

Analysis of the Ornithine Decarboxylase Antizyme RNA Structure and Function

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Abstract: Riboswitches are segments of non-coding, functional RNA that reside in mRNA. These structures are able to bind cellular metabolites and undergo a conformational change which affects the expression of downstream genes. This mechanism of genetic control is common throughout bacteria and is being researched by some for use as a novel antibiotic. The discovery of riboswitches in higher organisms would allow for a new method of gene therapy, most notably in humans. One gene of interest is Ornithine Decarboxylase Antizyme. This gene is essential for the synthesis of polyamines, like spermine, in the cell. In mice, the Ornithine Decarboxylase Antizyme mRNA contains a non-coding segment that creates a pseudoknot structure, a common element of multiple bacterial riboswitches. This segment of RNA has been shown capable of binding to spermine and inducing a structural change. This shape change does not occur in the presence of other polyamines. In addition to this specificity, the structural conformation change suggests that this mRNA segment is acting as a riboswitch. Development of techniques to further analyze this potential riboswitch has led to the utilization of Isothermal Titration Calorimetry for more complex binding assays and X-ray crystallography for structural analysis.

Introduction

The persistent threat of cancer is continuously leading to research into new techniques for treatment. Riboswitches are segments of non-coding mRNA that are capable of binding to cellular metabolites. These binding events are highly specific, and metabolite binding by the RNA results in a conformational change that affects the expression of downstream genes. This genetic control mechanism is common in bacteria and their manipulation is being studied by some as a novel technique to affect genetic expression in bacteria, potentially for use as an antibiotic.¹⁻⁵ Previous studies have found a riboswitch in a fungus, but none have yet identified one in a mammal.

In mice, Ornithine Decarboxylase (ODC) is important for the synthesis of cellular polyamines, such as spermine.^{6,7} High concentrations of spermine cause an increase in the level of Ornithine Decarboxylase Antizyme (AZ-1 or OAZ-1),

although the exact mechanism is unknown (see Figure 1).

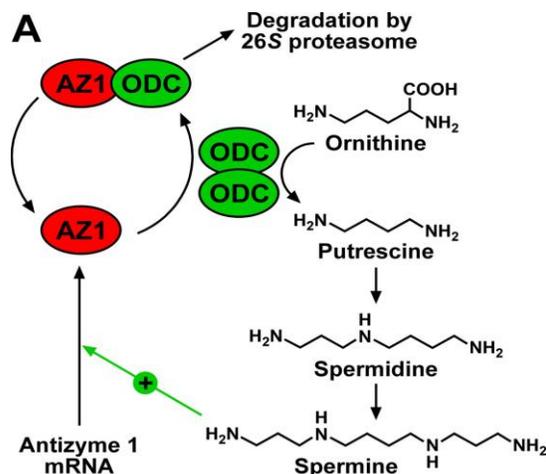


Figure 1. The production and regulation of cellular polyamines. The presence of spermine is necessary for the production of AZ-1 (previously published but without evidence of the mechanism). The production of AZ-1 leads to decreased ODC activity, thereby decreasing the rate at which new polyamines can be synthesized, effectively serving as a negative feedback mechanism.

