Pseudouridines in rRNA helix 69 play a role in loop stacking interactions†

Jean-Paul Desaulniers,‡ Yu-Cheng Chang,§ Raviprasad Aduri, Sanjaya C. Abeysirigunawardena, John SantaLucia, Jr. and Christine S. Chow*

Received 24th July 2008, Accepted 1st September 2008
First published as an Advance Article on the web 19th September 2008
DOI: 10.1039/b812731j

The ^{1}H NMR spectra of RNAs representing *E. coli* 23S rRNA helix 69 with [1,3-^{15}N]pseudouridine modification at specific sites reveal unique roles for pseudouridine in stabilizing base-stacking interactions in the hairpin loop region.

Helix 69 (H69) of *E. coli* 23S rRNA is an RNA hairpin motif located at the heart of the peptidyltransferase center of the ribosome, which is proposed to have a functional role in protein translation initiation, translation termination, and/or ribosome recycling.[1] As such, H69 has gained attention as a potential target for the development of new anti-bacterial, anti-infective agents. To date, several X-ray and cryo-EM structures have revealed that H69 adopts significantly different conformations and makes contacts with RNAs, proteins, and small molecules.[1,2] Despite a number of different static structures, little structural information exists for H69 within a solution-based environment. One challenge associated with generating models of H69 for solution studies is the presence of a highly abundant modified residue known as pseudouridine.[3]

Among the more than 100 known RNA modifications in nature, pseudouridine (Ψ) is the most common.[4] Three conserved Ψ residues are present in H69 (Fig. 1) at positions 1911, 1915, and 1917 (*E. coli* numbering). Pseudouridine plays an important role in fine tuning the stability of RNA hairpins and other structural motifs.[5] The most significant effect of pseudouridylation is the introduction of an additional imino group in the pyrimidine ring. Because of the significance of H69 within the ribosome, a primary goal of this work was to determine whether these imino protons contribute to the tertiary structure of H69.

^{1}H NMR spectroscopy is a useful starting point for RNA structure determination because the imino proton region (9–15 ppm) is often the simplest portion of an NMR spectrum to interpret.[6] Typically, the guanine and uracil imino protons that are involved in base pairing are observed (90% H_2O–10% D_2O) and assigned using 1D NOE difference spectroscopy or novel NMR techniques with ^{15}N, ^{13}C-labeled RNAs.[7] Non-traditional hydrogen-bonding patterns between imino resonances can also arise in RNA due to unique stacking interactions and/or structures. Additional imino proton resonances that are not associated with standard base-pairing schemes are much more difficult to assign and often involve complex 2D NMR techniques. Pseudouridine-containing RNAs (Fig. 1) contain additional imino protons that do not participate in traditional base-pairing schemes, but give rise to peaks in ^{1}H NMR spectra. Here, we report on the site-specific incorporation of ^{15}N-enriched Ψs at various positions of hairpin RNAs representing H69 in order to identify imino resonances within the ^{1}H NMR spectrum. By doing so, loop Ψ imino proton resonances in ^{1}H NMR spectra are assigned unambiguously, thus alleviating the need for complex 2D techniques.

In this study, H69 RNAs with ^{15}N-labeled Ψ at three different positions were produced (Fig. 1) by using chemical synthesis. The generation of these RNAs first required the synthesis of ^{15}N-labeled Ψ (see ESI†). Previously, routes were developed for [1-^{15}N]- or [3-^{15}N]-enrichment of Ψ.[8] Here, ^{15}N was incorporated at both the N1 and N3 positions of Ψ in order to examine both imino protons in ^{1}H NMR experiments. Due to the presence of both uridine and Ψ residues in H69, enzymatic synthesis with T7 RNA polymerase could not be used to produce ^{15}N-labeled RNAs.

