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Measurement of chemical exchange between RNA conformers by ^{19}F NMR

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ABSTRACT

Many noncoding RNA molecules adopt alternative secondary and tertiary conformations that are critical for their roles in gene expression. Although many of these rearrangements are mediated by other biomolecular components, it is important to evaluate the equilibrium relationship of the conformers. To measure the spontaneous interconversion in a bi-stable RNA stem loop sequence into which a single ^{19}F-uridine label was incorporated, a ^{19}F–^{19}F EXSY experiment was employed. The kinetic exchange rate measured from EXSY experiments for this system was 37.3 ± 2.8 s^{-1}. The advantage of this approach is that exchange kinetics can be monitored in any RNA sequence into which a single ^{19}F nucleotide is incorporated by commercial synthesis. This method is therefore suitable for application to biologically significant systems in which dynamic conformational rearrangement is important for function and may therefore facilitate studies of RNA structure–function relationships.

1. Introduction

Functional RNA molecules are often very plastic and may undergo changes in base pairing patterns, thus forming alternative secondary and tertiary conformations. This property is critical for multiple roles of some noncoding RNA molecules in gene expression [1]. For example, analysis of the chemical exchange rate for conformational interconversion is important for understanding ribozyme-mediated catalysis [2] as well as RNA-ligand interactions associated with riboswitch activity [3] and drug design [4]. In order to understand mechanisms of RNA-mediated activity, it is essential to probe the kinetics and thermodynamics associated with conformational rearrangement of functional RNA molecules. Here, we report the use of a new approach using two-dimensional NMR studies to determine rates of chemical exchange between RNA conformers.

Dynamic motions observed for RNA molecules span a wide range of timescales [5] from picoseconds [6] to hundreds of seconds [7]. Rapid motions, including librations, interhelical motions, and base flipping, have a significant impact on binding small molecules, proteins, or other RNA molecules, *e.g.* [8]. However, interconversion between alternative conformations, including those from lowest energy states to higher energy states and secondary structural transitions [5], which typically have timescales in the millisecond to second range, are of great interest as they are directly implicated in biological processes such as ribozyme activity [8].

Kinetics of the RNA folding landscape over a wide range of time scales has been analyzed by a number of experimental methods. Analysis of kinetics of the forward reaction induced by external stimuli such as light [9] or addition of an ion [10] or ligand [11] has been measured by hybridization with complementary RNAs [7], chemical structure probing [12], single molecule FRET [13,14], and NMR spectroscopy [15].

However, measurement of individual lifetimes associated with interconversion between alternative RNA folds in equilibrium in the bulk phase is more difficult as a result of relatively short lifetimes as well as the reversible nature of the interconversion. Solution NMR offers an excellent approach for investigation of dynamics of such exchange reactions because the timescale (ms → sec) is within the NMR detection range. For example, the dynamics of a bi-stable RNA were probed by longitudinal exchange and CPMG relaxation dispersion NMR spectroscopy monitoring a single ^{13}C-methyl label [16], and recent work from Al-Hashimi and coworkers discovered a low abundance (~0.4%) transient RNA conformer with a long lifetime (~2 ms) by use of ^{13}C and ^{15}N R1ρ relaxation dispersion experiment [17]. However, because of the ease of incorporating a single ^{19}F-labeled nucleotide by commercial chemical synthesis, the demonstrated lack of structural or energetic perturbation associated with the ^{19}F [18,19] as compared with the methyl group, and large spectral dispersion

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and sensitivity to chemical environment, monitoring of ^{19}F has unique advantages.

In this paper, we demonstrate the application of ^{19}F-NMR to observe a single substituted ^{19}F-pyrimidine in a bi-stable RNA molecule that adopts two conformations in the equilibrium state, and probe the dynamic parameters of conformational interconversion between conformers. Our data illustrate the application of this new approach, which can be readily applied to analysis of the timescale of conformational interconversion in biologically significant systems.