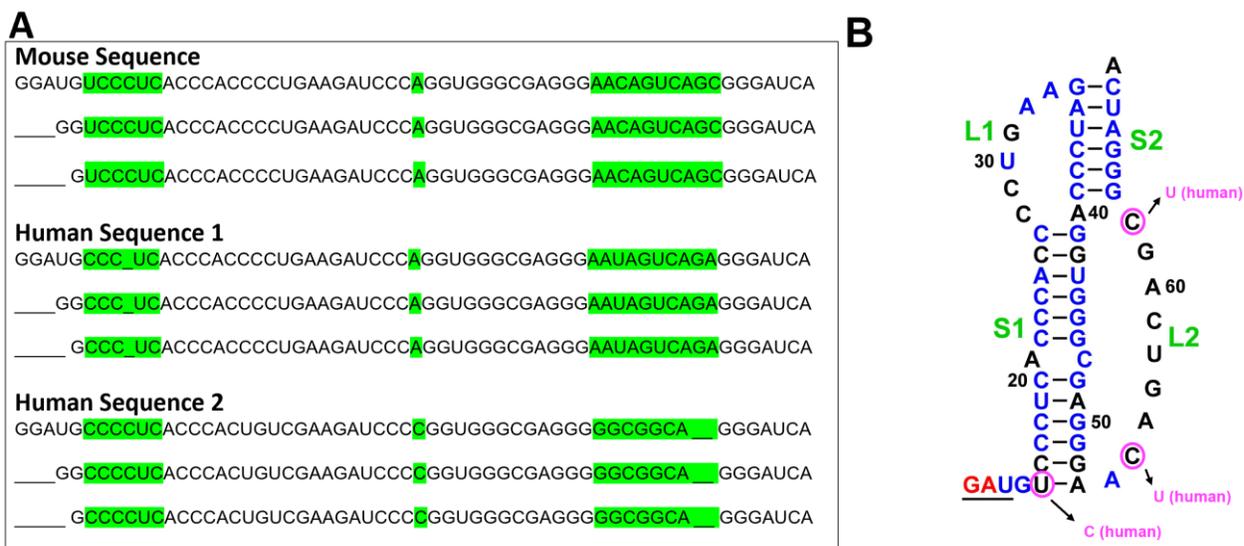


Figure 3. RNA sequences used for Crystallography. A) The nine sequences used for crystallography. Segments that vary are highlighted in green, with base pair omissions noted as $_$. B) Differences in sequence between mouse and human primarily occur in the 1st stem (S1) and the 2nd loop (L2).

Structural Studies

Determination of the three-dimensional structure was performed in collaboration with the Borgstahl lab at University of Nebraska Medical Center.

X-ray Crystallography

Due to the unexplored nature of RNA crystallography, a variety of RNA sequences were prepared, to maximize chances of creating an ideal solution for RNA crystallization. Due to RNA's inherent flexibility, the 5' and 3' tails were removed. Due to a believed importance of the frameshift site for binding, a varied length of 5' tail was left to maximize and enable binding. Human sequences were also used in the hope that the varied nucleotide sequence would increase the ability to crystallize (due to better/worse solvent interaction, more solid structure, etc.)

Commercial screens were used for initial setup of 96-well plates. Each well was loaded with 100 μ L of the screening solution, and drops were setup using the sitting drop method with a ratio of 1:1 mother liquor to RNA solution. 200 μ M RNA solutions were

prepared in buffers containing $MgCl_2$, Tris, and EDTA. Promising well conditions were then set up in 24 well plates with a gradient of the buffer, to fine tune crystal growth conditions. Upon crystal formation of sufficient size, the crystals will be placed in front of a X-ray beam to obtain the scattering pattern.

Results

ITC Results

ITC data has been obtained for three of the spermine analogs shown in Figure 4, with preliminary data for three others (data shown in Table 1).

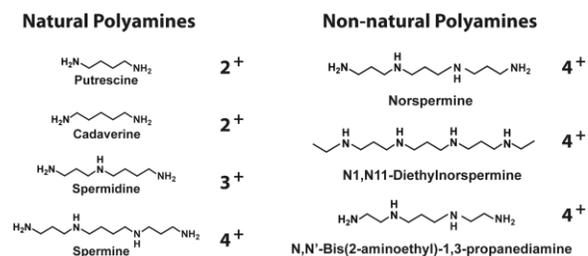


Figure 4. The ligands studied using ITC. Naturally occurring polyamines are shown on the left, with synthetically obtained analogs shown on the right. To the right of each molecule is the charge at physiological pH. N1,N11-Diethylnorspermine will be referred to as Diethylnorspermine and N,N'-Bis(2-aminoethyl)-1,3-propanediamine as propanediamine.

