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Probing Conformational States of Modified Helix 69 in 50S Ribosomes

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Supporting Information

ABSTRACT: The movement of the small ribosomal subunit (30S) relative to the large ribosomal subunit (50S) during translation is widely known, but many molecular details and roles of rRNA and proteins in this process are still undefined, especially in solution models. The functional relationship of modified nucleotides to ribosome activity is one such enigma. To better understand ribosome dynamics and the influence of modified nucleotides on such processes, the focus of this work was helix 69 of 23S rRNA, which contains three pseudouridine residues in its loop region. Ribosome probing experiments with dimethylsulfate revealed that specific base accessibilities and individual nucleotide conformations in helix 69 are influenced differently by pH, temperature, magnesium, and the presence of pseudouridine modifications.

In all living organisms, protein biosynthesis is carried out by ribosomes. The protein biosynthesis core domain is mainly composed of rRNA, whose activity is supported and enhanced by ribosomal proteins.[1] A number of nucleotide modifications are clustered within the functionally important core domain.[2] Although numerous high-resolution crystal structures of ribosomes exist, information regarding ribosome dynamics in aqueous solution is still lacking. In particular, the specific roles of modified nucleotides in regulating these processes remain unclear.

Helix 69 (H69) of the large ribosomal subunit (50S) together with helix 44 (h44) of the small ribosomal subunit (30S) forms intersubunit bridge B2a,[1a] which contains a number of modified nucleotides, including two pseudouridines (Ψ) and one 3-methylpseudouridine ($m^3\Psi$) (Figure 1a).[3] Interactions of H69 with h44 are important for normal ribosome activity, including subunit association,[4a,b] translational fidelity,[4c−e] ribosome recycling,[1d,e] and accurate ribosome translocation.[1f] In order to participate in dynamic functions, H69 should be conformationally flexible and able to adapt to twistlike movements of the ribosome, while still maintaining the key B2a interaction throughout ribosome translation.[5] Conformational variability of H69 is observed in crystal structures of 50S and 70S ribosomes under different experimental conditions.[1] Previous biophysical studies on small 19-nucleotide RNAs representing fully modified *Escherichia coli* H69 showed conformational changes in the loop region upon changing pH from 5.5 to 7.0.[6a] These data were consistent with the following models based on X-ray crystal structures: under low pH conditions, fully modified H69 forms a base-stacked conformation (closed) but a base-flipped conformation (open) at high pH (Figure 1b).[1f,g,6] No such pH-induced conformational change was observed in the corresponding unmodified H69 RNA, suggesting that Ψ plays a role in regulating H69 dynamics.

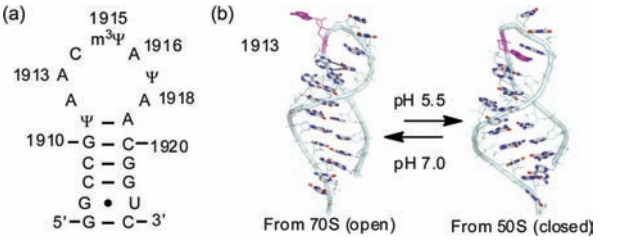

Figure 1. (a) *E. coli* H69 RNA sequence (Ψ is pseudouridine and $m^3\Psi$ is 3-methylpseudouridine). (b) H69 "open" conformation from *E. coli* 70S ribosomes (PDB: 3I1P)[1g] and "closed" conformation from *Deinococcus radiodurans* 50S subunits (PDB: 1NKW).[1f] Previous studies on H69 hairpins suggested that the open conformation was formed at pH 7.0 and the closed conformation was formed at pH 5.5.[6]

Our goal for this study was to determine whether different conformational states of H69 exist on full-length 23S rRNA within the context of 50S subunits, and to understand how these states are influenced by solution conditions. Dimethylsulfate (DMS) probing of positional differences (i.e., exposure or protection) of adenine residues (N1 position) in H69 under different pH, magnesium, and temperature conditions was used to provide information about the relative conformations of nucleotides within H69.[7]

DMS reactivity under various solution conditions was first confirmed to be pH independent by analyzing denatured 23S rRNA (Supporting Information [SI]). Reverse transcription stop sites generated from DMS reactions were determined by using primer binding at nucleotides 1929−1948 and denaturing gel electrophoresis. DMS reactivity was then analyzed on structured 23S rRNA in 50S subunits. DMS probing of wild-type *E. coli* 50S subunits under "on ice" and 1 mM Mg^{2+} conditions revealed clear conformational changes in H69 that were induced by pH; reduced reactivity of DMS at residues A1913 and A1916 (relative changes of 42 and 49%, respectively) at pH 5.5 compared to pH 7.0 was observed, suggestive of reduced exposure to solvent (Figure 2a and b (left panels); blue bars). These changes were independent of salt type.

