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Investigating the malleability of RNA aptamers

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ABSTRACT

Aptamers are short, single-stranded nucleic acids with structures that frequently change upon ligand binding and are sensitive to the ionic environment. To achieve facile application of aptamers in controlling cellular activities, a better understanding is needed of aptamer ligand binding parameters, structures, intramolecular mobilities and how these structures adapt to different ionic environments with consequent effects on their ligand binding characteristics. Here we discuss the integration of biochemical analysis with NMR spectroscopy and computational modeling to explore the relation between ligand binding and structural malleability of some well-studied aptamers. Several methods for determining aptamer binding affinity and specificity are discussed, including isothermal titration calorimetry, steady state fluorescence of 2-aminopurine substituted aptamers, and dye displacement assays. Also considered are aspects of molecular dynamics simulations specific to aptamers including adding ions and simulating aptamer structure in the absence of ligand when NMR spectroscopy or X-ray crystallography structures of the unoccupied aptamer are not available. We focus specifically on RNA aptamers that bind small molecule ligands as would be applied in sensors or integrated into riboswitches such as to measure the products of metabolic activity.

1. Introduction

Aptamers hold great promise as probes in a variety of sensors and as unique intracellular receptors such as in riboswitches. Although antibodies can compete with aptamers as probes in many situations, they cannot be applied inside cells due to their inability to properly fold in the intracellular reducing environment. This identifies a unique role for aptamers in the rapidly growing field of synthetic biology [1]. But, the use of aptamers in synthetic riboswitches and other regulators requires a good understanding of their structures, particularly as they relate to function [2]. Major challenges in applying aptamers as intracellular regulators are in understanding the structures of these small RNA molecules alone and when integrated into larger molecules like mRNAs, how the aptamer structures change with ligand binding and how aptamers can be harnessed for regulating cellular functions in metabolic engineering and in synthetic biology.

The current gold standards for molecular structure analysis are X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR). These biophysical approaches effectively produce well-resolved images of aptamers at the atomic scale. However, small RNAs are known to be structurally flexible, which is an important characteristic of their interactions with ligands. Molecular mobility at time resolutions from nanoseconds to seconds can be observed by NMR [3–6], but this requires specialized instrumentation and expertise. Consequently, NMR and X-ray crystallography are mostly used to determine ensemble structures of small RNAs. The general approach has been to identify conditions that promote the majority of molecules to adopt a resolvable structure that can be viewed as an ensemble of related structures. However, many of these chosen conditions are quite different from the conditions that have been used for measuring aptamer binding activities and are different from the intracellular environment in which the aptamer is to be applied [7]. Here we discuss several reliable experimental methods for measuring the strength of aptamer–ligand interactions and methods of simulating aptamer structures computationally. We also show how the ionic environment of aptamers can have large effects on aptamer structure and function and demonstrate some examples of the benefit of integrating biochemical, biophysical and computational approaches to better understand aptamer structural malleability and ligand interaction.

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2. Results and discussion

2.1. Methods for determining aptamer structure

Most measurements of aptamer structure provide an average of a variety of related structures adopted by the aptamer. In addition to X-ray crystallography and NMR, SHAPE [8,9] and nuclease sensitivity assays provide information about RNA structure. The latter two methods can be performed in less time than the biophysical analyses and are therefore more amenable to testing the effects of variations in nucleotide sequence and environmental conditions on aptamer structure. The results from X-ray crystallography and NMR can provide an atomistic view of an aptamer structure that has a high probability of being in the same valley of the energy landscape as is achieved in the presence of alternative buffer and ionic conditions such as inside the cell or as used for biochemical analysis of the binding parameters of the aptamer. However, this assumption should be made cautiously. As we demonstrate here, the ionic environment of the aptamer can have a large effect on its ligand interaction that may involve significant changes in its structure.

2.2. Methods for measuring aptamer–ligand binding parameters

We have evaluated many methods for determining the binding parameters of aptamers with their ligands. These include column and filter binding assays, fluorescence anisotropy, equilibrium dialysis, surface plasmon resonance (SPR), differential scanning calorimetry, isothermal titration calorimetry (ITC), fluorescent dye displacement, and 2-aminopurine (2-AP) reporters. In general, we have found that assays that require the ligand be modified (column binding assays, fluorescence anisotropy, SPR) are likely to give inaccurate values because aptamers tend to interact with most parts of the ligand molecule and binding interactions can be significantly influenced by molecular appendages such as the fluorophore or surface to which the ligand is linked. Of the remaining assays listed, ITC, fluorescent dye displacement and 2-AP reporter assays are the most reliable and generally provide consistent inter-assay results (Table 1).