Table 1. Dissociation Constants of analogs

Polyamine	K_d (μM)	Error ($\pm\mu\text{M}$)
Spermine	250	30
Spermidine	450	75
Putrescine	0 **	N/A
Norspermine*	5,000	1,500
Diethylnorspermine*	40	7
Propanediamine	30	7

*Preliminary results **No binding event

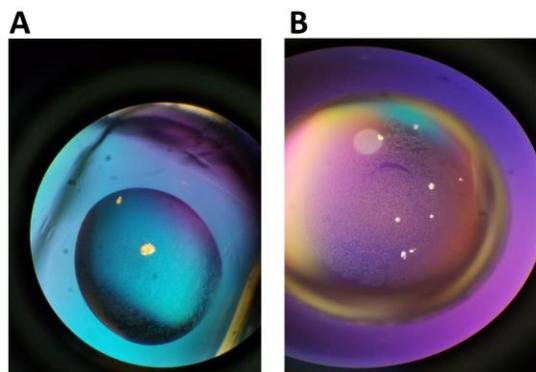
The differences in binding affinity between analogs can then be compared to spermine. The differences in charge and structure can then be correlated to binding affinity (K_d) and important motifs for binding can be determined.

Spermidine had a decreased binding affinity than spermine, which was expected due to the lesser positive charge, decreasing electrostatic interactions. Putrescine lacking any detectable interaction was not unexpected due to the much weaker charge and shorter length preventing alignment with hydrophobic regions.

Of interest, synthetic analogs diethylnorspermine and propanediamine demonstrated stronger binding affinity than the natural ligand spermine. The stronger interaction between diethylnorspermine likely results from increased potential for hydrophobic interactions due to the ethyl tails. For propanediamine this higher affinity likely results from the shorter carbon chains between the positively charged amino groups. These shorter chains may create a better electrostatic interaction with the RNA backbone as the carbon chain lengths may better align with the spacing between negatively charged groups in contrast to the interactions between the RNA and spermine.

X-Ray Crystallography Results

Initial screens have shown promising conditions with LiCl and Sodium HEPES,

**Figure 5. Examples of precipitation and microcrystals.**

The glowing white spots result from biological crystals being birefringent and reflecting light in the same manner, despite polarization. A) Precipitation and microcrystals with MgCl_2 , Na^+ HEPES pH 7, and LiCl. B) Precipitation and microcrystals with MgCl_2 , Na^+ HEPES pH 7, and LiCl.

but so far crystals have not developed enough to be placed in front of the x-ray. Over 5,000 initial conditions have been tested, with only 18 showing potential so far. As crystals are allowed to develop for longer periods of time (up to 6 months), new potential conditions may be found as well. Further conditions will be tested as new RNA sequences are developed for testing.

Conclusions

The binding studies determined stronger interactions between some of the synthetic analogs and the RNA than spermine. This data could be used in future studies to create synthetic binding partners that could outcompete spermine and interfere with the regulation of polyamine levels in the cell.

Although x-ray scatterings have been attempted, none so far have given reliable data; but, in the future once a crystal of sufficient quality has been grown, a three-dimensional structure will be generated. From that 3D structure, the binding interactions and binding pocket in general can be studied. From there an analog of spermine could be designed in a more targeted manner, due to knowing where electrostatic and hydrophobic interactions are occurring, and a general best fit model applied.

The mouse AZ-1 PK-RNA was the first potential eukaryotic riboswitch found by the Soukup Lab, with more being discovered in oysters, fungi, mold, and insects. For this study we focused only on the mouse sequence, with future studies looking to analyze the other sequences. In addition, having studied the riboswitch functionality of the AZ-1 RNA, future studies also plan to study the mechanism by which the frameshift event is occurring.

References

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