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Summary for the Website

(Research Stay Duke University)

Resolving structural and functional properties of the pistol ribozyme by solution NMR spectroscopy

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introduction in the research goal

During the first two years of my PhD I worked on the synthesis of atom-specific labelled phosphoramidites and their incorporation into DNA/RNA. These building blocks are modified with $^{13}C/^{15}N/^{2}D$ isotopes allowing state-of-the-art NMR experiments on different oligonucleotides. The solid phase synthesis makes site-specific labelling possible, which is not amenable with any other RNA production method. This is of special importance for various fields of application, to get a more detailed understanding of the dynamic features of different nucleic acids. Further, the site-specific introduction of NMR active residues will also alleviate assignment problems, which can occur in RNA constructs of this size (Chapter 2.1). A schematic illustration of a solid phase synthesis, a phosphor amidite and the possible labelling positions within the standard RNA bases is shown in figure 1.

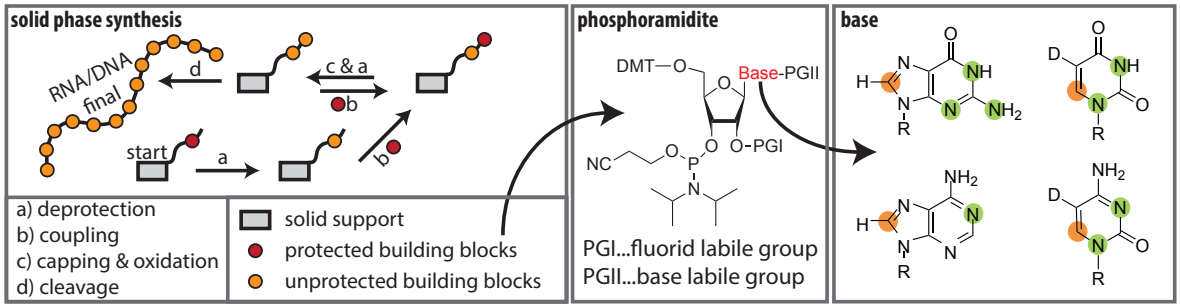

Figure 1: The schematic workflow of the solid phase synthesis, a schematic setting of phosphor amidites and possible labelling positions for all RNA bases is presented.

These selectively modified building blocks have been used for different approaches later on. It could be shown, that novel isotope labelled building blocks can be used to study dynamic properties of nucleic acids [1].

During the end of my PhD studies I focus on a detailed NMR spectroscopic investigation of the pistol ribozyme. In detail, structural and dynamic features of this self-cleaving ribozyme are in the focus of my research project [2]. After a few rounds of optimization an ideal construct of the pistol ribozyme was found. The aim for the pistol ribozyme was to resolve a 3D-NMR-solution structure in combination with a deeper insight into the dynamical behavior of his different segments. Especially a detailed investigation of the cleavage site and all during the cleaving mechanism involved residues should be implemented. But also, the opening of the pseudoknot

and its importance in the formation of the active cleavable structure has been investigated.

ribozymes – and the pistol ribozyme as part of the family

First findings, that can be seen as the beginning of modern ribozyme research have been reported in the 1950s. It has been shown that scission of the RNA strand takes place and a more prefund understanding of the mechanism was developed [3]. In the 1980s, Cech and his co-workers reported on the first ribozyme [4, 5]. Since these days, catalytic active RNAs have been intensively investigated. Up to now, especially scission and ligation reactions seem to be predominant in the field of nucleic acids [6].

Very recently, Roland R. Breaker and his co-workers reported on a new class of self-cleaving ribozymes found by a bioinformatics search strategy. In detail, they characterized three new RNA sequences called twister sister, hatchet and pistol ribozyme [7]. Self-cleaving ribozymes in general are ribonucleic acids in the range of 50 to 150 nucleotides in length, first discovered as domains of satellite RNAs [8].
After screening of several ribozymes, the pistol ribozyme proved to be the best investigated complex for the planned project. A comparison of several hundred pistol ribozymes occurring in natural systems indicated, that this motif has 10 highly conserved nucleotides and a broad range of modestly conserved nucleotides. The secondary structure in the presence of Mg^{2+}-cations includes three base-paired stems and a pseudoknot. First investigations on the cleavage mechanism of this class of self-cleaving ribozymes, indicate that it takes place via an internal phosphoester transfer [9].
As a starting point, we used a env25 pistol ribozyme sequence that was used in the crystal structure determination by Dinshaw J. Patel and co-workers. The secondary and the crystal structure of the env25 pistol ribozyme is shown in figure 2 [2]. For NMR investigations, some minor modifications of this used sequence have been mandatory, in order to improve the NMR spectra quality. Additionally, some parameters (sample concentration, relaxation delay d1, used bivalent cations, NMR buffer) in the measurement setup have been modified. These improvements of the original setup gave another increase in spectra quality.