In contrast, little or no reduction in DMS reactivity was observed at A1912 and A1918 (22 and <10%, respectively) upon lowering of pH. The overall reactivity at A1912 was, however, less than that of A1918 at both pH values, indicating less solvent exposure at either condition. Similarly, quantitative analysis of the gel data did not reveal any major changes at A1918 when the

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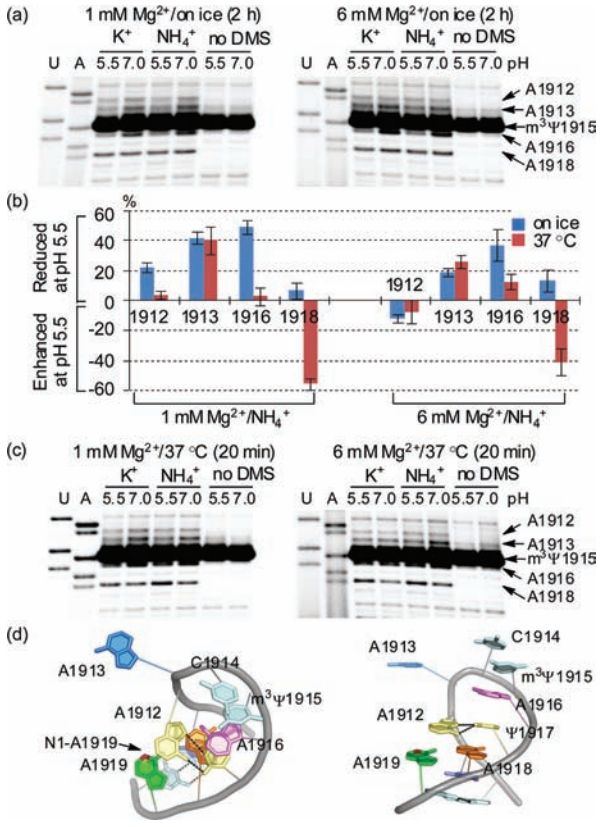

Figure 2. DMS probing on wild-type *E. coli* 50S subunits. (a) Autoradiograms for on ice conditions (50 mM DMS/2 h). DMS sites were determined by reverse transcriptase stop sites. (b) Percent change of reactivity at pH 5.5 relative to pH 7.0. Data were normalized to a nonspecific stop site (1920). The strong stop site at position 1915 occurred due to N3 methylation. Positive values indicate reduced reactivity and negative values reveal enhanced reactivity at pH 5.5 relative to pH 7.0. (c) Autoradiograms for 37 °C reactions (10 mM DMS/20 min). (d) Structures of H69 indicate exposure of A1919 N1 (red atom) from the loop and stem region of H69 (PDB: 3I1P).[1g].

Mg^{2+} concentration was increased from 1 to 6 mM, whereas the A1913 conformational change toward the closed conformation was suppressed (<20% reduction in reactivity as pH is lowered) (Figure 2a and b (right panels); blue bars). This result is consistent with previous biophysical model studies using 2-aminopurine (2AP)-substituted H69, which showed increased exposure of A1913 with increasing pH (2AP at position 1913), but suppression of the switching behavior with increased Mg^{2+}.[6c]

The effects of Mg^{2+} on the A1913 conformational change may be due to competitive metal-ion binding to a site in the H69 loop, which could reduce flipping of residue A1913 to the closed conformation at lower pH. Residue A1912 is even less exposed at higher Mg^{2+} concentrations, and pH-dependent conformational changes at this position are minimal at 6 mM Mg^{2+} (Figure 2a and b (right panels); blue bars), which is consistent with the formation of a reverse-Hoogsteen base pair with Ψ1917, as observed in some crystal structures.[1a,b] In contrast, A1918 in the loop remains exposed at both sets of on ice conditions.

