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Ribozyme-catalyzed tRNA aminoacylation

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The RNA world hypothesis implies that coded protein synthesis evolved from a set of ribozyme catalyzed acyl-transfer reactions, including those of aminoacyl-tRNA synthetase ribozymes. We report here that a bifunctional ribozyme generated by directed *in vitro* evolution can specifically recognize an activated glutaminyl ester and aminoacylate a targeted tRNA, via a covalent aminoacyl-ribozyme intermediate. The ribozyme consists of two distinct catalytic domains; one domain recognizes the glutamine substrate and self-aminoacylates its own 5'-hydroxyl group, and the other recognizes the tRNA and transfers the aminoacyl group to the 3'-end. The interaction of these domains results in a unique pseudoknotted structure, and the ribozyme requires a change in conformation to perform the sequential aminoacylation reactions. Our result supports the idea that aminoacyl-tRNA synthetase ribozymes could have played a key role in the evolution of the genetic code and RNA-directed translation.

According to the RNA world hypothesis, the evolution of coded protein synthesis was a critical step in the transition from the RNA world to modern biological systems¹⁻³. Several lines of experimental evidence support the postulate that ribosomal RNA (rRNA) participates directly in protein synthesis, and it has been argued that primitive versions of the translation apparatus were fully RNA-based^{4,5}. In nature, the aminoacylation of tRNA is now catalyzed solely by protein enzymes, the aminoacyl-tRNA synthetases (aaRSs), whose amino acid specificities determine

the genetic code⁶. Therefore, a completely RNA-based protein synthesis system would require a set of RNA molecules capable of synthesizing aminoacyl-tRNAs in place of the protein aaRSs.

The accuracy of aminoacyl-tRNA synthesis relies on the specificity of the synthetases for both amino acid and tRNA^{1,7-9}. Ribozyme synthetases should be capable of similar specificity. There are now many examples of specific amino acid recognition by RNA aptamers¹⁰⁻¹². Furthermore, a number of aminoacyl-transferase ribozymes have been isolated by directed *in vitro* evolution, including self-aminoacylating RNAs^{13,14}, 3'- to 2'- or 5'-acyl-transferases^{15,16}, and amide and peptide synthetases^{17,18}. However, no ribozyme has been reported with the ability to specifically aminoacylate a distinct substrate RNA, such as a tRNA. Here we report the *in vitro* evolution of such a ribozyme.

Aminoacyl-transfer from donor substrate to tRNA

In previous work^{16,19,20} we isolated and characterized an acyl-transferase ribozyme by selecting for enhanced transfer of an N-biotinyl-L-methionyl (Biotin-L-Met) group from the 3' end of a donor hexanucleotide, 5'-pCAACCA-3', to the 5'-hydroxyl group of the ribozyme. Further experiments allowed us to delete non-essential regions of the original sequence, leading to a smaller version of the acyl-transferase ribozyme (82 nt), referred to as ATRib (Fig. 1a), that retains essentially full catalytic activity. The secondary structure of ATRib consists of four stems (P1-P4) and three hairpin-loops (L2-L4) arranged in a cloverleaf structure.

Since ATRib was selected to self-modify its own 5'-hydroxyl group, it acts as a single-turnover ribozyme. However, since the acyl transfer reaction is energetically neutral, the ribozyme-catalyzed reaction should be readily reversible. This led us to test whether another oligoribonucleotide 'acyl-acceptor', containing a sequence that would allow it to interact with the internal guide sequence (IGS) on the ribozyme, could accept the acyl group from acylated ribozyme (Fig. 1b). This 'ping-pong' process would potentially allow the ribozyme to act as a multiple-turnover catalyst.