findings on the pistol ribozyme

One of the main goals for the investigation on the pistol ribozyme is the localisation of dynamic regions in the catalytic active complex. This can be realized by the application of state of the art RD NMR methods. However, as a precondition for a successful implementation of these NMR investigations, it is indispensable to have a full assignment of all residues of interest. For this reason, several atom specific RNAs have been synthesized by solid phase synthesis. The obtained spectra have been overlaid subsequently. This technique, illustrated in figure 2 with the U7 residue of the complex helped to realize a straight forward assignment procedure for the C8 purin and C6 pyrimidine atoms. Additionally, the imino proton signals have been assigned for the uridine and guanosine residues in base pairs.

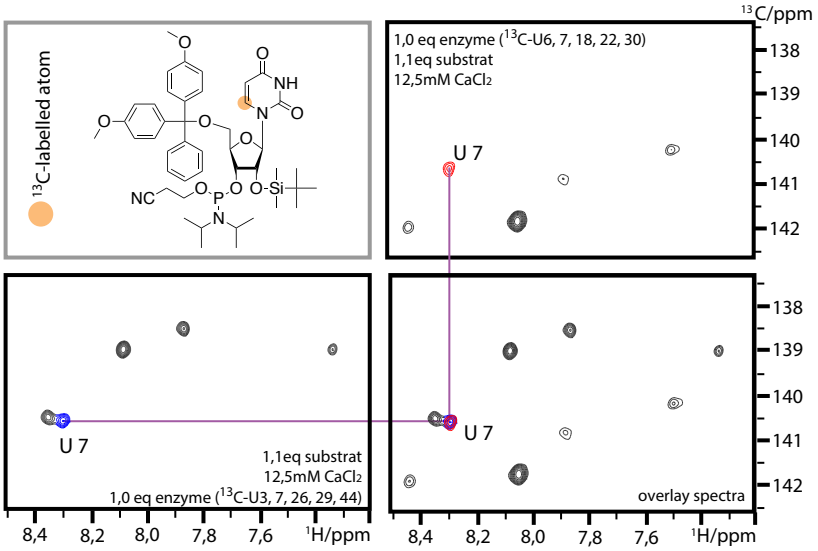

Figure 2 Figure 2 6-13C-uridine phosphoramidite building block used for solid-phase RNA synthesis. Orange dot =13C. Two 1H/13C HSQC spectrum of a pistol ribozyme complex with five selective 6-13C-uridine labels (U3, 7, 26, 29, 44 and U6, 7, 18, 22, 30) and an overlay of spectra of both HSQCs to assign residues (here: U7).

After optimizing and assigning the investigated pistol ribozyme, the folding mechanism was investigated in more detail. A deeper insight in these mechanisms is of tremendous importance to understand the different steps that are necessary for the catalytic activity in a system in more detail. During these investigations, we found out, that the enzyme on its own is present as a double hairpin in the *apo* form. After addition of Mg^{2+}-cations the formation of a weak pseudoknot fold could be observed. In the last step, the substrate strand was added to the sample leading to an increase in the signal to noise ratio. This strengthen the assumption that the preformed pseudoknot motif is highly flexible until the complex between the two RNAs is formed. Figure 3 shows the collected data on the folding mechanism of the investigated duplex.

