosomes isolated from wild-type MG1655 strain (Fig. 2B). Likewise, the base substitutions A1916C and A1919G did not have a significant effect on the RluD activity in vitro (Fig. 2B). This finding indicates that the mutated bases do not contribute to substrate recognition and pseudouridine formation by RluD on the timescale used. Replacement of A1916 by uridine caused two-fold reduction of pseudouridine formation at all three native positions (Fig. 2B). In our previous in vivo study, the single base replacements A1916G and A1916U in the loop region of H69 affected the ability of RluD to synthesize pseudouridines in H69, thus suggesting that A1916 as an important specificity determinant for RluD in cells [49].

To study whether the length of the stem of H69 is important for the pseudouridylation activity of RluD, 23S rRNA variant (H69 + 6G) with one extra GC base pair in the stem region of H69 (See Experimental procedures) was analyzed. The pseudouridine specific signals were detected at positions 1915 and 1917, albeit at a lower level compared to the wild-type MG1655 23S rRNA (Fig. 2C). Thus, the stem extension of H69 by one base pair does not abolish isomerization of uridines at these positions. In contrast, we did not detect a pseudouridine at position 1911 after 3 h of incubation with two-fold molar excess of RluD (Fig. 2C). It is noteworthy that all of the mutations in the loop regions of the H69 exhibited similar effects on pseudouridylation of all three native positions (1911, 1915, and 1917), while the extension of the stem affects mostly the position 1911. Thus, specificity determinants of RluD are different for uridines at different positions of H69. Moreover, these results suggest that the docking site of RluD is at least partially outside of the loop region of H69.

When the ability of the RluD enzyme to synthesize pseudouridines in H69 other than at the native positions (1911, 1915, and 1917) was analyzed, uridines introduced into the positions 1912, 1914, and 1919 were not isomerized by RluD during the extended (3 h) incubation of 50S subunit variants at 1:2 enzyme concentration (Fig. 2B). In contrast, replacement of A1916 with U resulted in the RluD-dependent synthesis of pseudouridine at that position (Fig. 2B). The level of pseudouridylation at position 1916 is comparable to pseudouridylation levels at native positions (Fig. 2B). As mentioned earlier, introducing U into the position 1916 leads to approximately two-fold reduction in the pseudouridylation levels of all three native pseudouridines. Apparently, aside from the three native substrate uridines, the uridine introduced into the position 1916 of H69 is the only one accessible to RluD. Isomerization of uridine 1916 is unexpected as U1916 of 23S rRNA was not isomerized by RluD in vivo albeit the mutations A1916U and A1916G exhibiting negative effects on the “native” pseudouridine formation in the H69 [49]. Isomerization of U1916 is not caused by the high enzyme concentration used in this experiment, as it occurs also at significantly lower concentrations of RluD (Fig. 3 and data not shown).

The more stringent specificity of the RluD protein in vivo can be a result of the presence of the ribosomal small subunit in cells. We therefore tested the effect of the 30S subunit on the RluD-directed isomerization of uridines at 12 mM and also at 20 mM magnesium ion concentrations which is known to favor subunit association (Fig. 3). The assay was carried out using the A1916U variant 50S subunits and 50S subunits of the ΔrluD/ΔrlmH double knockout strain as a control. The presence of the 30S subunit inhibits the RluD-dependent pseudouridine formation at all three native modification sites on both the ΔrluD/ΔrlmH and the A1916U variant ribosomes (Fig. 3). However, low level of pseudouridylation is still found at the position 1916 (Fig. 3). Thus, the different effect of the mutation A1916U on the RluD specificity in vivo and in vitro is not caused by the ribosomal small subunit.

3.3. Effect of the mutations on the RlmH activity

Following the RluD-directed isomerization of uridines 1911, 1915, and 1917, the pseudouridine at the position 1915 is methylated by RlmH. It has been proposed that in addition to interacting with the H69 region of the large subunit, RlmH also requires interactions with the ribosomal small subunit in order to perform its function [51,52]. However, the exact rRNA elements recognized by RlmH within and beyond H69 have not been identified.

In order to shed light onto the substrate recognition determinants of RlmH, we analyzed the effect of the single base alterations in the H69 region of 23S rRNA on the activity of RlmH protein in vitro. Variant 50S subunits were first treated with 10-fold molar excess of RluD protein for 10 min in order to introduce the pseudouridines (foremost the prerequisite Ψ at position 1915) into the H69. Similarly to RluD, the experimental conditions were designed such as to reveal major effects of the mutations on the activity of RlmH. Methyl group incorporation into the ribosomes was measured at various RlmH concentrations as described in experimental procedures. Efficiency of methylation of 23S rRNA with WT sequence was 80%. Control experiments revealed that the affinity tag used for 50S subunit purification did not affect the efficiency of RlmH-dependent methyl group incorporation (data not shown).