We demonstrated this concept using tRNA^{Met} with an ATRib having a 5'-UGGUU-3' IGS (Fig. 2a). The deletion of G82 of

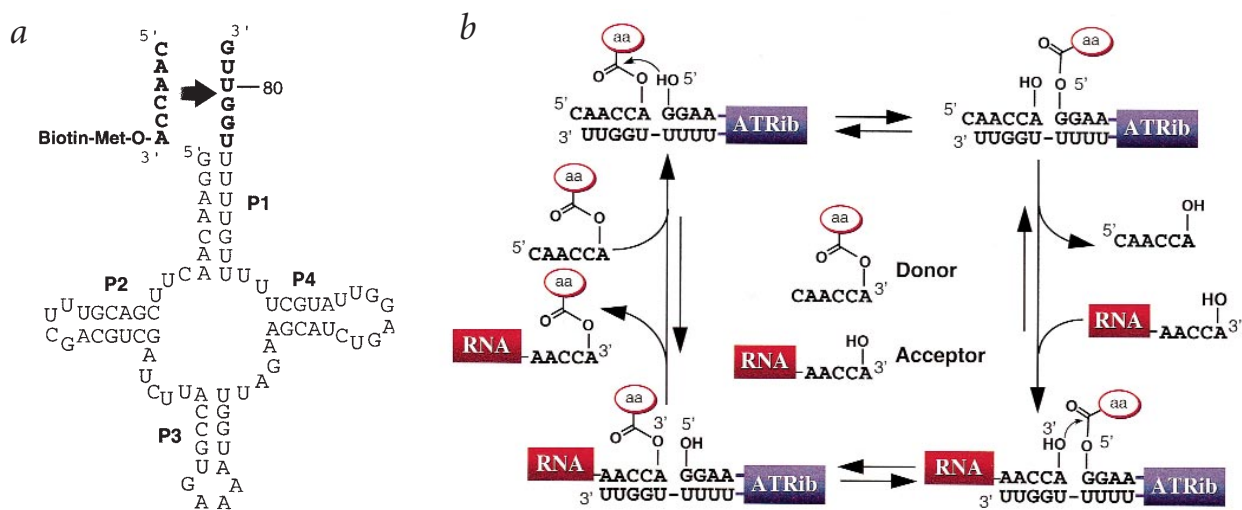


Fig. 1 Secondary structure of acyl-transferase ribozyme (ATRib) and schematic representation of a ATRib-catalyzed aminoacylation of acceptor RNA. **a**, Secondary structure. The ATRib self-aminoacylates the 5'-hydroxyl group in the presence of the acyl-donor hexanucleotide. Bold letters in the ribozyme denote the internal guide sequence (IGS), which is complementary to the sequence of the donor. **b**, Schematic representation of the reaction. The abbreviation 'aa' stands for amino acid. The G82 of the wild type ATRib is deleted for efficient aminoacylation on tRNA.