After ^{15}N-Ψ was generated, it was mixed with a sample of natural abundance (^{14}N-Ψ) such that the level of ^{15}N enrichment was 50%. Therefore, any imino proton attached to Ψ that exchanges slowly with water (N1H and N3H) is observed as a combination of a singlet and doublet peak. The use of a 1 : 1 mixture of ^{15}N : ^{14}N was essential in the assignment of various imino peaks, due to the presence of multiple imino peaks within the region. The mixed-label Ψ phosphoramidite was synthesized (see ESI†) and used to generate three RNA hairpins representing modified H69 bearing ^{15}N-labeled Ψs at each of the three positions, 1911, 1915, and 1917 (Fig. 1).[5d]

The stem-region proton resonances of H69 were previously assigned using 1D NOE difference spectroscopy.[5d] In the case of pseudouridine, the imino resonances are typically assigned based on the presence of an NOE from N1H to H6 (and lack of NOE when NH3 is irradiated). The three new RNAs, ^{15}NΨΨΨ, Ψ^{15}NΨΨ, and ΨΨ^{15}NΨ, show nearly identical 1D ^{1}H NMR spectra at 21 and 37 °C (data shown for ^{15}NΨΨΨ in the ESI†). As the temperature is lowered to 3 °C, several peaks become apparent in the imino region of the spectra (Fig. 2). Since the level of ^{15}N enrichment in labeled Ψs is 50%, the imino protons (N1H and N3H) are each observed as a combination of a singlet and doublet peak (these peaks will be referred to as a triplet). For the ^{15}NΨΨΨ RNA, ^{15}N-enrichment of Ψ_{1911} allows confirmation of the previously assigned resonances of N1H and N3H at 10.3 and 13.1 ppm, respectively. Irradiation of the 10.3 ppm peak gives rise to an NOE at 7.0 ppm, assigned to Ψ_{1911}H6. In the case of Ψ^{15}NΨΨ, peaks at 10.7 and 10.4 ppm, which are broad

Department of Chemistry, Wayne State University, Detroit, Michigan, 48202, USA. E-mail: csc@chem.wayne.edu; Fax: 313-577-8822; Tel: 313-577-2594

† Electronic supplementary information (ESI) available: Synthetic schemes, experimental details, ^{1}H, ^{13}C, ^{15}N, and MS spectra, RNA experimental methods, 1D NOE spectra, and temperature-dependent NMR spectra. See DOI: 10.1039/b812731j

‡ Present address: University of Ontario Institute of Technology, Faculty of Science, 2000 Simcoe Street North, Oshawa, Ontario, L1H 7K4, Canada.

§ Present address: The Methodist Hospital Research Institute, Weill Medical College of Cornell University, 6565 Fannin Street, Houston, Texas 77030, USA.

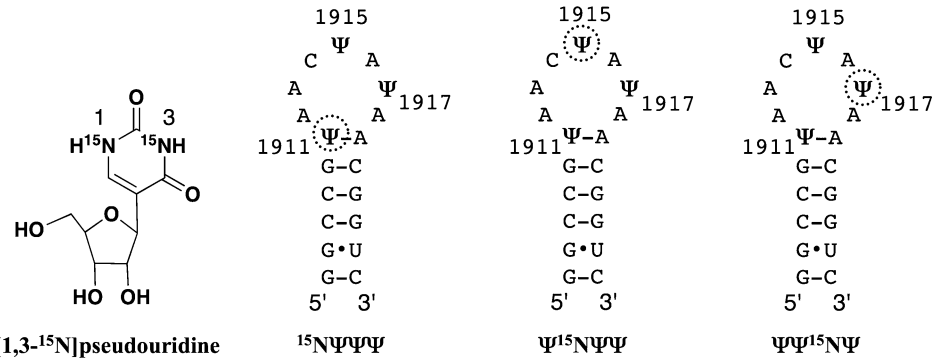

Fig. 1 The structure of pseudouridine (Ψ) and secondary structures of RNA hairpins representing H69 (positions 1906–1924) of *E. coli* 23S rRNA are shown. The nitrogens of Ψ are enriched with ^{15}N, and circles indicate [1,3-^{15}N]Ψ positions in the H69 hairpins.