2.2.1. Isothermal titration calorimetry for determining the thermodynamic parameters of RNA-small molecule interactions

Isothermal titration calorimetry (ITC) is based on simple physico-chemical parameters of aptamer–ligand interaction. An ITC instrument directly measures the heat gained or lost during a physical or chemical reaction that is equal to the enthalpy changes (ΔH) associated with that specific molecular interaction in solution at equilibrium. The binding isotherm, resulting from titration of one reactant into a cell containing the interacting partner, provides an estimate of the enthalpy change (ΔH), association constant (K_a) and stoichiometry (N). The Gibbs free energy change (ΔG) is calculated from the thermodynamic relationship between these terms: $\Delta G^0 = -RT \ln K_a$, where R is the gas constant, T is the absolute temperature and $K_a = 1/K_d$. The equation, $\Delta G = \Delta H - T\Delta S$, provides the means of calculating the entropy (ΔS). ITC is simple to perform and the analysis of most data is a trivial task.

Table 1
Comparison of ITC, steady-state 2-AP fluorescence and TO displacement assays for determining the dissociation constant of aptamer–ligand interactions.

Ligand	Isothermal titration calorimetry	TO-dye displacement	2-AP fluorescence
Neomycin-B	0.29 ± 0.058	0.35 ± 0.065	0.53 ± 0.090
Paromomycin	1.4 ± 0.19	1.2 ± 0.27	1.2 ± 0.25

NEO1A was analyzed in buffer A for its affinities for two ligands by the three assays identified in the table. For 2-AP fluorescence, the aptamer contained 2-AP in place of adenine at position 16 (see table 2 for numbering). Shown are the average values for K_d ± standard deviation.

2.2.2. Principles of ITC experiments

The change in enthalpy (ΔH) for almost any bimolecular binding interaction can be accurately measured by ITC at a constant temperature in a well-designed experiment with the appropriate concentrations of reactants. The ITC instrument contains a pair of identical cells (sample and reference cells) constituted of a highly efficient thermal conducting and chemically inert material surrounded by an adiabatic jacket. Several calorimeters are commercially available that operate on the same principle but vary in their parameters and in some operating procedures. Depending on the calorimeter the cell sample volume varies between 200 µL and 1.4 mL. Sensitive thermopile/thermocouple circuits detect temperature differences between the reference and the sample cells.

The sample cell generally holds the macromolecule into which the smaller interacting partner or another molecule is titrated during the ITC experiment at a fixed temperature in the range 5–60 °C. However, the reverse titration (macromolecule titrated into a cell containing the smaller partner) can also be performed. To ensure saturation, titrant concentrations in the injection syringe should be such that the final concentration of titrant in the sample cell is 2–5N times the concentration of its binding partner in the cell, where N is the stoichiometry of ligand/aptamer. Titration typically involves 21–31 injections every 3–5 min of 10–15 µL aliquots. During titration the temperature of the sample cell is monitored against the reference cell, which contains water or buffer. Depending on the nature of the interaction (endothermic or exothermic), energy in the form of heat is consumed or evolved. The instrument determines the absolute amount of change in heat of the reaction by measuring the power required to equalize temperatures between the sample and reference cells. To achieve this measurement with a stable baseline signal, the instrument applies constant power (<1 mW) to the reference cell and activates a heater located around the sample cell prior to initiation of the titration. Prior to the start of the experiment, samples should be degassed as the presence of gas bubbles may result in abnormal data points.

The results from the ITC run are plotted as a series of spikes (represented as peaks or dips depending on the software associated with the instrument) of measured changes in power units as a function of time that are required to adjust the sample cell to the reference cell temperature after each injection of titrant. It is important in setting the parameters of the experiment that sufficient time is given to allow complete equalization of the cell's temperature after each injection so as to obtain accurate measures of the heat change due to the injected ligand. The power inputs, converted to heat changes (µCal/s), are used to measure ΔH, which is calculated by the integrated area under each peak. Once the titrant has saturated the binding partner in the cell, the remaining heat signals are due to the heat of dilution. The titration curve is analyzed with the appropriate non-linear fitting models to calculate thermodynamic parameters; the enthalpy (ΔH), association constant (K_a) and stoichiometry (N) of the interaction.

2.2.3. Experimental parameters

The parameters for an ITC experiment are entered into the software program controlling data acquisition before the start of an experiment. They include (1) RNA concentration in the sample cell, (2) ligand concentration in the syringe, (3) volume of each serial injection, (4) time between injections (spacing), (5) duration of injections, and (6) experimental temperature. Typically 7- to 10-s injection durations give proper mixing of ligand, which is aided by a stirring device. The stirring speed should be chosen at approximately 400 rpm for good mixing and we found 310 rpm to be