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Proper NMR methods for studying RNA thermometers

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Abstract In some pathogenic bacteria, there are RNA thermometers, which regulate the production of virulence associated factors or heat shock proteins depending on temperature changes. Like a riboswitches, RNA thermometers are located in the 5'-untranslated region and involved translational gene regulatory mechanism. RNA thermometers block the ribosome-binding site and start codon area under the 37℃ within their secondary structure. After bacterial infection, increased the temperature in the host causes conformations changes of RNA, and the ribosome-binding site is exposed for translational initiation. Because structural differences between open and closed forms of RNA thermometers are mainly mediated by base pairing changes, NMR spectroscopy is a very useful method to study these thermodynamically changing RNA structure. In this review, we briefly provide a fundamental function of RNA thermometers, and also suggest a proper NMR experiments for studying RNA thermometers.

Keywords RNA thermometer, NMR methods, thermodynamic structure

Introduction

RNA plays diverse roles in the cell, including RNA processing, transcription, translation, and regulations. Among these roles, regulations are related to sensory RNAs, which can sense intracellular signals such as proteins, nucleic acids, small molecules, pH, and temperature, all of which can coordinate RNA structure.[1-3] RNAs have their own structures and fold into functional forms by adapting to their environmental conditions. Therefore, to study this structure-function relation is a key to understanding living systems.

In recent years, two kinds of regulatory RNAs possessing sensory function have been reported. One is riboswitches which interact with the small metabolite,[4, 5] the other is RNA thermometers which respond to temperature.[6] Both structured RNAs function by structural alterations, and involve in non-protein mediated regulation.

Riboswitches are typically present in the 5'-untranslated regions (5'-UTRs) of bacterial mRNAs that code for proteins related to biosynthesis or expression of bacterial virulence. Riboswitches consist of aptamer domains, high-affinity binding module with their specific ligands such as a vitamin, nucleotide, or amino acid, and determine ON or OFF operation for translation by structural rearrangement. OFF state of riboswitches masks the ribosomal binding site so called Shine-Dalgarno (SD) sequence.[5] Binding of ligands to the aptamer domain induces RNA conformational changes, which makes riboswitches ON state by opening the SD sequence.

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There are many researches about riboswitches, which mentioned ligand-binding structural studies using NMR or X-ray methods.

In this review, we focus on the other structured RNA regulator, RNA thermometers, which show similar aspects with riboswitches. RNA thermometers are also located in the 5'-UTRs of regulated genes, and also hinder the ribosome binding site (SD sequence) as well as the start codon area at low temperature. (Figure 1. (A)) However, unlike with riboswitches, RNA thermometers do not bind any molecules, but respond only to temperature changes. As temperature elevates to 37℃, the base pairing of RNA helices become unstable gradually, the ribosome binding site is exposed,[6, 7] which can initiates the translation of proteins. (Figure 1. (B)) This gradual manner also differs to the On-Off mechanism of riboswitches. Overall summary of riboswitch and RNA thermometer is shown in Table 1. [8]

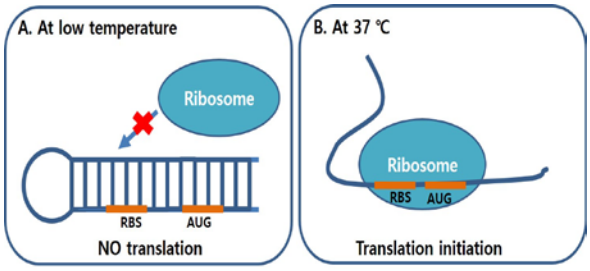

Figure 1. Operation mechanism of RNA thermometers. (A) At low temperature, ribosome binding site is blocked by stable base pair of RNA (no translation). (B) At 37℃, ribosome binding site is exposed by breaking base pairs of RNA (translation initiation).

For pathogenic bacterial, increasing temperature to 37℃ is an intuitive signal that means successful invasion to a mammalian host, and upregulation of heat shock protein is continued to survive in warm-blooded host organisms. [9, 10] It should be noted that many heat shock proteins of bacteria play a critical role for survival and virulence in the host. [11] And other virulence associated genes that contain RNA thermometers are also induced spontaneously.

Using NMR spectroscopy, a few cases of structural studies about RNA thermometers are reported. A well-known RNA thermometer, ROSE (Repression Of heat Shock gene Expression) from *Bradyrhizobium japonicum* was reported by Saheli Chowdhury et al. in 2006.[7] And other classes, the fourU RNA thermometer from *Salmonella* species[12, 13] and HSP17 RNA thermometer in cyanobacteria were thoroughly investigated at the base-pair resolution. [14] Interestingly, only NMR-based methods were used for analyzing the structure of RNA thermometers, not by X-ray crystallography. Both NMR spectroscopy and X-ray crystallography are the most useful tools for studying secondary or tertiary structure of biomolecules, but NMR is much more appropriate to thermodynamic data analysis. It is due to the fact that NMR spectroscopy is a unique technique providing the atomic-level structural information and dynamics of biomolecules in a liquid state.