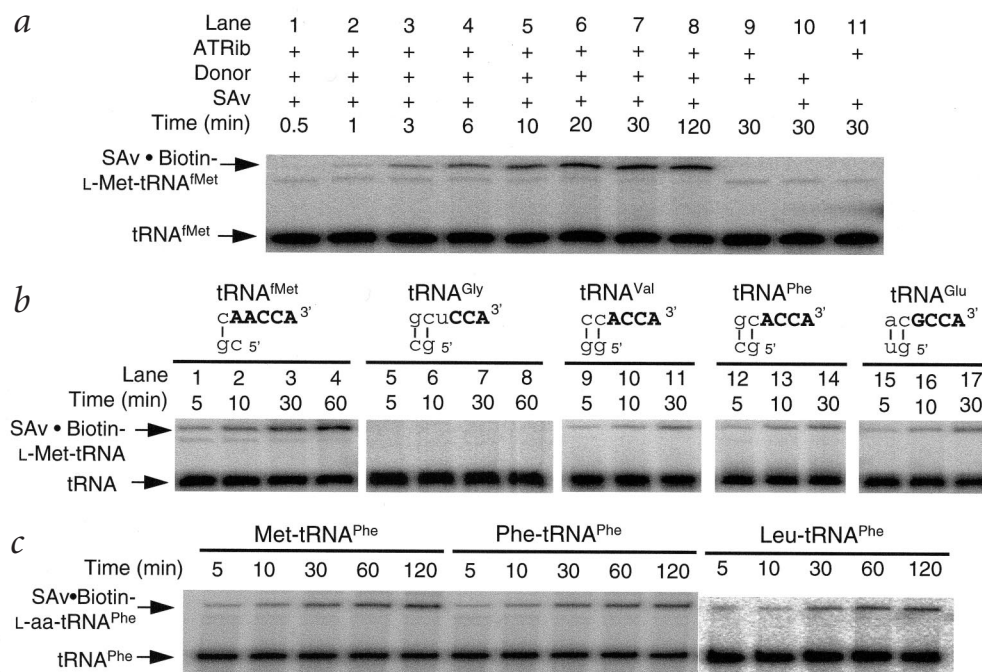


Fig. 2 Aminoacylation of tRNA catalyzed by ATRib. **a**, Methionylation of tRNA^{Met} catalyzed by ATRib, in which the IGS is 5'-UGGUU-3'. Single turnover conditions (2 μ M ATRib, 1 μ M tRNA^{Met}, and 10 μ M donor) were used in lanes 1–7, and the multiple turnover conditions (0.2 μ M ATRib, 1 μ M tRNA^{Met}, and 10 μ M donor) were used in lane 8. The abbreviation 'SAv' denotes streptavidin. **b**, Methionylation of cognate and non-cognate tRNAs catalyzed by ATRib. Sequence of the acyl-acceptor region of each tRNA is shown, and the bold letters denote the complementary sequence to that in the IGS of ATRib. Reactions were carried out under the multiple-turnover conditions (0.2 μ M ATRib, 1 μ M tRNA^{Met}, and 10 μ M donor). **c**, Aminoacylation of tRNA^{Phe} in the presence of various aminoacyl-hexanucleotides. Reactions were performed under the multiple-turnover conditions, where 0.5 μ M ATRib, 2 μ M tRNA^{Phe}, and 20 μ M donor for Biotin-L-Met- and Biotin-L-Phe-hexanucleotide or 10 μ M for Biotin-L-Leu-hexanucleotide.

ATRib (Fig. 1a) was essential for the ribozyme to exhibit catalytic activity for aminoacylation of tRNA. Under single turnover conditions (lanes 1–7), ~30% of tRNA^{Met} was acylated within 30 min. Under optimized multiple turnover conditions, conversion of 30–40% acyl-tRNA^{Met} was observed in 120 min (Fig. 2a, lane 8), corresponding to 1.5–2 turnovers. Because tRNA acylation results in only a small change in molecular weight, visualization of the new product by gel electrophoresis required the use of N-biotinyl-L-methionine as a substrate and addition of streptavidin to shift the mobility by binding to Biotin-L-Met-tRNA^{Met} (compare lanes 7 to lane 9). In the absence of either ribozyme or donor oligonucleotide, no acyl-tRNA^{Met} was detected (lanes 10 and 11), indicating that aminoacylation of tRNA^{Met} was catalyzed by the ribozyme.

The ribozyme-catalyzed tRNA charging reaction described above involves tRNA recognition by base-pairing to five complementary bases of the IGS. The five bases of the tRNA that are recognized include the CCA-3' sequence common to the 3' ends of all tRNAs, the so-called discriminator base (position 73, A73), and A72. On the basis of this proposed base-pairing, the ribozyme should be able to discriminate between some but not all tRNAs. The degree of ribozyme-catalyzed aminoacylation of tRNA is indeed directly related to the sequence complementarity (Fig. 2b). In contrast, the aminoacyl group of the substrate is not a critical determinant of binding. The ribozyme could transfer phenylalanyl and leucyl groups to tRNA^{Phe} with almost equal efficiency as the methionyl group (Fig. 2c).

Evolution of an amino acid recognition domain

Specificity of modern biological tRNA synthetases resides largely in the amino acid activation reaction. Addition of an amino acid-specific activation or charging domain to ATRib could therefore potentially generate a ribozyme with the fundamental properties of a true tRNA synthetase. In the scheme illustrated in Fig. 3a, the ribozyme reacts with an activated amino acid substrate to form a covalent acyl-intermediate that subsequently reacts with an accep-

tor tRNA. In principle this could be a multiple-turnover process.

In designing a selection procedure to implement this process, we wished to ensure that the ribozyme would recognize the activated amino acid substrate primarily through interactions with the amino acid, and not the activating group. We therefore avoided aminoacyl-adenylates as substrates, and focused instead on cyanomethyl ester (CME), a simple leaving group with no hydrogen bond donors or acceptors to interact with the ribozyme. Background acylation of pool RNA is negligible at 5 mM amino acid-CME and physiological pH¹⁹. We chose L-glutamyl-CME as our initial substrate, because its amide side chain should be a suitable recognition element for RNA (Fig. 3b). Selection was done with N-biotinyl-L-glutamyl-CME (Biotin-L-Gln-CME) in which the biotin is a selectable tag by streptavidin to isolate the rare active RNAs from the RNA population.

