RIBOZYMES and RNA MODIFICATIONS

CLAUDIA HÖBARTNER
Institute of Organic Chemistry, Julius-Maximilians-Universität Würzburg, Germany

Introduction

Nearly all classes of coding and non-coding RNAs undergo post-transcriptional modification, and methylated nucleotides belong to the evolutionary conserved features of RNA. Recent studies revealed a dynamic RNA modification landscape in mRNA and hint at important roles of methylated RNA in regulating cellular functions [1]. Synthetic RNA modifications, including methylated nucleotides are also important for emerging RNA-based medicine. For example, the modified nucleoside 1-methylpseudouridine (m^1Y) increased the effectiveness of COVID-19 mRNA vaccines [2], and 2'-*O*-methyl or 2'-*O*-methoxyethyl nucleotides are key components of therapeutic antisense oligonucleotides [3], while other modified nucleotides interfere with viral replication by preventing efficient translocation or introducing massive copying errors, as recently shown for SARS-CoV-2 RNA polymerase and the repurposed antiviral nucleosides prodrugs remdesivir [4] and molnupiravir [5].

In an era before modern life emerged, RNA was thought to function both as genetic material and as catalyst. *In vitro* directed molecular evolution experiments discovered ribozymes that function as RNA-dependent RNA polymerases and can copy themselves or their ancestors [6, 7]. RNA may have had additional catalytic roles during evolution, and nucleotide-containing cofactors used by contemporary metabolic enzymes may constitute molecular remnants of ancient ribozymes [8, 9]. The discovery of riboswitches as regulatory RNA elements that bind small molecules including various coenzymes [10] nourished the thoughts of more widespread catalytic competences of RNA [11, 12]. Indeed, in vitro selection endeavors have revealed aptamers and ribozymes that bind and utilize nucleotide cofactors, including redox cofactors and acetyl coenzyme A [13, 14].

Besides nucleotide-derived cofactors, the involvement of modified nucleosides in RNA catalysis is interesting to consider. Spontaneous nucleobase or ribose modification/ methylation may have occurred in reactive environments and the presence of non-canonical nucleotides in RNA could benefit or burden the evolution of RNA catalysts. Methylation of nucleic acids is a widespread modification found in all domains of life, and most methyl groups are installed by methyltransferase enzymes that use *S*-adenosyl-methionine (SAM) as the methyl donor. Methylated nucleosides and methyl group transfer reactions may have even occurred on the primitive Earth [15]. Directed

molecular evolution of methyltransferase ribozymes that catalyze the installation or selective removal of methyl groups from RNA nucleotides may shed light on potential catalytic abilities of primordial ribozymes and could reveal distinctive properties of now universally conserved methylated nucleotides in tRNA and rRNA.

Ribozymes that install RNA modifications

Earlier experimental attempts to address the question whether RNA can catalyze site-specific RNA methylation using *S*-adenosylmethionine (SAM) resulted in the enrichment of cofactor-binding aptamers without methyltransferase activity [16]. *In vitro* selection by enrichment for catalytic activity identified self-alkylating ribozymes using reactive iodo- or chloroacetyl derivatives [17-19], and later electrophilic epoxides [20], resulted in N^7-alkylation of guanine with the RNA. Based on the design of these experiments, these ribozymes could not be used as methyltransferases. The required methyl halogenides would be too reactive reagents, and epoxides would transfer at least two carbon atoms to the guanine. Searching for alternatives, we hypothesized that a methyltransferase ribozyme should use a cofactor that makes specific non-covalent contacts to the RNA, including H-bonding, π-stacking, and others. Our selection strategy was designed for the ribozyme to form a binding site for the "leaving group", and we chose O^6-methylguanine (m^6G) as potential methyl group donor. Transfer of the methyl group from m^6G to the target RNA would result in the release of guanine. Guanine is well established as an RNA ligand in natural riboswitches and in vitro selected aptamers [21]. A possible evolutionary relationship between ribozymes and riboswitches has previously been discussed [11, 12]; metabolite-binding riboswitches may resemble inactivated ribozymes that lost their catalytic activity during evolution from the RNA world.

Following the hypothesis outlined above, we have discovered the first methyltransferase ribozyme (MTR1). This ribozyme catalyzes a site-specific intermolecular methyl transfer to install 1-methyladenosine (m^1A) at a defined position in a target RNA by utilizing O^6-methylguanine as methyl group donor [22]. We showed that the ribozyme can be engineered to methylate natural RNA sequences, including tRNAs, which contain m^1A at conserved positions. The ribozyme showed accelerated transfer rates at pH 6 and could be split into two fragments that assemble on the target RNA into a functional ribozyme. We solved the crystal structure of MTR1 and investigated the mechanism of RNA-catalyzed methyl transfer [23]. The structure revealed the products of the reaction, i.e. the post-catalytic state, with m^1A and the free guanine bound in close proximity in the active site. The ligand is fully contacted by hydrogen bonds with the RNA, and the base pairing pattern is highly reminiscent to guanine binding observed in natural guanine riboswitches [24].

The MTR1 ribozyme utilizes general acid catalysis to enable the methyl group transfer. Structure probing in solution as well as activity assays of structure-guided mutants provided strong support for the mechanism, in which a protonated cytidine is involved in binding and activation of the cofactor. The mechanistic analyses revealed two

key nucleotides that act in concert to accelerate the methylation reaction. We found a synergistic effect of two methylated ribose residues (at C12 and U42), which enhanced the reaction rates by at least 120-fold over the rates obtained with unmodified RNA under in vitro selection conditions. This finding supports the speculation that modified nucleotides may have enhanced early RNA catalysis.

