NMR Spectroscopy

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Ligand-Detected Relaxation Dispersion NMR Spectroscopy: Dynamics of preQ$_1$–RNA Binding**

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Abstract: *An NMR-based approach to characterizing the binding kinetics of ligand molecules to biomolecules, like RNA or proteins, by ligand-detected Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion experiments is described. A ^{15}N-modified preQ$_1$ ligand is used to acquire relaxation dispersion experiments in the presence of low amounts of the Fsu class I preQ$_1$ aptamer RNA, and increasing ligand concentrations to probe the RNA small molecule interaction. Our experimental data strongly support the conformational selection mechanism postulated. The approach gives direct access to two parameters of a ligand–receptor interaction: the off rate and the population of the small molecule–receptor complex. A detailed description of the kinetics underlying the ligand binding process is of crucial importance to fully understanding a riboswitch's function and to evaluate potential new antibiotics candidates targeting the noncoding RNA species. Ligand-detected NMR relaxation dispersion experiments represent a valuable diagnostic tool for the characterization of binding mechanisms.*

A common mechanism in biological systems to pass on information or to trigger a specific event is the binding of a ligand to a host molecule. Detailed knowledge of the binding event between the ligand and the biopolymer is a crucial prerequisite for shedding light on the associated follow-up effects induced by the interaction of the two molecules. The recently discovered riboswitches, a class of noncoding RNAs which is involved in a protein-free gene regulation mechanism are a striking example of small-molecule binding to a biomolecular receptor.[1] Their function is based on divergent RNA folding pathways, which are selected by the cellular concentration of a metabolite. In the presence of a ligand the aptamer domain of the riboswitch selectively binds the small molecule with high affinity and leads to a signalling effect, which is passed to the expression platform of the mRNA, thus leading to either a down- or up-regulation of the gene under control. Thus, the ligand-binding mechanism is the crucial determinant as it is at the heart of the regulation process. For the growing RNA chain in transcriptionally acting riboswitches, a delicate balance between being responsive to the ligand for a certain time period and being able to fold into another conformational state has to be maintained and depends on the cellular metabolite concentration. Knowledge of the kinetics of the ligand-binding process is thus vital for fully understanding a riboswitch's function.[2] Furthermore, ligand-binding kinetics represent a benchmark for the potential of small molecules as novel RNA-targeting antibiotics, as the off-rate is directly correlated to the mean lifetime of the drug–target complex.[3] Herein, we report on an NMR-based approach which gives insights into the process of ligand recognition.

As a proof of concept system we picked the *Fsu* preQ$_1$ class I aptamer, which we have previously investigated using fluorescence and NMR spectroscopy (Figure 1 a).[2c,4]

We and others found evidence that the transcriptionally acting preQ$_1$ aptamer domain of *Fusobacterium nucleatum*

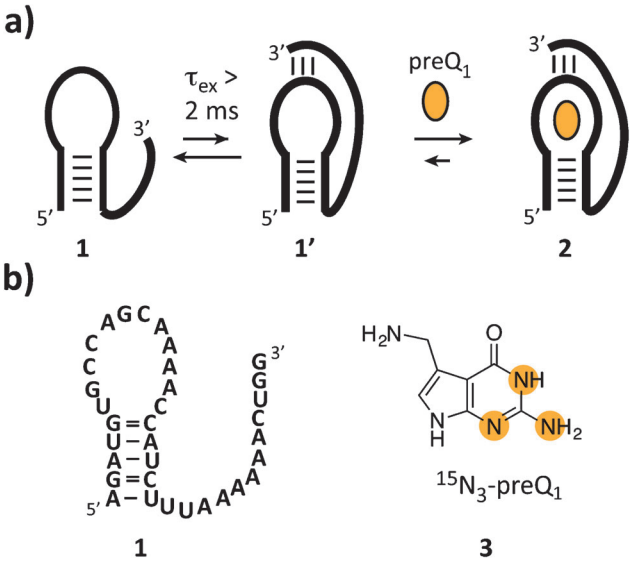

Figure 1. a) Proposed mechanism for the ligand recognition of the *Fsu* preQ$_1$ class I aptamer. The free RNA interconverts between a 5′-hairpin fold (**1**) and a pseudoknotted state (**1′**) which binds preQ$_1$. b) Secondary structure of **1** and ^{15}N-preQ$_1$ (**3**) which were used in this study for ligand-detected CPMG RD experiments. Orange circles represent ^{15}N nuclei. The synthesis of ^{15}N-labeled preQ$_1$ was reported elsewhere.[5]