Table 1. Comparing RNA thermometer with riboswitch. UTRs* - untranslated regions, RBS** -ribosome binding site

	RNA thermometers	Riboswitches
Location	In the 5'UTRs* of mRNA, upstream of protein coding gene	In the 5'UTRs* of mRNA, upstream of protein coding gene
Function	Regulation of translation by blocking the RBS** at < 37℃	Regulation of translation by blocking the RBS** when operation is OFF
Operation mechanism	Gradual conformational change as temperature rises, opening RBS sequence and start codon	ON or OFF operation with ligand or without ligand, opening RBS sequence when ligand binding
Binding partner	None	Small metabolites such as vitamins, nucleic acids
Sequence conservation	None	Sequence conservation in aptamer region

In the following text, we briefly summarize about the target selection, sample preparation and suitable NMR experiments for RNA thermometers. Understanding RNA thermometers of pathogenic bacteria based on structural analysis is might be helpful to overcome multidrug-resistant bacteria

problem.

Target selection and sample preparation

One of the most characteristic features of RNA thermometers distinguished from other biomolecules is that RNA thermometers have little or no sequence conservation.[8] Therefore, it is difficult to select or predict their presence from sequence based search. So, target selection is completely dependent on the previously discovered sequence. Recently about dozens of RNA thermometers have been studied through *in vivo* or *in vitro* experiments, and there are some well-organized review papers. [3, 6, 8] These works are directly useful for sample selection. In addition, some challenging methods for predicting or engineering RNA thermometers are developed based on bioinformatics strategies and physicochemical characteristics of known RNA thermometers.[15, 16] Very recently, using high-throughput sequencing, as known as Next Generation Sequencing (NGS) methods (so called RNA structurome) was also proposed to detect the presence of novel RNA thermometers.[2, 17] However, these approaches should be considered carefully because of the lack of experimental data.

Because NMR spectroscopy has a rather low sensitivity, a large amount of homogenous sample is required. *In vitro* transcription with T7 RNA polymerase using DNA template methods are usually proper for massive production of RNA except for the small RNA molecules. Chemical synthesis is recommended for the RNAs with less than 10 nucleotides in length. Since the smallest length of identified RNA thermometer has approximately 30 nucleotides, *in vitro* transcription method is recommended for preparation RNA thermometer samples. The general procedure of *in vitro* transcription with T7 RNA polymerase is described in our previous work by Kim et al. at some length.[18]

A large scale of transcription (could get milligram quantities) can be performed with enough DNA template, T7 RNA polymerase, transcription buffer, 2.5 mM DTT, 10-30 mM $MgCl_2$ and 2 - 4 mM rNTPs. The reaction mixture is incubated for 6-8 hours at 37℃, and reaction is quenched by adding EDTA at the same equivalent of Mg^{2+} ions. The ethanol precipitation is performed overnight at -20℃ after addition of 3M sodium acetate buffer. Following the centrifugation and resuspension, the acquired RNA is purified using 10-20% polyacrylamide denaturing gel made with 6-8 M urea. The target RNA band is cut and eluted in the elutrap system. Then, the filtered RNA from previous step is purified with an anion exchange column. Finally, the RNA has to be refolded into a homogenous form through heating and cooling of diluted RNA, and the optimal buffer and concentration for NMR experiments are controlled with centrifugal membrane tubes. In case of ^{13}C and ^{15}N labeled RNA production, the ^{13}C and ^{15}N labeled rNTPs are used instead of non-labeled rNTPs in the transcription procedure.[19]

NMR methods for RNA

NMR methods for studying the structure and dynamics of RNA molecules and their binding with proteins, small molecules, metals, and nucleic acids have been developed in recent decades. Although a lot of experiments are investigated up to date, most widely used experiments could be simply classified into four categories.[20]

a. RNA resonance assignment

First of all, the most essential step of NMR methods is the resonance assignment of RNA molecules. The protons from nucleobases are comprised of HN (imino H1/H3 in G/U, exchangeable), NH2 (amino H41/H42 in C, H61/62 in A, H21/H22 in G, exchangeable), H2 (in A), H5 (in C/U), H6 (in C/U), and H8 (in A/G). The protons from sugar region are H1', H2', H3', H4' and H5'/H5''. A series of NMR spectra are required for assignment of protons. 2D NOESY experiment is the most widely used for assignment, and also give information of secondary structure in helical region of RNA from the sequential walks in the imino region (H1/H3(N) to H1/H3(N+1), 9-15 ppm) and base-sugar region (H6/H8 to H1', 5-9 ppm). H2 (in A) and H5(C/U) can be assigned in 2D NOESY. The correlation between

H5 (in C/U) and H6 (in C/U) is well-shown in 2D TOCSY spectrum. For the ambiguous proton assignment, the isotope labeling methods are very useful. The uniformly labeled or base-selective ^{15}N, ^{13}C labeled, and deuterated RNA samples are additionally measured and analyzed. The assignment for the sugar moiety (H1',H2',H3', H4', and H5',/H5'') can be achieved by optimized 2D or 3D HCCH-COSY, HCCH-TOCSY, 3D NOESY HSQC and 3D HCP- TOCSY with ^{31}P spin system.