DMS probing of 50S subunits was also carried out at physiological temperature (37 °C) (Figure 2c and b; red bars). DMS reacts faster at 37 °C than at 0 °C, which affects the solution pH due to byproduct formation; therefore, the DMS concentration was lowered to 10 mM, and a shorter reaction time (20 min) was employed. As with the on ice conditions, reactions at 37 °C in 1 mM Mg^{2+} revealed A1913 protection at pH 5.5 and exposure at pH 7.0. In contrast, A1916 showed much different behavior than it did in the on ice experiments; specifically, it showed high reactivity at pH 7.0 as observed at low temperature, but the reactivity was not reduced at pH 5.5 (Figure 2b (left panel); red bar). This result is consistent with NMR experiments on hairpin RNAs in which raising the temperature reduced base-stacking interactions in the loop region at high pH, but had diminished effects at low pH, as well as different effects on A1916 and A1913.[6]

At 37 °C, the behavior of residue A1918 was clearly different than at lower temperature. Unlike the other adenosine residues in H69, A1918 was more reactive (increased exposure to DMS) at low pH and less reactive (protection from DMS) at high pH (55 and 40% enhanced reactivity at pH 5.5 relative to pH 7.0 in 1 mM and 6 mM Mg^{2+}, respectively) (Figure 2b; red bar). To rule out the possibility that lower DMS concentrations or shorter reaction times at 37 °C could result in different reaction profiles at A1918 from that of on ice conditions, two additional probing conditions were employed (10 mM DMS, 2 h, on ice and 50 mM DMS, 1 h, on ice). Neither condition led to any changes in the reaction profile at A1918 (SI). These results lead us to propose temperature-induced conformational changes of H69 that differ from the pH-induced changes.

Our previous thermal melting analyses using synthetic oligonucleotides showed that H69 forms a slightly more stable stem-loop structure at pH 5.5 (T_m: 66.3 °C, ΔG^o_{37}: −5.2 kcal/mol) compared to pH 7.0 (T_m: 65.1 °C, ΔG^o_{37}: −4.7 kcal/mol).[6a] Taking into account the model studies, temperature-induced H69 fluctuations in the 50S subunit could be explained as follows; at low temperatures, A1918 cannot overcome the small energetic barrier to stack inside the loop; whereas at higher temperatures the barrier can be overcome in order for A1918 to move inside of the H69 loop. It is quite possible that within the ribosome, association or interactions with other ribosome components could shift the equilibrium and relative nucleotide positions of H69. This dynamic behavior and range of nucleotide conformational states is not surprising, given the number of biological roles proposed for H69.[4]

In the case of A1919, the base was unreactive toward DMS under all conditions tested, even though it appears from X-ray crystal structures to have its N1 position exposed (Figure 2d). Imino proton NMR analysis of the model H69 stem−loop RNA indicated that A1919 forms a base pair with Ψ1911,[6] which is consistent with the lack of DMS reactivity in 50S ribosomes. Genetic studies with mutations at A1919 show lower translation activity and abnormal 70S formation,[4c] suggesting that A1919 positioning is important for these ribosome functions.[1]

To understand the influence of modified bases (Ψ1911, m^3Ψ1915, Ψ1917) on H69 conformational states, 50S subunits from Ψ synthase-deficient *E. coli* strains (RluD(-) 50S)[8] were employed in DMS-probing experiments. RluD(-) 50S subunits contain U1911, U1915 or m^3U1915, and U1917 in place of Ψs at the corresponding positions in wild-type ribosomes. At position 1915, a strong nonspecific primer extension stop was observed (Figure 3a) due to partial methylation at U1915.[9] The DMS-probing analysis of RluD(-) 50S showed little pH-induced change (<10%) at A1913 at 37 °C (Figure 3a and b; yellow bars). Although there was still a small pH-induced conformational change at A1913 under on ice conditions (21% reduction

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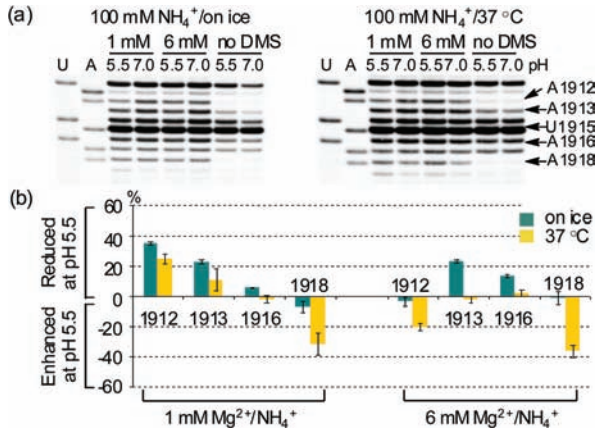

Figure 3. (a) DMS probing of RluD(-) 50S subunits under on ice (left) and 37 °C (right) conditions. (b) Percent change of probing efficiency on RluD(-) 50S subunits at pH 5.5 relative to pH 7.0.