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(and the closely related preQ$_1$ class I aptamer from *Bacillus subtilis*) very likely utilizes a conformational selection mechanism to recognize its ligand, preQ$_1$.[1g] By using NMR spectroscopy in combination with site-specific ^{15}N-RNA labeling we could show that the free aptamer interconverts between a 5′-stem-loop fold (**1**) and a pseudoknotted conformation (**1′**), with the latter being able to specifically recognize the ligand. In the absence of a ligand, separate resonance sets were observed for **1** and **1′**, suggesting that interconversion between the two folds is slow on the NMR chemical shift time scale. The reanalysis of the original data set suggests half-lives for the individual folds in the millisecond time regime (> 2 ms), and is based on the chemical shift difference of the resonances for N(3)–H of U20 in **1** and **1′**, that is, 0.55 ppm (^{1}H) and 1.17 ppm (^{15}N) at 298 K in the presence of Mg^{2+} ions and at 600 MHz proton larmor frequency.[4]

Here, we report on an NMR-based approach which enables the description of the ligand-binding process for the *Fsu* preQ$_1$ class I aptamer with unprecedented precision. We use ligand-detected Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion (RD) NMR spectroscopy as a function of ligand concentration to obtain insights into the binding kinetics at unperturbed equilibrium. RD NMR experiments have proven to be a useful tool for addressing biomolecular dynamics in the microsecond to millisecond regime. In favorable cases, important insights into the kinetics, thermodynamics, and structural features of conformational transitions between a ground state and transiently populated excited states can be obtained.[6] The common approach to studying biomolecular dynamics relies on stable ^{13}C/^{15}N-isotope labeling of the target protein or nucleic acid, followed by NMR relaxation experiments to detect and quantify (ligand binding) dynamics on various time scales.[7] Here, we propose an "out of the box" approach using a ligand-detected CPMG RD experiment to determine the kinetics of the ligand-binding process.[8]

In our setup, isotope-labeled preQ$_1$ **3** is employed as an experimental probe while unlabeled the 34 nt *Fsu* class I preQ$_1$ aptamer **1** is present only at low concentration (Figure 1 b). We explored ^{15}N labeling of preQ$_1$ since the exocyclic amino group of preQ$_1$ directly interacts with RNA in the preQ$_1$–RNA complexes by base-pairing with a cytosine.[9] As a consequence, the exocyclic amino group of preQ$_1$ displays substantially different ^{15}N-chemical shifts in the free and bound states, which is a prerequisite for RD experiments. In ^{15}N-filtered ^{1}H NMR spectra of the unbound **3** only a single resonance is visible, corresponding to the exocyclic $^{15}NH_2$ moiety (see Figure S1 a in the Supporting Information). A series of one-dimensional $^{15}NH_2$ CPMG RD experiments of **3** was acquired (with an experimental time of 0.5–1.5 h per CPMG field) at ligand/RNA concentration ratios ranging from 1:0.08 to 6:0.08 mM (Figure 2 and see Figure S1 in the Supporting Information).[10] We chose to vary the concentration of **3** rather than that of the RNA to minimize errors during sample preparation (e.g., pipetting errors). It is evident from Figure 2 that the ligand/RNA ratio modulates the ^{15}N-relaxation dispersion profiles, as the amplitude of the observed dispersion profile increases upon changing the ligand/RNA ratio from 6:0.08 to 1:0.08. For the ligand alone, a flat profile is obtained. The clear-cut ligand/RNA dependence strongly supports the fact that we indeed observe the ligand binding of **3** towards its cognate aptamer **1** in our assay. A negative control experiment using binding-incompetent RNA was performed to verify our experimental setup (see Figure S2 in the Supporting Information).