The design of the RNA pool was driven by the need to place the random sequence segment in a region accessible for the 5'-hydroxyl acylation without interfering with formation of the catalytic core of ATRib. On the basis of our structural studies of ATRib, the RNA pool was designed to have a 70 nt random sequence flanked by the 80 nt ATRib sequence at the 5'-end and a 20 nt constant sequence at the 3'-end (Fig. 3a).

The selection was designed in three phases (Fig. 3c), beginning with a simple selection for self-acylation of the ribozyme (phase I), followed by a more specific selection for acylation on the 5'-hydroxyl (phase II), and ending with selection for retention of the original oligonucleotide-ribozyme acyl-transfer reaction (phase III). Our goal was an RNA pool that is 'ambidextrously' active in self-aminoacylation of its own 5'-hydroxyl group using both substrates, and which would therefore be likely to contain ribozymes capable of transferring the glutamyl group first to itself and then to a tRNA. We carried out 12 rounds of the above selection (9, 1, and 2 rounds, respectively). The course of each phase of the selection was monitored by a streptavidin-dependent mobility-gel-shift assay (Fig. 3d–f). The 12th-round RNA

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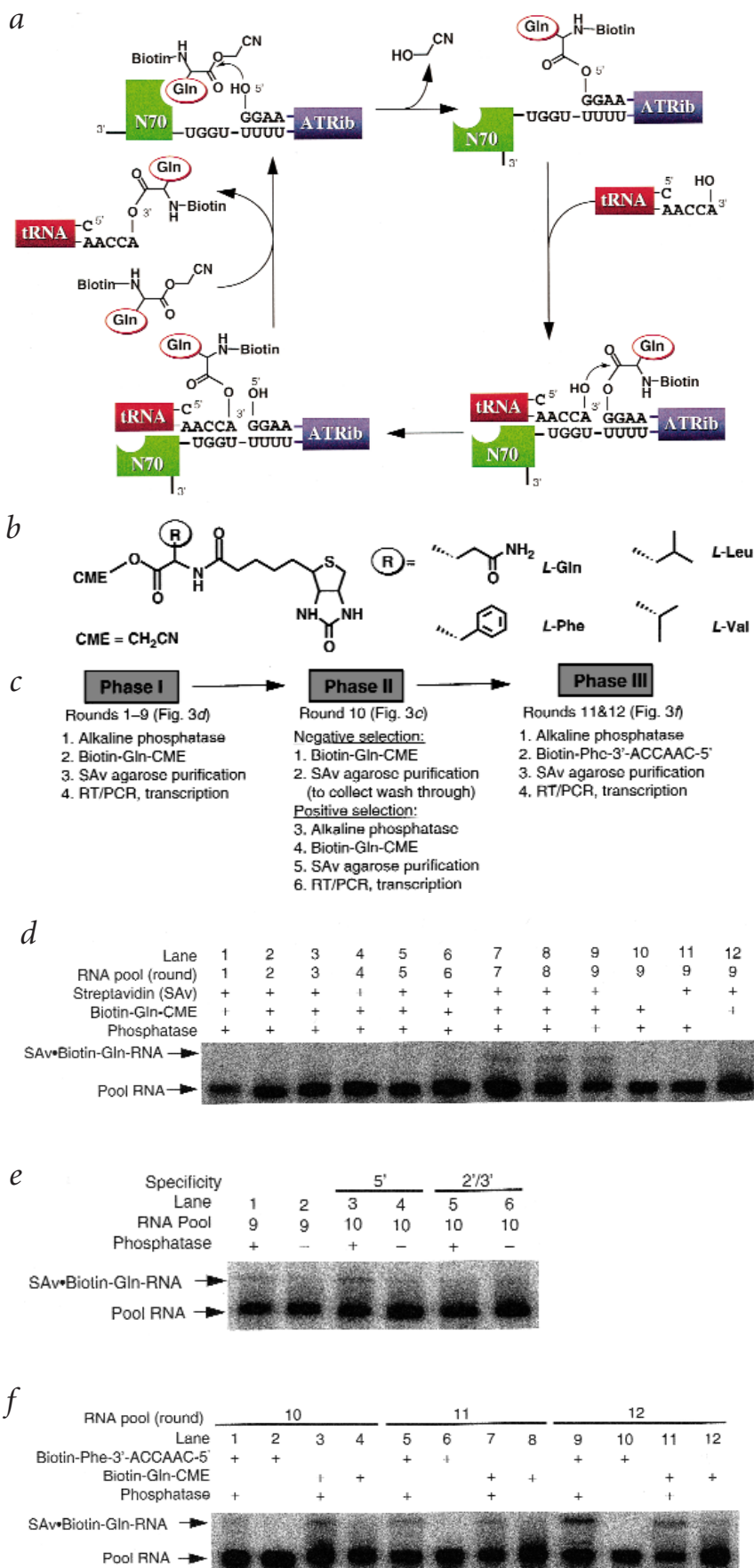


