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In vitro evolution of coenzyme-independent variants from the *glmS* ribozyme structural scaffold

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ABSTRACT

Uniquely among known natural ribozymes that cleave RNA sequence-specifically, the *glmS* ribozyme-riboswitch employs a small molecule, glucosamine-6-phosphate (GlcN6P) as a catalytic cofactor. *In vitro* selection was employed to search for coenzyme-independent variants of this ribozyme. In addition to shedding light on the catalytic mechanism of the ribozyme, such variants could resemble the evolutionary ancestors of the modern, GlcN6P-regulated ribozyme-riboswitch. A mutant pool was constructed such that the secondary structure elements, which define the triply-pseudoknotted global fold of the ribozyme, was preserved. A stringent selection scheme that relies on thiol-mercury affinity chromatography for separating active and inactive sequences ultimately yielded a triple mutant with a cleavage rate exceeding 3 min^{-1} that only requires divalent cations for activity. Mutational analysis demonstrated that a point reversion of the variant toward the wild-type sequence was sufficient to partially restore GlcN6P-dependence, suggesting that coenzyme dependence can be readily be acquired by RNAs that adopt the *glmS* ribozyme fold. The methods employed to perform this selection experiment are described in detail in this review.

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1. Introduction

1.1. Background

The *glmS* ribozyme-riboswitch is a catalytic, gene-regulatory mRNA domain widespread in Gram-positive bacteria. It is the only natural RNA known to be both a riboswitch and a ribozyme (reviewed in [1,2]). Riboswitches are mRNA elements that regulate gene expression in *cis* in response to direct binding to a cognate ligand [3–6]. The *glmS* gene encodes the protein enzyme glucosamine 6-phosphate (GlcN6P) synthetase, and the ribozyme-riboswitch lies in its 5′-untranslated region. The self-cleavage activity of the ribozyme-riboswitch is activated by binding to GlcN6P, and self-scission leads to degradation of the mRNA [7,8]. In this manner, the *glmS* ribozyme performs negative-feedback regulation of the intracellular levels of GlcN6P, an essential precursor for the synthesis of the bacterial cell wall. Because disruption of *glmS* ribozyme function is deleterious *in vivo* in the model organism *Bacillus subtilis*, this catalytic RNA is a candidate antibiotic target, and its molecular mechanism of action has been studied intensively [2].

Activation of the *glmS* ribozyme by GlcN6P could result from either of two distinct mechanisms. The small molecule could function as an allosteric effector whose binding allows the RNA to achieve a catalytically competent conformation. Alternatively, GlcN6P could be a coenzyme, providing functional groups essential for chemical catalysis to an otherwise incomplete ribozyme active site. Biochemical and crystallographic studies support the latter mechanism. The amine of GlcN6P is positioned to protonate the 5′-OH leaving group of the internal transesterification through which the ribozyme cleaves RNA, thus functioning as a general acid catalyst [9,10]. Binding to the ribozyme lowers the pK_a of the amine group, thereby improving its catalytic efficiency [11–13]. Further arguing against an allosteric mechanism, the ribozyme has been shown readily to adopt its active conformation under physiological Mg^{2+} concentrations [14,15], and its rigid, prefolded structure is not perturbed by binding of GlcN6P, or cleavage of the substrate strand [9,16–19]. The catalytic mechanism of the *glmS* ribozyme, which relies on binding of a small molecule

Abbreviations: APM, *N*-acryloylaminophenylmercuric acetate; dNTPs, deoxyribonucleotide triphosphates; DTT, dithiothreitol; GlcN6P, glucosamine-6-phosphate; $glmS^{WT}$, wild-type *glmS* ribozyme; ^{6S}GMP, 6-thioguanosine monophosphate; $glmS^{AAA}$, triple adenosine-mutant *glmS* ribozyme; nt, nucleotides; NTPs, ribonucleotide triphosphates; PAGE, polyacrylamide gel electrophoresis.

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coenzyme, is unique among all known ribozymes that cleave RNA through internal transesterification, and it suggests the possible involvement of catalytic cofactors in a primordial RNA world [20].

1.2. In vitro selection of glmS ribozyme variants

Several studies have employed *in vitro* selection to isolate sequence variants of the *glmS* ribozyme [21,22]. These studies aimed to investigate the coenzyme selectivity of the ribozyme, to discover variants that could efficiently employ molecules other than GlcN6P as catalytic cofactors, and to explore possible evolutionary origins of this gene-regulatory RNA. Because GlcN6P is a metabolite present in all kingdoms of life, it is difficult to selectively activate the *glmS* ribozyme in a heterologous context. Identification of a *glmS* ribozyme variant that employs a coenzyme other than GlcN6P, and which is not activated by GlcN6P itself, would provide a ribozyme and coenzyme set that is orthogonal [23] to the natural ribozyme, and thus be useful for synthetic biology applications. Moreover, characterization of such an orthogonal system may also shed light on the molecular basis of coenzyme selectivity by the wild-type ribozyme-riboswitch.

It is thought that complex biological functions arise step-wise, with each step providing a selective advantage [24]. Thus, in the case of the *glmS* ribozyme-riboswich, it is conceivable that an ancestral molecule existed that was either a ribozyme or a riboswitch, but not both [22]. Phylogenetic analyses have thus far failed to uncover possible molecular ancestors of the *glmS* ribozyme-riboswitch, but because none of the other known natural ribozymes that cleave RNA through internal transesterification employ a coenzyme, and because other riboswitches that bind sugar derivatives are unknown, a "ribozyme-first" scenario is more likely. That is, the molecular ancestor of this gene-regulatory RNA could have been an RNA-cleaving ribozyme that only subsequently acquired dependence on GlcN6P, and thus the ability to regulate cellular levels of this metabolite.