According to our results, all of the alterations introduced into the H69 region had an inhibitory effect on the activity of RlmH in vitro (Fig. 4A–C). The strongest negative effect was observed with replacements at the position 1919; changing A to either G or U rendered the ribosomes very poor substrates to RlmH (Fig. 4C). A strong inhibitory effect was also seen upon replacement of A1912 with U (Fig. 4A). Somewhat less severe inhibition of RlmH activity was seen with replacing A1916 with C and U, and C1914 with U (Fig. 4A and B).

Since we have previously shown that RlmH methylates H69 only in the 70S ribosomes and very likely requires interactions with the
30S subunit for its activity [50]. any alteration that affects the ability of the ribosomal subunits to associate would also be likely to affect the activity of the RlmH protein. We therefore sought to estimate to what extent the impairment in the methyl group incorporation by RlmH on the variant 50S subunits was caused by defects in subunit association as opposed to the effects of the mutations on the structure of H69 per se. To that end, we analyzed the ability of the variant 50S subunits to form 70S ribosomes under the conditions used (20 mM Mg\(^2\+)
concentration) in the methylation assay. The formation of the 70S ribosomes was monitored on a 10%–25% sucrose density gradient. As seen from the gradient profiles, compared to the 50S subunits with wild-type 23S rRNA sequence (WT), the re-association efficiency was decreased with most of the 50S variants that exhibited significant inhibitory effects on RlmH activity (Fig. 4E). The strongest defect in subunit association was observed with the A1919U variant 50S subunit that was also one of the poorest substrates for RlmH in the methylation assay (Fig. 4). The extent of the association defect of the A1919U variant 50S subunits was comparable to the one caused by the A1960G mutation in H71 (located away from the H69 and forming a part of the

Fig. 4. Effect of mutations in the 23S rRNA on the activity of RlmH in vitro and on the association of the ribosome subunits. Affinity purified mutant 1912U, 1914U, 1916U, 1916C, 1919G, 1919U, and 1960G 50S subunits (10 pmol) pre-treated with RluD protein were incubated with increasing concentrations of RlmH protein (pre-incubated with 30S subunits and [\(^3\)H]SAM 1500 dpm/pmol) as described in experimental procedures. Affinity purified 50S subunits with native 23S rRNA sequence were used for comparison (WT). RlmH-dependent incorporation of the methyl group (A–D) and the ability of the variant 50S subunits to form 70S ribosomes (E) were monitored.
inter-subunit bridge B3), which was used as a control for a mutation known to cause an association defect [58]. The results of the subunit re-association assay alone would suggest that the association defect is the main cause of the poor methylation activity of RlmH on the A1919U variant ribosomes. However, while the association defects of the A1919U and A1960G variant ribosomes were comparable, the inhibitory effect of the A1919U mutation on the RlmH activity was significantly higher than that caused by the A1960G mutation (Fig. 4). The slightly lower methylation activity of RlmH on the A1960G variant ribosomes relative to the WT was not due to a lesser amount of pseudouridine at the 1915 position as the A1960G mutations did not inhibit the RluD-dependent pseudouridylation of H69 (Fig. 2B). The lack of correlation between the effects of the mutations in H69 on subunit association on one hand and on the methylation activity of RlmH on the other is further underscored by the A1912U replacement that significantly inhibited RlmH activity but did not affect the association of subunits (Fig. 4A and E). Together, these findings argue against subunit association defect being the primary cause behind the impairment in the methylation activity of RlmH on the H69 variant ribosomes. The impairment in the activity of RlmH may instead be attributed to the altered conformation of the H69 within the variant 70S ribosomes.

4. Discussion

In order to assess the significance of individual bases for guiding the modification enzymes RluD and RlmH to modify their target sites, the effect of mutations at selected positions in the H69 of 23S rRNA on the activities of both enzymes was monitored. Because the co-crystals of RluD and RlmH enzymes with their substrate H69 are currently not available, the structural basis of our results can be discussed only tentatively.