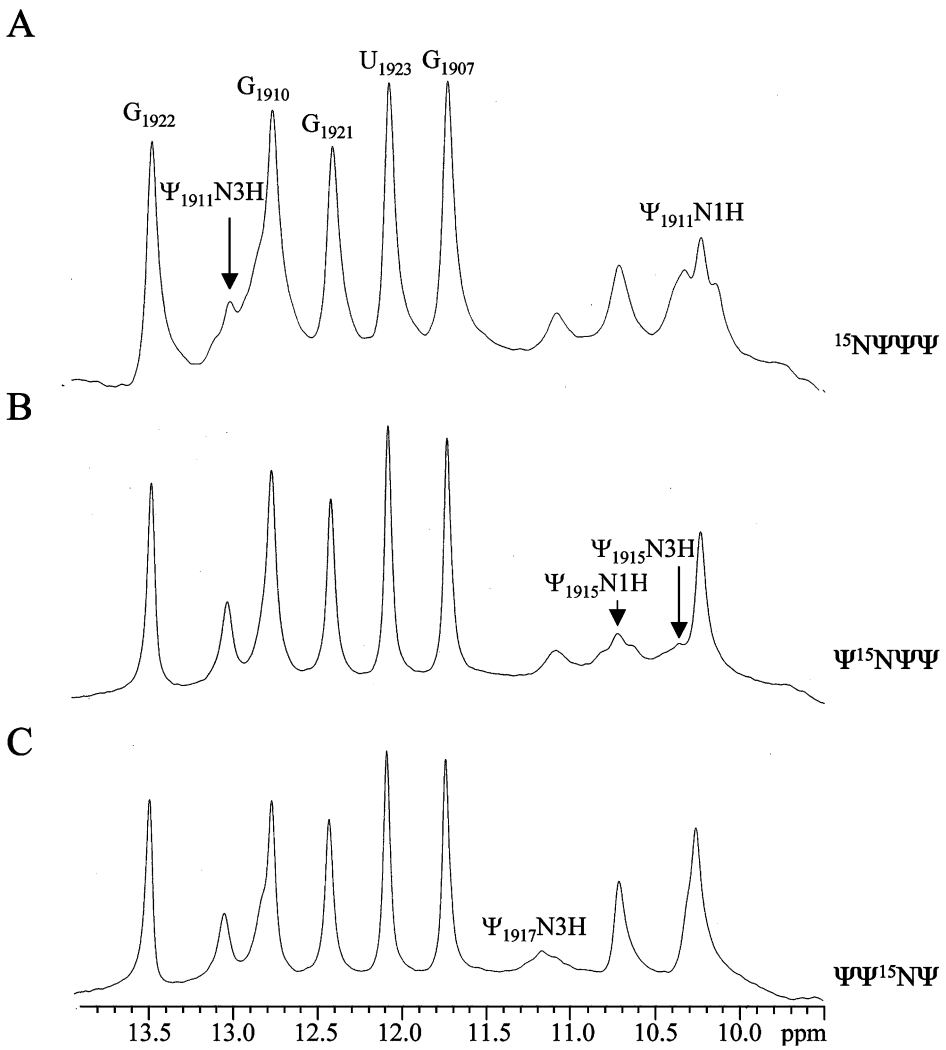

Fig. 2 The 1D imino proton (uridine H3/guanine H1/pseudouridine H1 and H3) NMR spectra at 3 °C (500 MHz) of ^{15}NΨΨΨ (A), Ψ^{15}NΨΨ (B), and ΨΨ^{15}NΨ (C) RNAs (90% H_2O–10% D_2O, 0.5–1 mM) in 30 mM NaCl, 10 mM sodium phosphate, 0.5 mM Na_2EDTA, pH 5.8, are shown.

