A novel ribozyme with ester transferase activity

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Background: The 'RNA world' hypothesis proposes that the early history of life on earth consisted of a period in which chemical transformations were catalyzed exclusively by ribozymes. Ribozymes that act as acyl transferases, or catalyze the formation of amide or peptide bonds seem particularly attractive candidates to link the RNA world to the modern protein–nucleic acid world. The central role played by aminoacylated RNAs in today's processes of translating RNA into protein suggests that aminoacyl transfer reactions catalyzed by RNA might have facilitated the development and optimization of the translation apparatus during early evolution.

Results: We describe the isolation and characterization of a novel ribozyme that catalyzes the transfer of an amino-acid ester from an aminoacyl donor substrate onto the ribozyme itself. The site of aminoacylation was determined to be at an internal 2′-OH group of a cytosine residue. The aminoacylation depends on the presence of Mg^{2+} and can be competitively inhibited by the AMP moiety of the aminoacyl donor substrate, suggesting that there is a specific binding pocket for this substrate. The originally selected ribozyme was engineered to act as an intermolecular catalyst that transfers the amino acid onto an external 28-residue oligonucleotide. The aminoacylated oligonucleotide was further used to quantify the reverse reaction catalyzed by the ribozyme.

Conclusions: The ribozyme we have isolated is an example of a catalytic RNA with ester transferase activity which uses a substrate that is not templated by Watson–Crick-pairing hydrogen bonds. The reaction catalyzed by the ribozyme expands the scope of RNA catalysis to include acyl transferase activity from an RNA 3′ end to an internal 2′ position and the reverse. Ribozymes with such activity have been posulated to be evolutionary precursors of ribosomal RNA.

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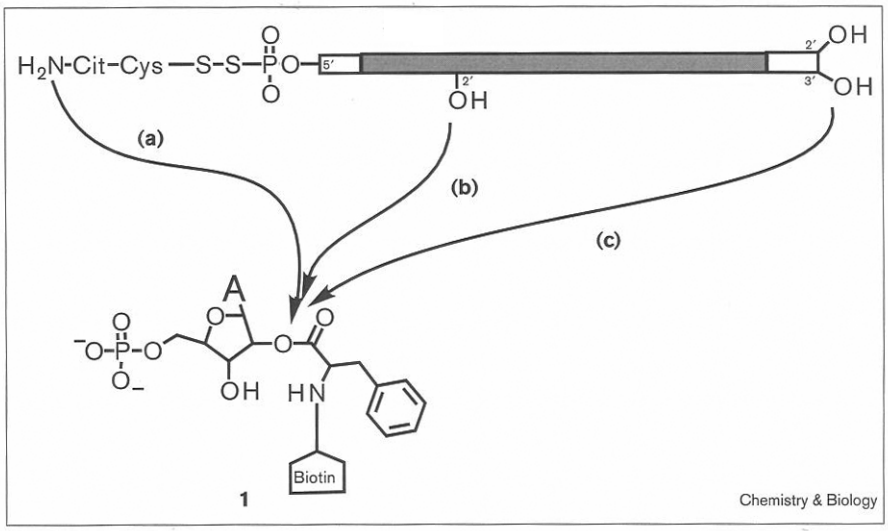
Introduction

RNA or DNA molecules with specific binding functionalities for a given target or with new catalytic activities can be isolated by screening combinatorial nucleic acid libraries [1,2]. The latest *in vitro* selection results clearly demonstrate that ribozymes or deoxyribozymes are able to catalyze a wide range of chemical reactions [3–9]. The generation of novel catalysts based on nucleic acids is important not only for possible biotechnological [7] or biomedical applications [10] but also because these molecules provide support for theories of an RNA world, in which the metabolism and replication of primitive organisms were accomplished and controlled by RNA enzymes [11,12].

Of particular interest are ribozyme-catalyzed reactions that link the RNA world to the 'protein world' of today — reactions demonstrating how protein translation systems could have evolved from a pre-existing RNA world. In a recent review, Hager *et al.* [4] have outlined five functions required of RNA if this transition could have occurred during evolution. RNA must be able to complex amino acids specifically [13], catalyze RNA aminoacylation [14,15], amide- or peptide-bond formation [16–18], and acyl-transfer reactions [16], as well as be able to activate amino acids. Of these activities for RNA, only the last has not yet been proven experimentally. In addition to their prebiotic relevance, RNAs that catalyze chemical transformations of these kinds may facilitate our understanding of enzymatic, ribozymatic and even ribosomal function. This notion is justified, for example, by strong evidence that 23S rRNA alone might be responsible for the peptidyl transferase activity of the 50S ribosomal subunit [19,20].