Fig. 3 *In vitro* evolution of aaRS-like ribozymes. **a**, Schematic representation of aminoacylation of tRNA catalyzed by an aaRS-like ribozyme. The 70 nt random region is shown in the green box. The 3'-end 20 nt constant region is indicated by a solid line. **b**, Structure of amino acid cyanomethyl ester (CME) substrates used in this study. The synthesis of substrates were performed by methods described elsewhere¹⁹ with minor modifications. **c**, Flowchart of the *in vitro* evolution of new catalytic domain for glutamine in ATRib. Procedures in each phase of the selection are listed. An initial RNA pool used in the experiment contained approximately 10¹⁵ different molecules. **d**, Phase I. SAV-dependent mobility-gel-shift assay was performed in each round of selected RNA (lanes 1–9). Control experiments for pool 9 RNA were no streptavidin (lane 10), no substrate (lane 11), and non-phosphatase (that is, 5'-triphosphate) pool 9 RNA. **e**, Phase II. The gel-shift assay shows the activity of pool 9 RNA with 5'-OH (lane 1) and 5'-triphosphate (lane 2), pool 10 (lanes 3 and 4) isolated from the negative-positive selection, and pool 10 (lanes 5 and 6) isolated from the negative selection. **f**, Phase III. Reactions were done in the presence of ~1 μ M pool RNA, 5 μ M Biotin-L-Phe-hexanucleotide or 5 mM Biotin-L-Gln-CME for 3 h at 25 °C. The gel-shift assay shows the activity of pool 10 RNA with 5'-OH and 5'-triphosphate (lanes 1–4), pool 11 (lanes 5–8), and pool 12 (lanes 9–12).

pool exhibited almost equal activity toward both Biotin-L-Gln-CME and Biotin-L-Phe-3'-ACCAAC-5' (Fig. 3f).

A total of 27 clones from pool 12 RNA were sequenced and aligned (Fig. 4a). We identified three sequence classes (classes I, II and III) and 14 unique sequences. Two clones from each of the classes, and all unique clones, were individually tested for activity with both substrates. Class I ribozymes exhibited activity with both substrates, whereas those in classes II and III exhibited preferential activity for the acyl-hexanucleotide rather than the glutamine substrate. We also identified five ambidextrous clones from the unique sequences. The remaining clones showed activity solely, or predominantly towards one substrate. We selected the AD02 ribozyme in class I for further studies.

The AD02 ribozyme exhibits self-aminoacylating activity with both Biotin-L-Gln-CME and Biotin-L-Phe-3'-ACCAAC-5' substrates (Fig. 4b, lanes 1 and 6, and 2 and 7, respectively). To explore the amino acid specificity of the ribozyme, we tested self-aminoacylation with three non-cognate Biotin-L-aminoacyl-CME substrates, phenylalanine, leucine, and valine (Fig. 4b, lanes 3–5, and lanes 8–10). Self-aminoacylating activity with all of these substrates is considerably reduced relative to the cognate substrate, indicating that the new catalytic domain has specificity for the