4.1. RlmH

Our results indicate that several nucleotides in the H69 of 23S rRNA are important for the activity of RlmH in vitro (Figs. 1 and 4). While the adenines at the positions 1912 and 1919 seem to be almost a prerequisite for the action of RlmH (Fig. 4A and C), mutations at positions 1914 (Fig. 4A) and 1916 (Fig. 4B) also exhibited a significant effect on the RlmH activity. Interestingly, compared to the 1914 and 1916 positions the most important nucleotides for the activity of RlmH, A1919 and A1912, are more distant to the actual modification site at position 1915 (see Figs. 1 and 5B and D). Whether the RlmH substrate pseudouridine 1915 was synthesized in vivo or in vitro did not affect the methylation efficiency, as ribosomes isolated from ΔrlmH strain behaved similarly to ΔrluD/ΔrlmH strain ribosomes treated with RluD in vitro (data not shown).

One possible explanation for the effects caused by the mutations is to assume that the RlmH protein makes extensive and specific contacts with nearly all of the bases of the H69. This assumption is in apparent conflict with the results of Purta and coworkers [52] who modeled RlmH into the crystal structure of the 70S ribosome. While in their model for the RlmH70S ribosome complex the

Fig. 5. Structure of the loop region of H69. Orientation of nucleotides 1910–1920 (standard E. coli numbering) in the tertiary structure of free 50S subunit of Deinococcus radiodurans (PDB ID 1NKW) (side view on A and top view on C panel) and in the 70S ribosome of Escherichia coli (PDB ID 3R8S) (side view on B and top view on D panel). Pseudouridines at the positions 1911, 1915, and 1917 are shown in bold black lines. Adenosine at the position 1916 is shown in thin black lines. Structures were generated with PyMOL (DeLano Scientific).
RlmH protein indeed interacts with both ribosomal subunits, the contacts with the 30S subunit seem to be much more extensive than the contacts with the substrate 50S subunit [52]. The contacts between RlmH and the 50S subunit are confined mainly to the tip of the H69 and do not seem to involve the nucleotides A1912 and A1919 that form an extension of the stem region [52]. However, our molecular dynamics simulations have shown that mutations at the positions 1912 and 1919 affect the conformation of the H69 [30]. This conformational change in H69 may therefore displace the ψ1915 from its native position, explaining the severe effect of the mutations at the 1912 and 1919 positions on the RlmH-directed methylation in spite of their distance from the actual modification site. Nucleotides A1914 and A1916 in the loop of the H69, whose replacement exhibited less severe effects, can either interact with the RlmH protein directly or, alternatively, be important for the presentation of the ψ1915 in the proper conformation. Taken together, the fact that base replacements at the 1914 and 1916 positions had a smaller impact on the RlmH activity than the mutations at the more distant positions 1912 and 1919 indicates that rather than interacting directly with the nucleosides neighboring its substrate ψ1915, RlmH is sensitive to the conformation of the H69 within the 70S ribosome.

Mutations at the component of the inter-subunit bridge B2a [10], have been shown to severely affect ribosomal subunit association (Fig. 4E) [30]. We have also shown that subunit association is essential for RlmH activity [50,51]. Thus, it is possible that the mutations in the H69 primarily inhibit subunit association and the effect on RlmH activity is a consequence of that. To test this possibility, we analyzed the effect of the mutation A1960G within another inter-subunit bridge (B3) on the methylation activity of RlmH. Previous [58] and present results show that this mutation impedes ribosome subunit association to the same extent as the aforementioned mutations in the B2a (Fig. 4). However, the mutation A1960G has only a mild inhibitory effect on the activity of RlmH (Fig. 4D). In addition, mutation A1912U has a severe effect on RlmH activity but only a moderate effect on subunit association. Therefore, the inhibition of ribosome subunit–subunit interaction cannot be the sole explanation for the severe effect of nucleotide substitutions at A1912 and A1919 on the methylation activity of RlmH. While the exact contribution of the 30S subunit in determining the RlmH substrate specificity remains unknown, a proper conformation of the H69 of 23S rRNA in addition to subunit association is likely necessary for an efficient methylation of ψ1915 by RlmH.

The modeled complex with the 70S ribosomes also suggest that only a minor change in the H69 conformation is required for swinging the target pseudouridine into the active site of RlmH [52]. Such base flipping mechanism is known to be used by other RNA methyltransferases and pseudouridine synthases [59,60].