2. Materials and methods

2.1. Sample preparation

The RNA oligomer 5′-GAAGGCAACUUCGG(^{5}FU)UG-3′, into which a single 5-^{19}F-uridine (U) was incorporated, was purchased from Dharmacon and deprotected according to their protocols. The sequence was modified from that used by Kreutz et al. [19] to form shorter stems in each of the two major conformations and thus to facilitate more rapid interconversion. The gel purified bi-stable sample was heated to 90 °C, rapidly cooled on ice for 5 min, dried, and re-suspended in 95% H_2O/5% ^2D_2O (Cambridge Isotope Laboratories). The buffer used for NMR experiments contained 5 mM NaP_i (pH 6.5), 50 mM NaCl, and 0.1 mM EDTA. Sample concentration was approximately 0.53 mM.

2.2. Acquisition parameters

2.2.1. 1D ^{19}F NMR

One-dimensional ^{19}F NMR experiments were performed on a Varian INOVA 500 MHz (spectrometer frequency is 470.220 MHz for ^{19}F) spectrometer equipped with a broadband probe. Spectra were collected at 25 °C on the bi-stable RNA sample with similar acquisition parameters as described previously for the U2–U6 snRNA complex [20] and referenced with external neat trifluoroacetic acid (TFA) to −78.5 ppm. Briefly, the ^{19}F excitation pulse length was set to 15 μs, spectral width was 60015.0 Hz, acquisition time was 1.063 s, and relaxation delay was 7.5 s. The number of scans was 8000 and experiment time was ~19 h.

2.2.2. 2D ^{19}F–^{19}F EXSY experiments

Two-dimensional ^{19}F–^{19}F EXSY experiments of the bi-stable sequence were performed on the same Varian INOVA 500 MHz spectrometer equipped with a broadband probe as used for 1D ^{19}F NMR experiments. EXSY spectra with different mixing times ranging from 2 to 760 ms were collected at 25 °C. ^{19}F excitation pulse length was 15 μs, spectral width was 10527.7 Hz, acquisition time was 0.195s, and relaxation delay was 2.5 s. The number of points was 4096 × 200. The number of scans was 200 and experiment time was ~24 h.

3. Results

The bi-stable RNA sequence adopts two conformations characterized by GCAA or UUCG tetraloops, in which the 5-^{19}F labeled uridine (U) is located in a single- or double-stranded environment, respectively (Fig. 1). The two environments result in ^{19}F peaks of chemical shifts at −165.2 ppm and −169.0 ppm, respectively, assigned according to published values [18] with a distribution of 0.57:1 at 25 °C (Fig. 2). Observation of sharp peaks at the predicted locations implies that interconversion between conformers is in the slow exchange realm of the NMR timescale. Observation of exchangeable imino ^1H peaks corresponding to the G·A and G·U pairs in the proposed tetraloops is consistent with formation of the two conformations (data not shown).

In order to characterize the kinetics of the interconversion, time-dependent ^{19}F–^{19}F exchange was measured in a series of 2D EXSY spectra [15,21,22] with mixing times ranging from 2 ms to 760 ms (Fig. 3A). These spectra exhibit a build-up and decay of cross peaks (Fig. 3B).

We observed no evidence of any intermediate between the two conformers in either ^1H or ^{19}F spectra, which would have been detected by additional peaks or relatively broader peaks with respect of the molecular weight of this molecule. We therefore applied a two-state exchange model to analyze the rates of exchange between each of the major conformations. The intensity of the auto (diagonal) peaks for the two states and the exchange peaks between them can be expressed by the following equations [15,22]:

$$I_{SS}(T) = I_S(0)\{-(\lambda_2 - a_{11})e^{-\lambda_1 T} + (\lambda_1 - a_{11})e^{-\lambda_2 T}\}/(\lambda_1 - \lambda_2) \quad (1)$$

$$I_{DD}(T) = I_D(0)\{-(\lambda_2 - a_{22})e^{-\lambda_1 T} + (\lambda_1 - a_{22})e^{-\lambda_2 T}\}/(\lambda_1 - \lambda_2) \quad (2)$$

$$I_{SD}(T) = I_S(0)(a_{21}e^{-\lambda_1 T} - a_{21}e^{-\lambda_2 T})/(\lambda_1 - \lambda_2) \quad (3)$$

$$I_{DS}(T) = I_D(0)(a_{12}e^{-\lambda_1 T} - a_{12}e^{-\lambda_2 T})/(\lambda_1 - \lambda_2) \quad (4)$$

where $a_{11} = R_s + k_{SD}$, $a_{12} = -k_{DS}$, $a_{21} = -k_{SD}$, $a_{22} = R_D + k_{DS}$, and $\lambda_{1,2} = 1/2\{(a_{11} + a_{22}) + [(a_{11} - a_{22})^2 + 4k_{SD}k_{DS}]^{1/2}\}$. R_S and R_D represent the relaxation rate of single-stranded and double-stranded conformations, respectively. k_{SD} and k_{DS} are kinetics of the exchange.