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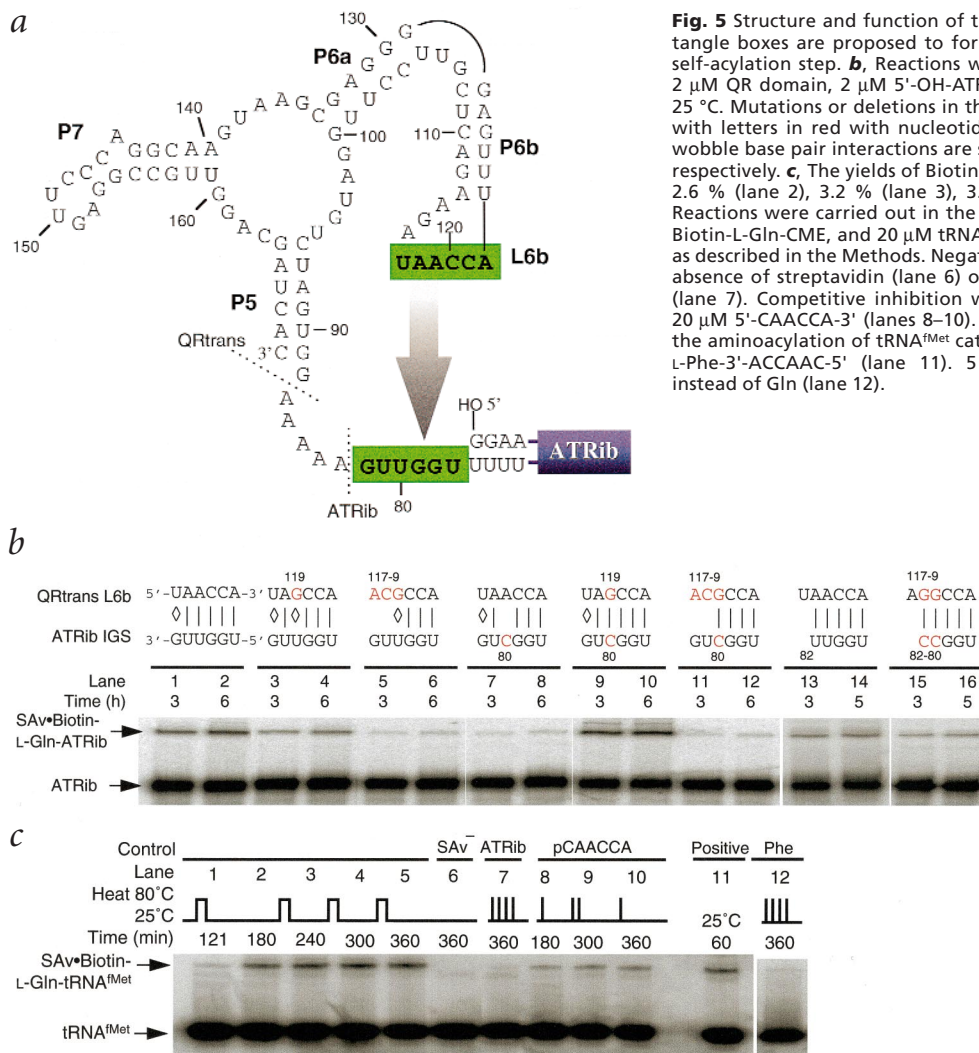


Fig. 5 Structure and function of the ribozyme. **a**, Bases in green rectangle boxes are proposed to form base pairs in the QR-dependent self-acylation step. **b**, Reactions were carried out in the presence of 2 μ M QR domain, 2 μ M 5'-OH-ATRib, and 5 mM Biotin-L-Gln-CME at 25 °C. Mutations or deletions in the ATRib and QRtrans are indicated with letters in red with nucleotide numbers. Watson-Crick and G:U wobble base pair interactions are shown by solid lines and diamonds, respectively. **c**, The yields of Biotin-L-Gln-tRNA^{fMet} were 0.1 % (lane 1), 2.6 % (lane 2), 3.2 % (lane 3), 3.6 % (lane 4), and 4.0 % (lane 5). Reactions were carried out in the presence of 2 μ M ribozyme, 5 mM Biotin-L-Gln-CME, and 20 μ M tRNA^{fMet}. Thermocycling was performed as described in the Methods. Negative controls were performed in the absence of streptavidin (lane 6) or replacing AD02 with 2 μ M ATRib (lane 7). Competitive inhibition was carried out in the presence of 20 μ M 5'-CAACCA-3' (lanes 8–10). Positive control was performed by the aminoacylation of tRNA^{fMet} catalyzed by ATRib using 5 μ M Biotin-L-Phe-3'-ACCAAC-5' (lane 11). 5 mM Biotin-L-Phe-CME was used instead of Gln (lane 12).