singlets in ^{15}NΨΨΨ and unlabeled ΨΨΨ spectra, are triplets, indicating that both imino protons of Ψ_{1915} exchange slowly with solvent. The resonance at 10.7 ppm is assigned to Ψ_{1915}N1H, based on the fact that it has a strong NOE to Ψ_{1915}H6 at 7.4 ppm (see ESI†). Therefore, the 10.4 ppm triplet peak is assigned to Ψ_{1915}N3H. This result is confirmed by the ^{15}N HMQC spectrum of Ψ^{15}NΨΨ, which reveals two cross peaks (see ESI†) at 135 (N1) and 159 ppm (N3). These chemical shifts are consistent with earlier studies in which [1-^{15}N]-Ψ had ^{15}N peaks ~132–136 ppm and ^{1}H peaks ~10.4–10.6 ppm.[8a] The ΨΨ^{15}NΨ RNA spectrum has a broad triplet at 11.2 ppm, resulting from ^{15}N labeling of Ψ_{1917}. Irradiation of this resonance does not show any

significant NOEs, suggesting that it corresponds to Ψ_{1917}N3H. In previous NMR studies, resonances between 10.5–10.7 ppm have been assigned to ΨN1Hs.[5] Thus, the assignment of the 10.4 and 11.2 ppm resonances as loop Ψ_{1915}N3H and Ψ_{1917}N3H is unique, and suggests a novel structural environment for these imino protons.

The 1D ^{1}H NMR spectra of ^{15}NΨΨΨ reveal slight upfield shifts (Δppm ~ 0.1–0.2) of several imino proton peaks as the temperature is raised from 3 to 37 °C (ESI†). In contrast, the imino resonances of Ψ_{1915} and Ψ_{1917} are no longer observed when the temperature is increased to 15 °C. These observations are consistent with the H69 loop region undergoing changes in stacking upon heating.

The Ψ imino protons of H69 are clearly observed in the ^{1}H NMR spectra shown in Fig. 2. Typically, imino protons of RNA loops are not observed, unless they are involved with base-stacking or hydrogen-bonding interactions, due to rapid exchange with solvent on the NMR timescale. Of the five Ψ imino proton resonances, those from Ψ_{1911}N1H and N3H are the most prominent. This result is consistent with the formation of an A-Ψ loop-closing base pair through N3H and a putative water-mediated hydrogen bond between N1H and the phosphate backbone, which contribute to H69 stability.[5d] Imino proton resonances from Ψ_{1915}N1H, Ψ_{1915}N3H, and Ψ_{1917}N3H are also observed (Ψ_{1917}N1H is the only Ψ imino proton that is not observed), indicating a specific conformation of the H69 loop that leads to protection of the Ψ imino protons from solvent exchange. Previous work by Davis showed that Ψ stacks favorably with adjacent adenines in a short AAΨA oligomer, and the ΨN1H (but not N3H) resonance was observed.[5b] The presence of multiple adenines in the H69 sequence surrounding Ψ_{1915} and Ψ_{1917} suggests a similar type of stacking interaction as the AAΨA tetramer. However, the Ψ_{1915} of H69 has unique stacking and/or hydrogen-bonding interactions, such that both N3H and N1H are protected from solvent. Although N3H uridine resonances have been observed by NMR in uridine-containing RNAs,[9] the unmodified H69 construct (containing U_{1911}, U_{1915}, U_{1917}) does not show imino resonances from any of the N3H uridines.[5d] Other NMR studies have shown imino resonances in RNA loop regions, but these typically arise from non-standard hydrogen-bonding interactions across loops.[10] In the case of H69, there is no evidence for hydrogen-bonding interactions of Ψs with the other loop residues.

Our data reveal that the extra imino protons in the loop region of the Ψ-containing H69 RNA lead to a structure with increased base stacking and decreased solvent accessibility. Also of note is the fact that the unmodified, or uridine, analog of H69 does not exhibit additional imino proton resonances in 1D ^{1}H NMR spectra.[5d] Therefore, the presence of Ψ residues is clearly important for the formation of a specific tertiary structure of H69. Within duplex regions, the effect of Ψ is well-defined, as ΨN3H participates in hydrogen-bonding interactions with an adenine partner; whereas, ΨN1H has typically been observed to participate in water-mediated hydrogen bonds with the phosphate backbone.[5e,11] However, Ψs in single-stranded regions have been shown either to destabilize or stabilize RNA structures, and this observation depends on their specific locations and sequence contexts.[5d] In this case, specific conformations in the H69 loop lead it to be inaccessible to solvent exchange on the NMR timescale (ms). We present a model in Fig. 3 that is based on the available X-ray crystal structures,[1,2] in which modified nucleotides on the 3′ side