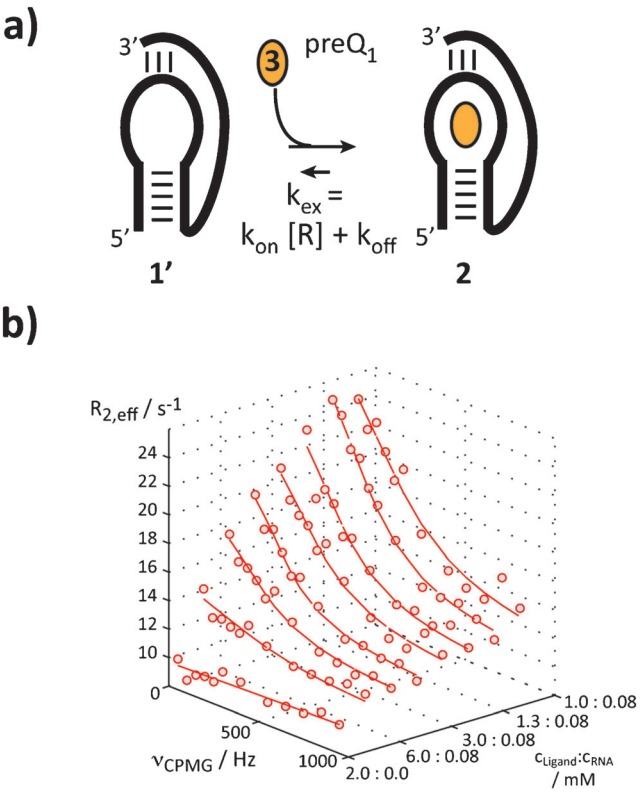

Figure 2. a) Ligand-binding subprocess, for which the kinetics were observed by one-dimensional $^{15}NH_2$ CPMG RD experiments of **3**. b) Relaxation dispersion profiles at 50 MHz ^{15}N larmor frequency for the exocyclic amino group of ^{15}N-modified preQ$_1$ (**3**). In the absence of RNA a flat relaxation dispersion profile is observed and $R_{2,eff}$ equals the intrinsic $^{15}NH_2$ CPMG relaxation rate of unbound preQ$_1$ (10 s^{-1}). In the presence of **1**, the dispersion profiles display a pronounced dependence on the ligand/RNA concentration ratio. Experimental data (dots) are shown, along with lines representing two-state numerical fits.

From the relaxation dispersion data at variable ligand/RNA concentration ratios it is possible to extract kinetic parameters for the ligand binding process.[11] Of note, for the ligand-based experimental setup the number of adjustable parameters to be included in the fitting procedure is limited: The intrinsic $^{15}NH_2$ CPMG relaxation rate of unbound **3** is directly accessible from the flat relaxation dispersion profile of unbound **3** (Figure 2 and Supporting Figure S3), while the ^{15}N-chemical shift difference for **3**, between the free and bound conformation, can be determined with ^{1}H ^{15}N HSQC experiments (Figure 3).

In a numerical two-state exchange fit of the seven non-flat relaxation dispersion profiles an off rate [$k_{off} = (1483 \pm 76)\ s^{-1}$] was determined, along with values of $k_{on} \times [R]$ (where k_{on} and [R] are the on rate and the concentration of

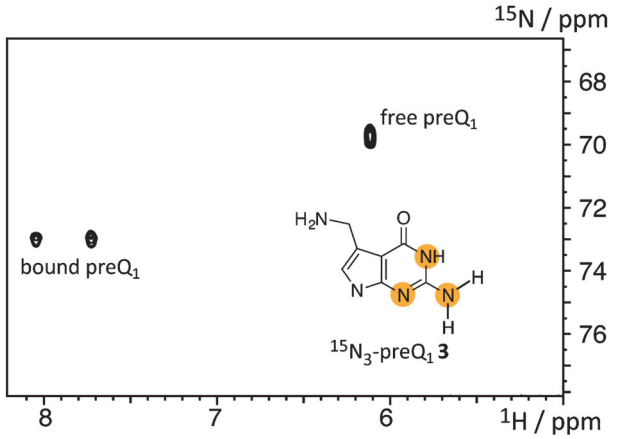

Figure 3. Ligand binding of **3** to **1**. Overlay of ^{1}H ^{15}N HSQC spectra for **3** in the free and in the RNA-bound state. The ^{15}N-chemical shift difference of $preQ_1$'s exocyclic amino group in the free and bound conformation amounts to a down-field shift of 3.23 ppm. A detailed description of the spectrum in the bound state is given in the Supporting Information (see Figure S4). Experimental conditions: 0.1 mM **3** in 10 mM sodium cacodylate (pH 6.4), 2 mM $MgCl_2$, plus 2 equivalents of RNA for the bound state $preQ_1$ spectrum.

free RNA, respectively), and the ^{15}N-transverse relaxation rate of bound **3** [$(51 \pm 6)\ s^{-1}$]. Values of the population of **3** which is bound to RNA, $p_{bound} = k_{on} \times [R]/(k_{on} \times [R] + k_{off})$, are given in Table 1. As expected, variation of the ligand/RNA ratio from 1:0.08 to 6:0.08, (i.e. an increase in excess of **3**), is accompanied by a measurable decrease of p_{bound}. It is evident from the experimental data, however, that the RD-derived

Table 1: Results from one-dimensional ^{15}N CPMG RD experiments of **3** in the presence of 80 μM **1**.