strate and with ATRib in place of the AD02 ribozyme (lane 7) yielded only the background level of aminoacyl-tRNA, showing that the QR domain of the ribozyme is essential for aminoacylation activity with glutaminyl-CME as substrate. Similarly, using Biotin-L-Phe-CME as substrate, which is inactive for self-acylation of the AD02, yielded only a background level of aminoacylated tRNA (lane 12). Addition of a competitor, non-radiolabeled pCAACCA, for the IGS strongly inhibited the aminoacylation on

$k_{cat} = 1.95 \times 10^{-3} \text{ min}^{-1}$ and $K_m = 158 \mu\text{M}$. For subsequent work, we set the concentration of the glutamine substrate at 5 mM. However, even when the ribozyme was pre-incubated with the glutamine substrate for 3 h (during which time it becomes largely self-acylated) and then mixed with a 10-fold excess of tRNA^{fMet}, the yield of aminoacylated tRNA was barely above background. One possible explanation for the observed low activity is that the ribozyme, once aminoacylated at its 5'-hydroxyl group, is incapable of binding tRNA due to cooperative interactions of the QR domain with the 5'-glutaminyl group and the IGS. Attempts to use mutations to decrease the strength of the QR domain interaction with the IGS unfortunately resulted in loss of self-acylating activity. We therefore used heat-induced denaturation to make the IGS open for binding to tRNA.

After four rounds of thermocycling, ~4% of the total input tRNA^{fMet} was aminoacylated (Fig. 5c, lanes 1–5), corresponding to 0.4 ribozyme equivalents. Appearance of the product band on the gel was dependent on the presence of streptavidin (lane 6), consistent with the product being the expected biotinyl-L-glutaminyl-tRNA^{fMet}. A product of identical mobility was generated by ATRib-catalyzed phenylalanyl transfer from the hexanucleotide to tRNA^{fMet} (lane 11). A control experiment that was performed with glutaminyl-CME as sub-

tRNA (lanes 8–10), consistent with the necessity of the ATRib domain in catalysis.

Conclusion

We have evolved a ribozyme that contains two distinct catalytic domains with different activities. These domains act sequentially to transfer an aminoacyl group first to the ribozyme itself, and then to tRNA, thus acting as an aminoacyl-tRNA synthetase. Two novel strategies were employed in the course of generating this ribozyme. First, a ribozyme that had been previously isolated as a standard self-modifying ribozyme was shown to act as a true multiple turnover catalyst simply by providing distinct donor and acceptor substrate oligonucleotides. The approach demonstrated here may be widely applicable for readily reversible reactions. Second, we generated a complex ribozyme with two active sites having distinct activities by performing two sequential stages of directed evolution. This may be a useful strategy for evolving ribozymes that catalyze sequential transformations on a substrate.

A conformational change must occur between the two sequential reactions catalyzed by the bifunctional ribozyme, since both reactions require base-pairing to the IGS. This conformational rearrangement is reminiscent of the changes that must occur

between the first and second reactions of the self-splicing group I and group II introns^{22–26}, as well as the more complex rearrangements that occur within the ribosome and the spliceosome^{27–31}. In our selected ribozyme, the necessary conformational change appears to be rate limiting overall. This raises an intriguing and challenging problem for future experiments to evolve ribozymes capable of switching between two conformations.

Although the activity of our aminoacyl tRNA synthetase-like ribozyme is modest, it is likely a sub-optimal catalyst because it was selected from a population that represented a very sparse sample of sequence space. Further directed evolution should yield ribozymes with greater catalytic activity and possibly higher amino acid and tRNA specificities. Since the *in vitro* evolution of aaRS-like ribozymes can be performed with any desired amino acid and tRNA, this strategy is a powerful method for the generation of useful catalysts for the synthesis of non-natural aminoacyl-tRNAs^{32,33}.

The ribozyme described here executes two of the key functions of an aminoacyl tRNA synthetase — specific amino acid recognition and charging of tRNA. Our results, coupled with previous demonstrations of ribozyme catalyzed amide and peptide bond formation, strongly support the idea that a translation system could have evolved in the RNA world from an initial set of simple ribozymes involved in acyl-transfer functions.