A simultaneous non-linear least squares fit to Eqs. (1)–(4) was applied to extract the exchange rates, from which we obtained the values of $k_{SD} = 24.2 \pm 1.8\ s^{-1}$, $k_{DS} = 13.1 \pm 1.0$ s, where k_{SD} is the rate constant of interconversion from single-stranded 5F-U to double-stranded 5-F-U and k_{DS} is the rate constant of the reverse reaction. The equilibrium constant $K_{eq} = k_{DS}/k_{SD} = 0.54{:}1$, and the exchange rate $k_{ex} = k_{SD} + k_{DS} = 37.3 \pm 2.8$ s. Other extracted parameters are shown in Table 1.

Fig. 1. Proposed secondary structures of the bi-stable RNA sequence. The 5-^{19}F labeled uridine (U, in red) was in a single- (A) or double-stranded (B) environment, depending upon the fold. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

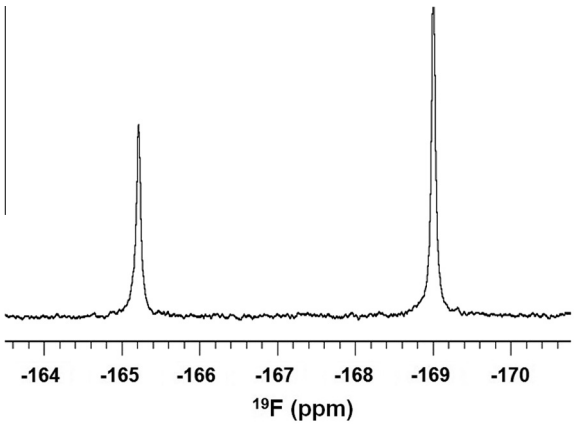

Fig. 2. One-dimensional ^{19}F spectrum of the bi-stable sequence shown in Fig. 1. The downfield peak at −165.2 ppm corresponds to the structure in Fig. 1A and the upfield peak at −169.0 ppm corresponds to the fold in Fig. 1B.