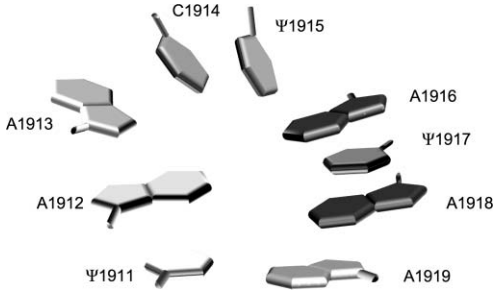

Fig. 3 A schematic representation of the H69 loop region is shown with base-stacking interactions between C_{1914} and Ψ_{1915} and A_{1916}, Ψ_{1917}, and A_{1918}, and base-pairing interactions between Ψ_{1911} and A_{1919}.

of the H69 loop participate in significant stacking interactions with A_{1916}. Evidence for this stacking model is also observed in solution NMR studies of the ΨΨΨ RNA 3D structure (see ESI† for a list of the key NOEs).[12] More specifically, the NOE data is consistent with strong stacking of C_{1914} with Ψ_{1915} and tri-nucleotide stacking with A_{1916}, Ψ_{1917}, and A_{1918}. Such a structure may be important for fine-tuning the biological function of H69. For example, O'Connor and Dahlberg showed that mutations in H69 (deletion of A_{1916} and insertion of additional adenosines after A_{1916}) cause frame-shifting and read-through of stop codons.[13]

The site-specific incorporation of [1,3-^{15}N]-enriched Ψ at positions 1911, 1915, and 1917 of H69 has allowed for the unambiguous assignment of the imino proton resonances in the 1D ^{1}H NMR and 1D NOE difference spectra, and determination of non-Watson–Crick loop interactions that are dependent on the presence of the modified nucleotide. The ^{15}N-enriched RNAs will be used for future ^{15}N NMR experiments, including complete structure analysis of H69, determination of Ψ pK_a values in H69, and monitoring of H69–ligand interactions.

Acknowledgements

We are grateful to Bashar Ksebati and Larry Clos II for helpful discussions and technical assistance. This work was supported by the NIH (GM54632).

Notes and references

1 (*a*) M. M. Yusupov, G. Zh. Yusupova, A. Baucom, K. Lieberman, T. N. Earnest, J. H. D. Cate and H. F. Noller, *Science*, 2001, **292**, 883; (*b*) B. S. Schuwirth, M. A. Borovinskaya, C. W. Hau, W. Zhang, A. Vila-Sanjurjo, J. M. Holton and J. H. D. Cate, *Science*, 2005, **310**, 827; (*c*) M. Selmer, C. M. Dunham, F. V. Murphy IV, A. Weixlbaumer, S. Petry, A. C. Kelley, J. R. Weir and V. Ramakrishnan, *Science*, 2006, **313**, 1935; (*d*) I. K. Ali, L. Lancaster, J. Feinberg, S. Joseph and H. F. Noller, *Mol. Cell*, 2006, **23**, 865.

2 (*a*) R. K. Agrawal, M. R. Sharma, M. C. Kiel, G. Hirokawa, T. M. Booth, C. M. T. Spahn, R. A. Grassucci, A. Kaji and J. Frank, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 8900; (*b*) D. N. Wilson, F. Schluenzen, J. M. Harms, T. Yoshida, T. Ohkubo, R. Albrecht, J. Buerger, Y. Kobayashi and P. Fucini, *EMBO J.*, 2005, **24**, 251; (*c*) S. Petry, D. E. Brodersen, F. V. Murphy IV, C. M. Dunham, M. Selmer, M. J. Tarry, A. C. Kelley and V. Ramakrishnan, *Cell*, 2005, **123**, 1255; (*d*) A. Weixlbaumer, S. Petry, C. M. Dunham, M. Selmer, A. C. Kelley and V. Ramakrishnan, *Nat. Struct. Mol. Biol.*, 2007, **14**, 733; (*e*) M. A. Borovinskaya, R. D. Pai, W. Zhang, B. S. Schuwirth, J. M. Holton, G. Hirokawa, H. Kaji, A. Kaji and J. H. D. Cate, *Nat. Struct. Mol. Biol.*, 2007, **14**, 727; (*f*) R. D. Pai, W. Zhang, B. S. Schuwirth, G. Hirokawa, H. Kaji, A. Kaji and J. H. D. Cate, *J. Mol. Biol.*, 2008, **376**, 1334.