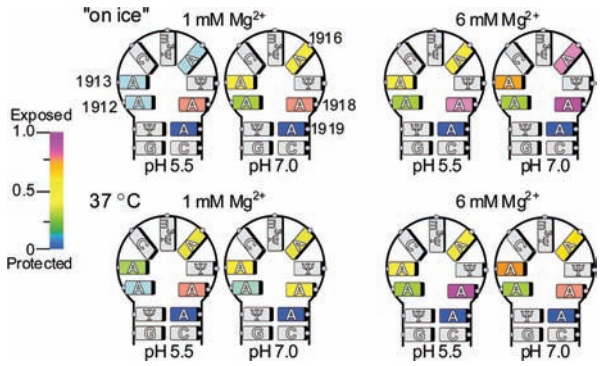

Figure 4. DMS probing maps of wild-type *E. coli* 23S rRNA at H69 with varying solution conditions.

of exposure at pH 5.5) (Figure 3b; green bars), the influence of the Ψ residues on A1913 exposure was clearly observed, especially under 37 °C conditions. At low Mg^{2+}, the same temperature-dependent change in exposure at A1918 was observed in RluD(-) 50S as with wild-type ribosomes, but the effect was diminished by 30% (Figures 2b and 3b; red and yellow bars). At high Mg^{2+}, the same level of enhancement in DMS reactivity at A1918 was observed at pH 5.5 as with wild-type 50S, suggesting that some effects due to Ψ deficiency in 23S rRNA may be overcome by Mg^{2+} binding.

The most noticeable difference between the Ψ-deficient and wild-type 50S ribosomes occurs at position 1916. The conformational change identified in wild-type 50S under on ice conditions is almost completely diminished in RluD(-) 50S. These results are also consistent with NMR and circular dichroism spectroscopic analyses that showed the influences of Ψs on base-stacking interactions in H69.[6] In the model systems, base-stacking interactions in the H69 loop are less favored at high pH when Ψs are present. Similarly, the pH-dependent conformation changes at A1916 are diminished by as much as 40% in RluD(-) 50S at low Mg^{2+} and 20% in high Mg^{2+} concentrations. Position A1912 appears to be more exposed in the Ψ-deficient 50S compared to wild-type ribosomes, which is also consistent with the model studies. In unmodified H69 hairpins, the loop-closing base pair (U1911-A1919) was not observed, which could influence the exposure of A1912 to solvent (and therefore DMS), although the exposure of A1919 in RluD(-) 50S did not change (SI).

DMS probing and previous studies[6] on model RNAs reveal that H69 is structurally dynamic and can exist in multiple conformational states in which the individual nucleotide positions vary. Ψ modifications play a role in organizing the H69 structure. Figure 4 shows a summary of the DMS-probing results on wild-type 50S subunits. The probing results are supported by the fact that mutations (e.g., A1916G) and Ψ-deficient ribosomes are both defective in ribosome assembly.[4c,d] The fact that some nucleotide positions remain constant is also likely important for ribosome function. For example, little or no variability is observed at residues A1912 and A1919, which have important functional roles. Mutations A1912G and A1919G both have strong growth phenotypes and inhibit translation.[4d] The relative position of A1919 is still unclear because crystal structures do not show base-pair formation with this nucleotide (Figure 2d), but an A1919-Ψ1911 pair is observed in solution NMR studies with model RNAs.[6] It is possible that 70S ribosome formation causes rearrangement of the H69 conformation, such that the position of A1919 is altered. Future probing studies on 70S ribosomes may help to clarify this discrepancy between the solution data and X-ray structures.

In summary, DMS probing under different solutions conditions revealed that H69 within 50S subunits can exist in multiple conformational states and Ψ modifications play a role in regulation of these states. A variety of H69 conformation states are expected to exist during translation, suggesting that point mutations, loss of modification, or ligand binding at this site could influence H69 conformation and result in disruption of this process. Such information will be useful for future antibiotic drug development, with specific targeting of these conformational states.

■ ASSOCIATED CONTENT

Supporting Information. Materials and methods, confirmation of pH-independent DMS reactivity, probing data for short reaction times and low DMS concentrations under on ice conditions, and comparison of DMS reactivity of A1918 and A1919. This material is available free of charge via the Internet at http://pubs.acs.org.

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