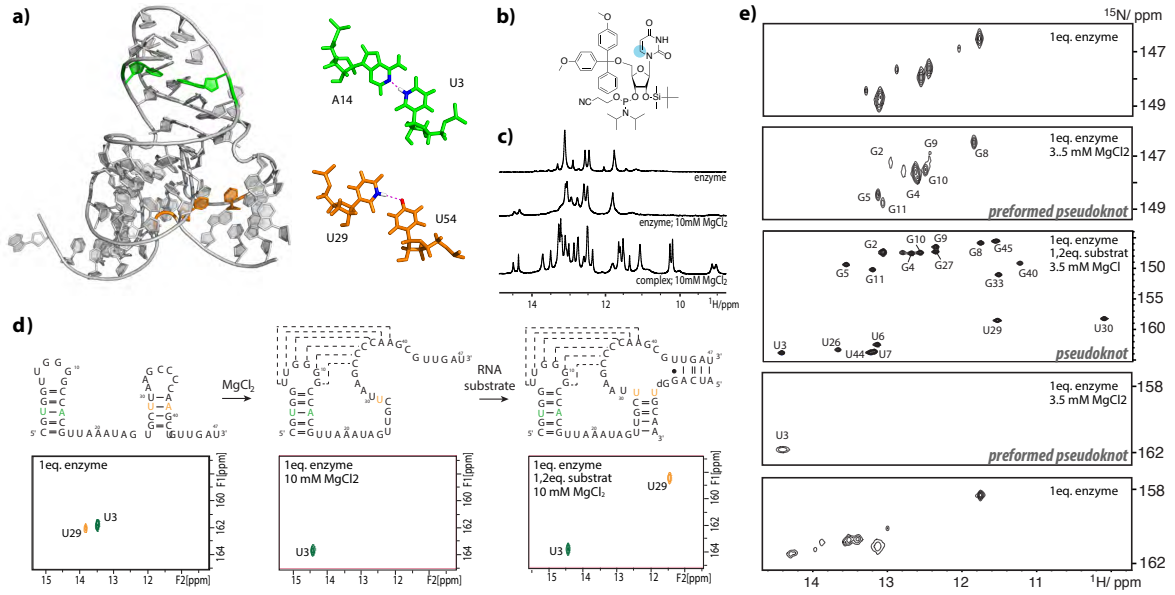

Figure 3: a) Crystal structure of the env25 pistol ribozyme showing the position of the selectively $^{15}N^3$-labeled U3 and U29 residue and their base pairing partner and a close-up on the base pairs U3-A14 and the non- canonical U29-U54. b) $^{15}N^3$-uridine phosphoramidite building block used for solid-phase synthesis. c) Influence of magnesium ions on the folding behaviour and complex formation with the substrate strand via imino proton NMR spectroscopy. d) Complex formation monitored by $^1H/^{15}N$-HSQC spectra making use of the two $^{15}N^3$-labeled uridine residues. e) Complex formation monitored by $^1H/^{15}N$-HSQC spectra of an uniformly ^{15}N-labeled enzyme strand.

A very interesting observation was, that the complex formation of the enzyme and the substrate strand can only be observed in the presence of bivalent cations (Ca^{2+}, Mg^{2+}). This was confirmed by collecting NMR spectra in the absence of bivalent cations and the subsequent titration of the same sample with calcium cations.

For a huge variation of investigated systems, it is known that metal cations can have crucial influences on structural and functional properties of nucleic acids. Especially for the class of self-cleaving ribozymes, the necessity of bivalent cations has always been postulated [7, 9]. Recent studies even raised the question if the metal ions are not only a key player in the folding process of the catalytic active complex. Rather it has been suggested that magnesia could be directly included in the cleavage process as an elementary part of the cleavage side [10].

Due to this fact and the result presented above, a more detailed investigation on the binding sides of bivalent cations were performed. NMR spectroscopy in general offers two different approaches. First manganese cations can be used to detect inner-sphere magnesia binding sites. Additionally $[Co(NH_3)_6]^{3+}$ is used as a mimic for magnesia hexahydrate and therefore the detection of outer sphere binding is accessible [11]. Preliminary experiments showed very promising influences of manganese on the

imino signals and the N7 regions of the purin residues. Presumable, one binding position could be localized in the cleavage side and a second one seems to be present within the pseudoknot. For a more detailed insight in the binding characteristics, measurements in the presence of $[Co(NH_3)_6]^{3+}$ are already planned.