4.2. RluD

In contrast to RlmH, whose methylation activity was affected by the mutations in H69, majority of those mutations had little impact on the activity of the pseudouridine synthase RluD (Fig. 2). As the mutagenized positions together span a significant part of the H69, it appears that the main specificity determinant of RluD lies outside of the H69. It has been suggested that the N-terminal 54 domain of the RluD protein binds the junction of three helices of 23S rRNA, namely H68, H69, and H70 [48]. This three-way junction may therefore be the determinant of the initial docking of RluD to the large subunit precursor particle. If that is the case, binding of the catalytic domain of RluD to the H69 is only a secondary determinant of specificity, positioning the catalytic cleft of the ribosome-bound RluD in a way that allows an easy access the target uridines in H69. In this scenario, the distance between the initial docking site of RluD and the actual modification sites in H69 would be more important for the activity of RluD than the actual identity of the bases in H69. Extending the stem region of H69 by one basepair strongly inhibits isomerization of U1911, while pseudouridines at positions 1915 and 1917 are still made by RluD, albeit at a reduced level (Fig. 2C). This suggests that on the mutant 50S subunit, the docking site of RluD is not optimal for isomerization of uridines 1915/1917 and uridine at position 1911 is out of reach. Based on the structure of H69 in the 50S subunit [26] our results do not support the base of H69 as the primary docking site of RluD like previously proposed [48].

In contrast to the base replacements at other H69 positions, uridine at position 1916 (in the loop of H69) affects the pseudouridylation activity of RluD (Fig. 2B). This result mirrors the effects of the base replacements at the 1916 position in vivo [49] and indicates that the position 1916 is involved in targeting RluD to its modification sites in H69. As the impairment in the activity of RluD in vivo has been observed with the A1916U and A1916G but not with the A1916C mutation [49], we propose that the extra-cyclic amino group of A and C nucleotide at 1916 makes a contact with RluD during the binding of the catalytic domain of the enzyme. Lack of the hydrogen bond donor in case of G and U makes the corresponding 23S rRNA variants poor substrates for RluD in vivo.

In addition to studying the effects of the mutations in the H69 on the pseudouridylation activity of RluD at its native modification sites, we also analyzed whether uridines introduced into other positions in H69 can be converted into pseudouridines by RluD. In agreement with our previous in vivo results [49], most of the uridines introduced into non-native positions in the H69 could not be isomerized by RluD (Fig. 2B). The only exception was again the position 1916 since pseudouridine formation at this position was detected on the A1916U variant 50S subunits (Fig. 2B). The latter result stands in contrast to our previous in vivo results where RluD was found to be unable to isomerize the uridine at the 1916 position [49]. The difference between in vitro and in vivo results concerning pseudouridylation at the 1916 position can be explained by different reaction conditions in vivo and in vitro. In vivo the post-transcriptional modification of rRNA occurs concurrently with the subunit assembly and the window for introducing the specific modifications is limited [44]. A comparatively longer incubation time and higher concentration of RluD was used in the in vitro pseudouridylation assay as compared to the conditions in the living cells. The residue at the position 1916 is located between the two native substrates for RluD (uridines at the positions 1915 and 1917) and shares their orientation with respect to the backbone of the H69 (Fig. 5A and C). The location and the orientation of the nucleotide at the position 1916 therefore places it close to the catalytic cleft of RluD and is a probable cause why the uridine introduced into this position can be isomerized to pseudouridine, although the efficiency of this modification appears to be low under the more stringent reaction conditions in the cellular milieu [49].

Overall, the observation that the ability of RluD to convert uridines into pseudouridines is largely limited to the positions 1911, 1915, and 1917 in the H69 indicates, that the uridines at those positions share structural features allowing RluD to distinguish them from uridines introduced into other positions of the H69. An inspection of the 50S subunit crystal structures for the placement of the residues in the H69 indeed shows that all the native substrate uridines are located on the side of H69 that faces the peptidyltransferase cleft, whereas residues at the positions where uridines were artificially inserted tend to be located on the opposite side (Fig. 5). The distinctive location of the substrate uridines may be one of the traits that earmark them for the recognition by the catalytic domain of RluD.

The importance of neighboring residues in specifying the site of pseudouridylation has been demonstrated for the pseudouridine
synthases TruB and RluA. In case of TruB, the formation of the $\Psi$55 in tRNA is determined by the neighboring U54:A58 reversed Hoogsteen base pair [60] and a similar base pair between U33 and A36 is crucial for making $\Psi$32 in the anticodon stem-loop of tRNA by RluA [61]. It is unlikely that RluD induces similar A-U reversed Hoogsteen base pair. All native U residues of H69 are isomerized to pseudouridines, and mutation of any of the uridines does not affect RluD activity at other two uridines.

Taken together, the two enzymes synthesizing modifications at the same site in the H69 have different sensitivities to the mutations in the vicinity of their substrate nucleotide(s). Results of the mutational analysis support the view that the substrate recognition elements for these enzymes lie outside of the H69. RlmH enzyme recognizes the 30's subunit and RluD enzyme the 50's subunit outside of the H69.

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References


Further reading