b. Base-pairing information of RNA

The signal range of imino proton resonance is very distinguishable from other protons, shown in 9 to 15 ppm. Because the exchangeable protons could be protected by hydrogen bond formation (so called, Watson-Crick base pairs) in water, the number of appeared signals from imino region is closely related to the number of base pairs. The imino protons are clearly shown in this area of the NMR spectra, and very useful for analyzing secondary structure based on base pairing information. The homonuclear 2D NOESY experiment is the first approach to assign imino protons sequentially. In the spectrum, various strength of cross peaks are observed depending on the distances between two protons. Moreover, it is possible to distinguish Watson-Crick G-C, A-U, and non-canonical base pairings (e.g. G-U wobble base pairs) using NOE information. The chemical shifts of H1 imino proton of G from G-C pairs generally are shown on 12-13.5 ppm, H3 of U from A-U pairs on 13-15 ppm, and H1, H3 from G-U wobble pairs on 10-12 ppm.[21, 22] Direct hydrogen bonding information could be easily recognized by analyzing 2D ^{1}H-^{15}N HSQC and ^{1}H-^{15}N HNN COSY experiments developed based on the through-space $^{2H}J(N,N)$ coupling correlation.[23, 24]

c. Local and global conformation of RNA

In the solution state, there is a conformational equilibrium on RNA molecules. The various factors such as temperature, salt, pH value, and concentration are correlated with RNA conformation. In some cases, two separate data sets co-exist in the NMR spectra from one homogenous sample. This phenomenon is because RNA molecules are in slow exchange on the NMR time scale. Slow or fast exchange is influenced by the rate relative to chemical shift difference between the sets of resonance. So, the local conformation could be determined by chemical shift deviation based analysis. Through the ^{3}J (H, H) coupling constants measurement from 2D DQF-COSY, the sugar conformation is determined. The dihedral angles in RNA (α, β, γ, δ, ε, ζ, χ, P, ν) can also be calculated with homo- and heteronuclear coupling constants(^{3}J) and cross-correlated relaxation rates. [20] Residual dipolar coupling (RDC) provides global structural information from IPAP-HSQC experiments. RDC is also useful for structure calculation and RNA dynamics.[25]

d. Interacting sites of RNA with other molecules

Many RNA molecules perform their own function by interacting other molecules such as proteins, other RNAs, small metabolites or metal ions. The structural configuration of the RNA-ligand complexes could be significant for developing drug candidate. Solving a complete structure of complex is the best way to study their structure, but it is impossible sometimes due to their physicochemical properties. The most traditional method is the observation of chemical shift perturbation of the RNA upon titration of the ligand. 2D ^{15}N- or ^{13}C HSQC experiments are most widely used for analysis. Cross-saturation experiments is an alternative method to study binding interfaces of RNA-ligand complex.[26]

NMR methods for RNA thermometers

Among the 4 categories described in above paragraph, base pairing information is most suited for studying RNA thermometers since RNA thermometers sense environmental temperature by thermo-stability of base pairs. The melting point of hydrogen bond from each base pair is directly related with *in vivo* activities of RNA thermometers.[27] At the low temperature, ribosome binding site and start codon area of RNA thermometers are protected with stable

base pairs. As temperature rises up to the active temperature, hydrogen bonds are broken, and the unstable base pairs expose the ribosome binding site gradually. Therefore, observation of the imino protons from the each base pair with gradual temperature change is the key to study RNA thermometers. As described in our text, analyses of 1D imino spectra, 2D ^{1}H-^{1}H NOESY, 2D ^{1}H-^{15}N HSQC, and ^{1}H-^{15}N HNN COSY experiments with temperature variation is required to explain how RNA thermometers change their conformations at the atomic level.

Conclusion

In the previous works, there are four papers which contain structural analysis of RNA thermometers. In all four papers, various RNA thermometers were thoroughly studied at the base-pair resolution by NMR spectroscopy, not by X-ray based study. Especially, 1D imino spectra, 2D ^{1}H, ^{1}H NOESY, 2D ^{1}H, ^{15}N HSQC, and ^{1}H ^{15}N HNN COSY experiments are used in common in all four papers.[7, 12-14] NMR spectroscopy is highly optimized to understand RNA thermometers. Other thermo-basic methods, such as, CD (circular dichroism), DSC (differential scanning calorimetry), or UV thermal melting studies are also useful. Regulation of heat shock protein and virulence factors is very essential for pathogenic bacteria survival in host infection. Because RNA thermometers have no binding pocket, it is thought that RNA thermometers have no relationship with drug development. However, recently, medical treatments of genetic manipulation such as stem cell therapy, or CRISPR based therapy are in the spotlight. Understanding RNA thermometers of pathogenic bacteria based on atomic level structural analysis might be helpful to develop alternative treatment for multidrug-resistant bacteria problem.

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