Methods

Aminoacyl-transfer reaction. Reactions were performed under the following conditions: 2 μ M ATRib (single-turnover) or 0.2 μ M (multiple-turnover), 1 μ M 5'-[³²P]-labeled tRNA (Sigma), and 10 μ M Biotin-L-aminoacyl-3'-ACCAAC-5' in a buffer containing 25 mM HEPES (pH 8.0), 100 mM KCl, 50 mM MgCl₂ at 25 °C. The ribozyme was preincubated with buffer in the absence of MgCl₂, heated at 95 °C for 1–2 min, and cooled to 25 °C. MgCl₂ was then added followed by a 5 min equilibration. The reaction was initiated by the addition of pre-mixed tRNA and donor hexanucleotide. At each time point, a 2 μ L aliquot was removed from 20 μ L reaction mixture, and quenched with 4 μ L MEUS buffer (25 mM MOPS, 5 mM EDTA, 8 M urea, 10 μ M streptavidin, pH 6.5). The resulting solution was analyzed by 6% polyacrylamide gel electrophoresis (PAGE) in a cold room to keep the gel temperature below 20 °C.

Pool construction. The oligonucleotides, DNA template corresponding to the sequence of ATRib, the 5'-primer containing T7 promoter sequence (5'-GGTAACACGCATATGT-AATACGACTCATATAGGAACAACCTGCAGCTTTC-3'), the random pool DNA (5'-GTGATCGTCCAACGGCCTC-N₇₀-ACCAAAAACAAAAGCATAACC-3'), and the 3'-primer (5'-GTGATCGTCCAACGGCCTC-3') were synthesized on an automated DNA synthesizer. After *Taq* polymerase-extension of these synthetic DNA templates, the full-length product was amplified by eight cycles of large-scale PCR in the presence of the 5'- and 3'-primers. Four equivalents of the pool DNA were transcribed by T7 RNA polymerase in the presence of α -[³²P]-UTP, and purified by PAGE. The resulting pool RNA was treated with calf intestinal phosphatase to remove the 5'-triphosphate.

Selection. The folded 1 μ M pool RNA (10 μ M for the first round of the selection) was incubated with 5 mM Biotin-L-Gln-CME in a EKM buffer (50 mM EPPS, 500 mM KCl, 100 mM MgCl₂, pH 7.5) at 25 °C for 3 h. This RNA was ethanol-precipitated twice, and the pellet was dissolved into a EKE buffer (50 mM EPPS, 500 mM KCl, 5 mM EDTA, pH 7.0), and applied to a column containing 0.25 ml (1 ml for the first round) of streptavidin-agarose gel. The slurry mixture was gently suspended for 30 min at 25 °C, and the resulting resin was washed 10-resin volumes of the EKE buffer, 4 M urea, followed by 5-resin volumes of water. Bound RNA was eluted with

10 mM biotin by heating at 95 °C for 10 min. The collected RNA was ethanol-precipitated and dissolved in 10 μ L of water. The remaining procedures were carried out as described¹⁶.

Construction of QRtrans and its mutants. The PCR-DNA template of AD02 ribozyme was passed through G-50 Sephadex columns (Boehringer Mannheim) to remove remaining primers from the PCR reaction. The purified DNA was used in the PCR reaction for generating the wild type QRtrans^{UAACCA} DNA using the corresponding internal 5'-primer containing T7 promoter sequence and the 3'-primer. Two mutant QRtrans^{UAGCCA} and QRtrans^{ACGCCA} DNAs were generated by the PCR reaction with the purified DNA using the internal 5'-primer and the 3'-primers containing the corresponding mutations.

Aminoacylation of tRNA. The folded 2 μ M AD02 ribozyme was equilibrated with MgCl₂ for 5 min, and the reaction initiated by the addition of pre-mixed 5 mM Biotin-L-Gln-CME and 20 μ M 5'-[³²P]-tRNA^{Met}. After incubating for 120 min at 25 °C, the reaction was subjected to PCR at 80 °C for 30 sec and 25 °C for 60 min. At each time point, a 2 μ L aliquot was removed from 20 μ L reaction mixture, was ethanol-precipitated twice, and the pellet was dissolved into 4 μ L MEUS buffer. The remaining procedures were the same as those in the acyl-transfer reaction.

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