We recently employed *in vitro* selection to search for coenzyme-independent variants of the wild-type *glmS* ribozyme (hereafter $glmS^{WT}$) [22]. In order to preserve the overall structure of the ribozyme, only nucleotides in the catalytic core that do not participate in forming the characteristic triply-pseudoknotted *glmS* ribozyme fold were mutagenized. The resulting library of sequence variants was subjected to *in vitro* selection, allowing us to identify an RNA with three adenosine mutations (hereafter $glmS^{AAA}$) that is active in cleaving RNA in the absence of GlcN6P. This mutant RNA achieved cleavage rates as high as 3 min^{-1} in the presence of the cations Ca^{2+} or Mg^{2+}, but its activity was not enhanced by GlcN6P. SAXS and crystallographic analyses confirmed that $glmS^{AAA}$ adopts the same fold as the wild-type ribozyme. The three adenosine mutations surround the scissile phosphate and abrogate RNA functional groups that coordinate GlcN6P in the wild-type. Biochemical characterization of point mutants of $glmS^{AAA}$ demonstrated that a single nucleotide reversion to the wild-type sequence is sufficient to confer GlcN6P-dependence (neither $glmS^{AAA}$ nor the various revertants have yet been tested *in vivo*). Thus, in addition to pinpointing the molecular requirements for GlcN6P utilization by the *glmS* ribozyme, this work revealed a possible path for the evolution of the bacterial ribozyme-riboswitch from an unregulated RNA-cleaving ribozyme that only requires divalent cations for full activity. Because the wild-type *glmS* ribozyme is very active (close to 100 min^{-1}) and can employ adventitious coenzymes (such as the buffer Tris) in the absence of GlcN6P [11], stringent conditions were needed for *in vitro* selection. The methods employed for the selection of $glmS^{AAA}$ could be useful for other *in vitro* ribozyme evolution experiments and are detailed in this article.

2. Methodology

2.1. Mutagenized pool construction

A mutagenized DNA pool based on $glmS^{WT}$ was designed to retain sequences forming the helical elements (secondary structure) of the RNA (Fig. 1A). Nucleotides from the core of the ribozyme, along with those from loops and bulges (43 positions in total, Note 1), were each mutagenized by 30% relative to $glmS^{WT}$ (Fig. 1A [22,25]). Flanking the pool sequences are constant regions. The 5′ constant region contains a bacteriophage T7 RNA polymerase promoter, followed by a 40-nt leader ("substrate") sequence (optimal length, Note 2). Following transcription, this 40-nt RNA leader can be cleaved in *cis* if its downstream sequence encodes an active ribozyme. The 3′ constant region contains a primer-binding site for reverse transcription and PCR. For our selection, the 205-nt single-stranded DNA pool was synthesized at the one-micromole scale using standard cyanoethyl phosphoramidite chemistry. For the synthesis, a molar ratio of 0.28:0.23:0.27:0.22 (dA:dG:dC:dT) was used to compensate for differences in coupling efficiencies [26,27]. Following synthesis, ~60 random isolates from the DNA pool were sequenced (Note 3) to provide an estimate of the actual sequence diversity and mutation rate per position.

2.2. Synthesis of double-stranded DNA template pool

The single-stranded DNA pool (~5 nmol, $\sim 3 \times 10^{15}$ unique sequences) was converted to double-stranded and amplified in a 5 mL PCR reaction containing 20 mM Tris-HCl pH 8.4, 50 mM KCl, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 2.5 μM of 5′ selection DNA primer (5′-TTC TAA TAC GAC TCA CTA TAG GAA ACC ATC ATA ACA GTT CTT GCT ACA AAT CAT TTA TCA GGG CCT GGA CTT AAA GCC GCG AGG-3′), 2.5 μM 3′ reverse selection primer (5′-TTC CTG CCC GGA CTG-3′), 0.03 U/μL Taq DNA polymerase (Invitrogen), using the following cycling program in a thermal cycler with a heated lid: (1) 72 °C for 5 min (3′-primer extension); (2) 94 °C for 4 min (denaturation); (3) 55 °C for 5 min (annealing); (4) 72 °C for 8 min (primer extension). Steps (2) to (4) were repeated for 4 cycles, which should produce 8 copies of each unique sequence in the starting population (2^{n-1}, where n is the number of PCR cycles). The resulting DNA pool was purified by extraction with one volume of 50% (v/v) Tris-saturated phenol in chloroform, and concentrated by precipitation by addition of 2.5 volumes of ethanol.

2.3. Transcription and purification of RNA pools for selection

2.3.1. First selection round

Approximately 20 nmol of DNA were transcribed into RNA (50 mM HEPES-KCl pH 7.5, 25 mM $MgCl_2$, 5 mM of each NTPs, 5 U/μL of T7 RNA polymerase, Fig. 1B), in the presence of 200 μM of a 33-nt DNA oligonucleotide (5′-GCT TTA AGT CCA GGC GCT GAT AAA TGA TTT GTA-3′) that hybridizes to the RNA region encoding the potential cleavage site. This functions to inhibit ribozyme cleavage and retain full-length RNA constructs during the transcription process where millimolar concentrations of Mg^{2+} as well as other molecules that may serve as adventitious cofactors are present (Fig. 2). Transcription was terminated after an incubation of 2 h at 37 °C by adding one volume of loading buffer (90% formamide, 25 mM EDTA), and purified by electrophoresis through a denaturing 8% PAGE. The 185-nt RNA was visualized by UV shadowing, excised, and eluted overnight by electro-elution or passively with water. The eluted RNA was brought up to 100 mM NaCl and precipitated with 2.5 volumes of chilled ethanol.

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