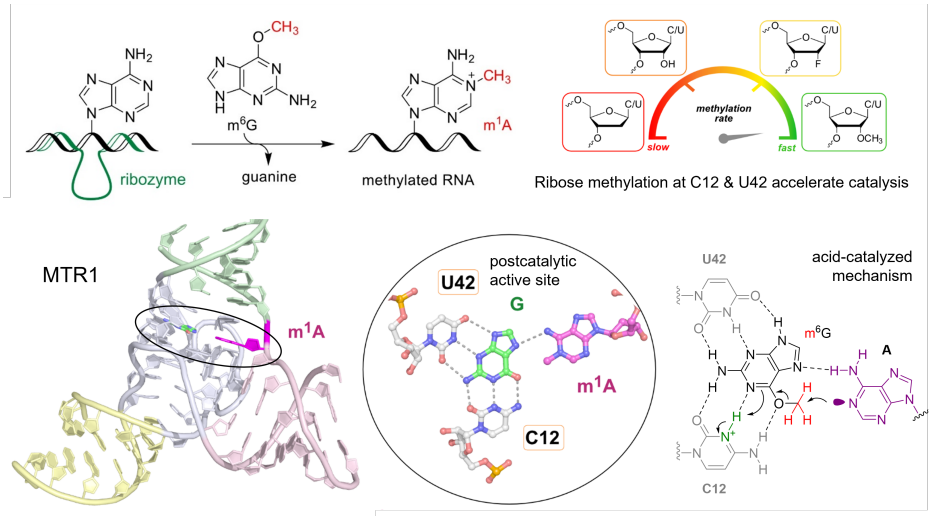

Fig. 1. The methyltransferase ribozyme MTR1 catalyzes site-specific RNA methylation using m^6G to install m^1A in RNA. The architecture of the active site is reminiscent of natural purine riboswitches. The catalytic mechanism involves a protonated cytidine. 2'-OMe nucleotides in the active site synergistically accelerate methyl transfer.

In analogy to MTR1, it seems feasible to evolve additional methyltransferase ribozymes and explore potential preferences for generation of various methylated nucleotides in RNA-catalyzed reactions. It may also be rewarding to perform in vitro evolution experiments starting from native RNAs (riboswitches) that are already known to bind methyltransferase cofactors or derivatives thereof, such as *S*-adenosylmethionine (SAM), cobalamine, or tetrahydrofolate. Indeed, last year two additional RNA-catalyzed RNA methylation reactions were described. Micura and coworkers demonstrated that a natural $preQ_1$ riboswitch RNA can bind the synthetic cofactor m^6preQ_1 and mediate the transfer of the methyl group to a cytidine in the binding site, thus generating 3-methylcytidine (m^3C) [25]. Murchie and coworkers found a ribozyme that uses SAM and Cu^{2+} to generate 7-methylguanosine (m^7G) in the ligand binding site [26]. The active sequence identified by in vitro selection in the laboratory was then also found in genomic sequences from all domains of life, suggesting that it may have ancient origins. Recently we found another SAM-utilizing ribozyme that alkylates the minor groove side of adenosine (T. Okuda, C. Höbartner, *submitted*) and we expect that more RNA-modifying ribozymes will be discovered in the future.

Perspectives for the future

More than 70 different methylated nucleosides are known in natural RNA, and it is an open question which fraction of methylation sites can be accessible by RNA-catalyzed RNA methylation. While some *N*- and *O*-alkylation sites seem more easily addressed than others, the whole family of C5-methylated pyrimidine nucleosides requires more sophisticated reaction mechanisms and likely additional cofactors. Nevertheless, the recent advances in the field of methyltransferase ribozymes and the insights into catalytic mechanisms beyond RNA-catalyzed phosphotransfer reactions suggest that other cofactor-utilizing ribozymes may be found in the laboratory and possibly in natural RNAs to catalyze more diverse reactions than currently known. The added benefit of modified nucleotides shaping the active sites of ribozymes is worthy of further exploration. This may lead to the first XNAzymes catalyzing reactions other than cleavage or ligation of RNA. The key challenges for finding new ribozymes by directed molecular *in vitro* evolution experiments lay in the design of the selection strategies for enrichment and amplification. Novel high-throughput analyses of ribozyme activities directly from sequencing data may accelerate the discovery rate. A bold speculation on possible cofactor-assisted RNA-catalyzed reactions could address radical reactions, for example to explore the potential transition from RNA to DNA, which would require cleavage of a carbon-oxygen bond, mimicking the enzymatic activity of ribonucleotide reductase in Nature or Barton-McCombie deoxygenation in the organic chemistry laboratory. In another direction, we are looking for RNA-catalyzed site-specific nucleobase deamination, akin to enzymatic RNA editing that converts adenosine to inosine. Fundamental studies in these and other directions will continue to explore the potential of ribozymes in shaping early RNA-based life and its evolution. In addition, the development of nucleic acid catalysts as research tools in RNA biology and as potential future RNA-based therapeutics are exciting and challenging research lines ahead.

Acknowledgments

This work was supported by the European Research Council (ERC consolidator grant 682586), the Deutsche Forschungsgemeinschaft (DFG SPP1784 and HO4436/3-1) and the University of Würzburg.

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