3 F. F. Davis and F. W. Allen, *J. Biol. Chem.*, 1957, **227**, 907.

4 (*a*) J. Ofengand, *FEBS Lett.*, 2002, **514**, 17; (*b*) M. Charette and M. W. Gray, *IUBMB Life*, 2000, **49**, 341.
5 (*a*) C. S. Yarian, M. M. Basti, R. J. Cain, G. Ansari, R. H. Guenther, E. Sochacka, G. Czerwinska, A. Malkiewicz and P. F. Agris, *Nucleic Acids Res.*, 1999, **27**, 3543; (*b*) D. R. Davis, *Nucleic Acids Res.*, 1995, **23**, 5020; (*c*) P. C. Durant and D. R. Davis, *J. Mol. Biol.*, 1999, **285**, 115; (*d*) M. Meroueh, P. J. Grohar, J. Qiu, J. SantaLucia, Jr., S. A. Scaringe and C. S. Chow, *Nucleic Acids Res.*, 2000, **28**, 2075; (*e*) M. I. Newby and N. L. Greenbaum, *RNA*, 2001, **7**, 833.
6 B. Fürtig, C. Richter, J. Wöhnert and H. Schwalbe, *ChemBioChem*, 2003, **4**, 936.
7 (*a*) J.-P. Simorre, G. R. Zimmermann, A. Pardi, B. T. Farmer, II and L. Mueller, *J. Biomol. NMR*, 1995, **6**, 427; (*b*) V. Sklenár, T. Dieckmann, S. E. Butcher and J. Feigon, *J. Biomol. NMR*, 1996, **7**, 83; (*c*) J.-P. Simorre, G. R. Zimmermann, L. Mueller and A. Pardi, *J. Biomol. NMR*, 1996, **7**, 153; (*d*) J.-P. Simorre, G. R. Zimmerman, L. Mueller and A. Pardi, *J. Am. Chem. Soc.*, 1996, **118**, 5316.
8 (*a*) R. H. Griffey, D. Davis, Z. Yamaizumi, S. Nishimura, A. Bax, B. Hawkins and C. D. Poulter, *J. Biol. Chem.*, 1985, **260**, 9734; (*b*) J.-P. Desaulniers, B. Ksebati and C. S. Chow, *Org. Lett.*, 2003, **5**, 4093.
9 K. M. Koshlap, R. Guenther, E. Sochacka, A. Malkiewicz and P. F. Agris, *Biochemistry*, 1999, **38**, 8647.
10 (*a*) F. M. Jucker and A. Pardi, *Biochemistry*, 1995, **34**, 14416; (*b*) M. Sundaram, P. F. Crain and D. R. Davis, *J. Org. Chem.*, 2000, **65**, 5609; (*c*) M. Sundaram, P. C. Durant and D. R. Davis, *Biochemistry*, 2000, **39**, 12575; (*d*) H. Zhang, M. A. Fountain and T. R. Krugh, *Biochemistry*, 2001, **40**, 9879.
11 (*a*) J. G. Arnez and T. Steitz, *Biochemistry*, 1994, **33**, 7560; (*b*) M. I. Newby and N. L. Greenbaum, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 12697.
12 R. Aduri, Ph.D. Thesis, Wayne State University, Detroit, MI, 2007, pp. 151–201.
13 M. O'Connor and A. E. Dahlberg, *J. Mol. Biol.*, 1995, **254**, 838.