Further, preliminary data on the dynamic features of the pistol ribozyme have been collected. These show that the pistol ribozyme is a highly dynamic nucleic acid duplex. Especially the fact that different regions of the investigated macromolecule show different dynamic features, present in diverse time domains underline the extremely high complexity of the target of interest. Figure 4a show the postulated secondary structure known from X-ray crystallography [2]. A closer look at a uniformly residue specific $^{15}N^{13}C$ guanosine labeled sample gave a deeper insight into these dynamic hotspots. Additional two samples with uniformly ^{15}N labeled base pairs (G/C and A/U residues labeled) gave further information. Combining all the obtained data, the localization of three main dynamic areas was possible. These areas are highlighted in figure 4b. In detail, dynamic processes have been located in the pseudoknot area (orang), the substrate binding branches (red) and the catalytically active cleavage side (green).

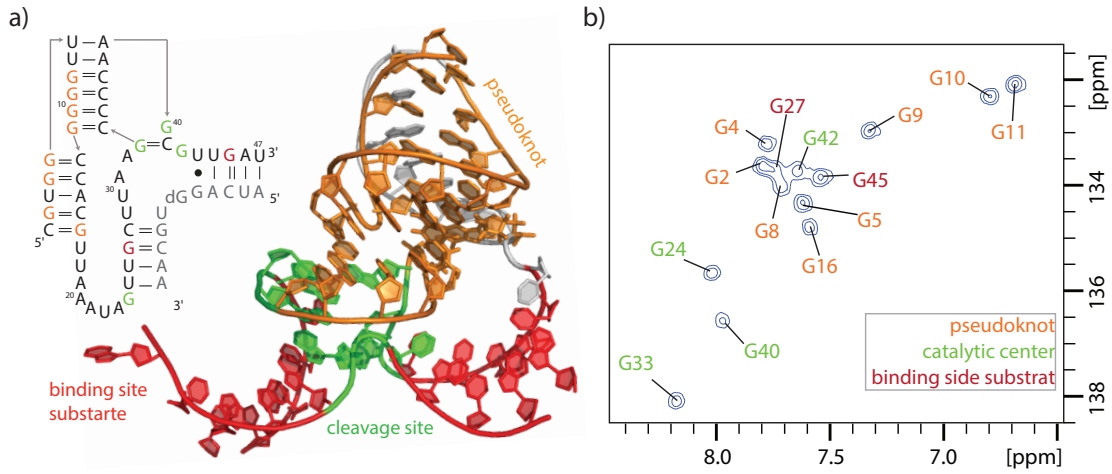

Figure 4 Localized dynamic processes in the pistol ribozyme color-coded in the secondary structure and the crystal structure a) and in a 1H/13C HSQC spectra of the C8 position in a 13C labelled enzyme strand.

conclusion

In our basic understanding of the pistol ribozyme complex so far, we assume that the three presented dynamic key steps combined with some structural features are of fundamental importance. The preliminary results are also indicators that these three dynamic motions cannot be seen individually. Rather they are all part of the big “self-splicing” story in the pistol ribozyme. In detail, we assume that the pseudoknot is weakly formed in the presence of bivalent cations (Ca^{2+}, Mg^{2+}). This preformed pseudoknot structure possesses a perfect binding side for the substrate. After this binding step took place, dynamic motions in the catalytic center generate the perfect cleavage geometry and carry out the cleavage mechanism. Attaching to these steps, the two generated parts of the substrate RNA are released and the enzyme can act again as a host for future substrates and their self-cleavage reaction.

To realize all these steps, pistol ribozymes are highly complex systems. This is also underlined by the first published structure of the investigated complex by Ronald R. Breaker [7, 9] and the results we obtained during the development of the perfect sequence and by numerous cleavage-assays implemented in our group and by others [2, 10].

The next milestone will be to collect a clean and fitable data set. This information should help to postulate a mechanism based on excited states obtained from relaxation dispersion (RD) NMR and chemical exchange saturation transfer (CEST) experiments. Additional, after assigning the imino proton signals and parts of the carbon signals, a solution structure of the pistol ribozyme will be calculated in collaboration with Jens Wöhnert [12].

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