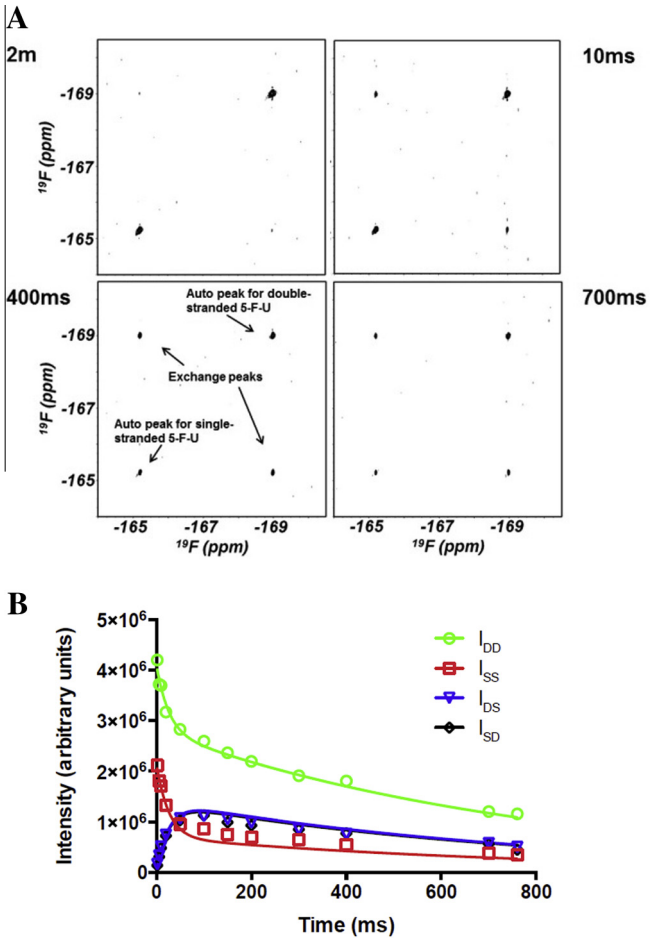

Fig. 3. Representative two-dimensional ^{19}F–^{19}F EXSY spectra and plot of intensities vs mixing times. (A) Two-dimensional ^{19}F–^{19}F EXSY spectra at mixing times of 2 ms, 10 ms, 400 ms and 700 ms, as marked. Spectra were acquired at 470.2 MHz. Auto- and cross peaks are labeled in the figure. (B) Plot of the intensities of exchange peaks vs. mixing times (2–760 ms) from which exchange values were calculated. I_{DD} and I_{SS} represent intensities of the auto peaks for double-stranded 5-^{19}F-U and single-stranded 5-^{19}F-U, respectively; I_{SD} and I_{DS} represent the intensities of exchange peaks from "S" to "D" and vice versa. Note that the curves corresponding to I_{DS} and I_{SD} are overlapped. Fitting of the curve was achieved using Prism 6 suite (GraphPad Software Inc.) with in-house written scripts.

Table 1
Extracted parameters from 2D ^{19}F–^{19}F EXSY experiments of bi-stable sample.

$I_S(0) * 10^6$	$I_D(0) * 10^6$	R_S (s^{-1})	R_D (s^{-1})	k_{SD} (s^{-1})	k_{DS} (s^{-1})
2.2 ± 0.7	4.1 ± 0.6	3.0 ± 1.4	0.4 ± 0.7	24.2 ± 1.8	13.1 ± 1.0

4. Discussion

The calculated K_{eq} value is in close agreement with the value we measured from a fully relaxed condition in a 1D ^{19}F spectrum (I_S/I_D = 0.57:1). Also, the rates we have extracted imply that the interconversion between the two states was cooperative. This latter finding is consistent with the result by ^{13}C NMR studies on a related sequence [16], which (as in this case) did not display any evidence of a fully open single-stranded state or other intermediates. These findings indicate that the ^{19}F–^{19}F EXSY experiment is a valid approach to measure the kinetics of dynamic exchange between RNA conformations in equilibrium.

The bi-stable RNA stem loop examined here represents a single model RNA sequence; however, we note that this same approach can be applied to a wide range of RNA sequences in order to gain further insight into the dynamics and thermodynamics associated with RNA folding and function. Such examples may include, but not be limited to, studies of small nuclear RNAs, ribozymes, and riboswitches.

^{19}F NMR has been widely used in study of structure and dynamics of biological molecules, such as proteins [23] and nucleic acids [18,19] and, more recently, to probe the secondary structure of a large RNA complex of human spliceosomal RNA [20]. Compared to the traditional NMR experiments that observe ^{1}H, the ^{19}F nucleus is far more sensitive to its environment because it has 19 electrons, resulting in greater chemical shift dispersion and environment-dependent change than observed for ^{1}H, and thus simplifies spectral analysis. Substitution of a single ^{19}F in the 5 position of a pyrimidine base by solid phase synthesis permits monitoring of the local environment of that nucleotide. NMR measurement of the ^{19}F-labeled pyrimidine does not involve any two-bond H–F couplings, and three-bond H–F couplings are within the line width of the peak; thus no decoupling is necessary. It is noted that at higher field strength or for larger biomolecules, chemical shift anisotropy may contribute to peak broadening. However, the high sensitivity of the ^{19}F nucleus (83.3%), which is similar to that of ^{1}H and much greater than that of ^{13}C (1.59%) or ^{15}N (0.104%), makes it a good candidate for NMR observation.

In conclusion, we have demonstrated the application of ^{19}F NMR spectroscopy to probe the dynamics of RNA conformational exchange. The novelty, experimental advantage, and impact of this approach is the ability to determine kinetic rates of RNA conformational exchange from data derived from ^{19}F spectra, using oligomers with single ^{19}F label made by commercial nucleic acid synthesis. This technique provides a valuable and unambiguous means to study rearrangement of functional RNA associated with biochemical function, and can readily be applied to biologically significant RNA systems.

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