c_{Ligand}/c_{RNA} [mM][a]	p_{bound} [%][b]	p_{bound}^{Lit} [%][c]
6.0:0.08	0.7 ± 0.02	1.4
4.0:0.08	1.4 ± 0.02	2.0
3.0:0.08	1.9 ± 0.04	2.7
2.7:0.08	2.3 ± 0.04	3.0
1.3:0.08	2.5 ± 0.06	6.2
1.1:0.08	3.2 ± 0.08	7.3
1.0:0.08	3.3 ± 0.06	8.0

[a] Ligand/RNA concentration ratios of $preQ_1$ and *Fsu* aptamer. [b] Population of $preQ_1$ ligand that is bound to RNA, as determined from ligand-detected NMR RD experiments. [c] Population of RNA-bound $preQ_1$ calculated from K_D^{app} (283 nM).[2c]

p_{bound} values are systematically below the values which are calculated from the apparent dissociation constant (K_D^{app}) of the $preQ_1$ binary complex with the *Fsu* $preQ_1$ aptamer, a value which is available in the literature ($K_D^{app} = 283$ nM).[2c] K_D^{app} was determined using fluorescently labeled RNA, where both components of the fold pre-equilibrium, **1** and **1′**, contribute to the experimentally observed RNA fluorescence. In our ligand-detected CPMG RD approach, however, only the transition between the free ligand molecule $preQ_1$ and the binary complex of $preQ_1$ with the aptamer is monitored. The fold pre-equilibrium between **1** and **1′**, described by the rate constants k_r and k_{-r} (Figure 1), does not contribute to the relaxation dispersion data. However, if only one component of the fold pre-equilibrium (the pseudoknotted conformer **1′** in Figure 1) is capable of binding the ligand, p_{bound} values which are derived from the ligand-detected RD data are smaller than the K_D^{app}-derived values, p_{bound}^{Lit}.[12] Our ligand-detected RD data therefore support the proposed conformational selection mechanism of ligand binding to the *Fsu* class I $preQ_1$ aptamer.

Moreover, this data illustrates that the p_{bound} values which are obtained from the ligand's dispersion profiles can be employed as a diagnostic tool for the ligand-binding mechanism. In case of a lock and key mechanism, p_{bound} values from the dispersion experiment and values which are calculated from K_D^{app} should be equal. However, in the case of conformational selection, a lower (as found here) or in case of induced fit mechanism, a higher p_{bound} value should be obtained by ligand-detected RD, because the ligand can be "stored" in the form of complexes that are not susceptible to dissociation.[12] In addition, the k_{off} rate constant which is accessible by ligand-based RD measurements is a highly desired quantity for drug development, as the lifetime ($1/k_{off}$) of the drug–target complex, relates to the drug–target residence time.[3a]

Our experimental setup relies on low amounts of an unlabeled biomolecule. This aspect is a particular strength of the methodology as isotope labeling of biomolecules can be very labor-intensive and expensive. In addition, because RNA can tend to homodimer formation, the requirement of only low concentrations represents another advantage of the methodology. While we have used ^{15}N-labeled ligand to record data on a room-temperature probe, we are confident that unlabeled ligands can be used in combination with currently available high-standard NMR hardware (e.g. > 800 MHz NMR with a cryogenic probe).[8c] As ^{15}N or ^{13}C CPMG RD experiments for small-ligand molecules can be recorded in a one-dimensional manner, datasets at natural abundance can be obtained in a reasonable amount of time on such NMR systems. This feature renders the approach an attractive alternative to other biophysical methods, like surface plasmon resonance (SPR) or fluorescence spectroscopy, for which labeling/modifications steps or a more elaborated sample handling (e.g. use of a stopped-flow apparatus) are necessary.[13] Moreover, the time resolution in the millisecond regime in some experiments can be limiting, whereas by means of relaxation dispersion it is possible to characterize processes with lifetimes on the order of milliseconds and below.

Taken together, we have presented an NMR-based approach to directly explore the binding of a small-ligand molecule to its macromolecular receptor. Contrary to the established experimental setup, that is, isotope labeling and NMR detection of macromolecules, we used ligand-detected experiments to probe the dynamics which are associated with the binding of a ligand ($preQ_1$) to its biomolecular receptor ($preQ_1$ class I aptamer) in unperturbed equilibrium. The CPMG RD profiles we acquired for $preQ_1$ at varying ligand/receptor concentration ratios directly reflect the association/

dissociation kinetics of the binding event. From these experiments, the intrinsic k_{off} rate of the binary complex is accessible, along with its population. Because $1/k_{off}$ is a measure of the lifetime of the complex, and as such is strongly dependent on the nature of the interactions between ligand and receptor, this parameter is of particular importance for drug design. The powerful NMR methodology provides, in combination with other experimental techniques that yield apparent dissociation constants, like isothermal titration calorimetry, an effective means to obtaining deeper insights into ligand binding processes.

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