In order to expand our knowledge of RNA catalysis, we have designed and applied an *in vitro* selection scheme to isolate RNA molecules with peptidyl transferase activity. Our strategy involved generating a randomized RNA pool coupled by a disulfide linkage to a dipeptide containing a free α-amino group. The resulting amino-functionalized RNA pool was then incubated with an *N*-biotinylated phenylalanyl-2′(3′)-adenosine-5′-ester (Bio–Phe–AMP **1**; Figure 1) to select RNA molecules that catalyze peptide-bond formation between a dipeptide and the aminoacyl-donor substrate (Figure 1a). We anticipated that the primary amino group, an exposed nucleophilic site in these RNAs, would be the main position to react with the

Figure 1



Several nucleophilic sites within the RNA pool can potentially react with the aminoacyl ester group of the substrate molecule **1**, Bio–Phe–AMP. **(a)** Attack of the amino group leads to the desired peptide bond formation. **(b)** and **(c)** show that internal and terminal hydroxyl groups are competing sites for the peptidyl transferase activity: attack of an oxy-anion leads to aminoacylation of the RNA (covalent attachment of the biotinylated phenylalanine to the ribozyme). Cit, citrulline.

activated amino acid ester **1**. The newly formed biotinylated tripeptide would then be covalently attached to the RNA via the disulfide linker. After removal of all non-biotinylated members of the pool by streptavidin–agarose chromatography, active RNA molecules would be eluted by reduction of the disulfide bond with 2-mercaptoethanol, then amplified and used in iterative selection cycles. We assumed that elution with 2-mercaptoethanol would provide a stringent selection criterion, because RNAs catalyzing other possible side reactions (Figure 1b, c) would not be eluted. Instead of the desired peptide-bond formation activity, however, we obtained ribozymes that catalyzed the transfer of the ester from the Bio–Phe–AMP substrate **1** onto an internal 2′-OH group of the ribozyme.

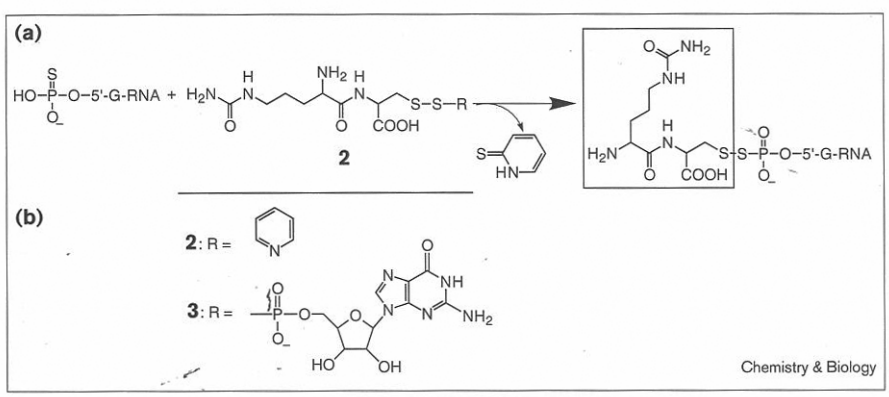
Results

Library construction

We generated a library of RNAs containing 120 randomized nucleotides, flanked by 20-residue constant regions and primed with guanosine monophosphorothioate (GMPS) at the 5′ end [21]. Differential migration on polyacrylamide gels containing [(β-acryloylamino)-phenyl]-mercuric chloride (APM–PAGE) demonstrated that more than 90% of the *in vitro* transcribed RNAs were primed with GMPS [22]. These RNAs were quantitatively reacted with citrulline–cysteine–S–S–pyridine (**2**; Figure 2) so as to covalently attach the dipeptide H_2N–Cit–Cys–COOH to the 5′ end of the RNA library via a disulfide linkage.

Stability of the disulfide linkage is critical to ensure that the dipeptide as well as the final biotin-tagged reaction product remains linked to the catalytic RNA during the incubation period, as the biotin unit is used as a tag with which to purify RNAs with the desired enzymatic activity. We therefore analyzed the stability of the amino-acid–S–S–RNA linkage using H_2N–Cit–Cys–S–S–(5′)–PO_3–guanosine (**3**; Figure 2) as a model compound. We incubated compound **3** in the selection buffer (200 mM NaCl, 5 mM $MgCl_2$, 50 mM

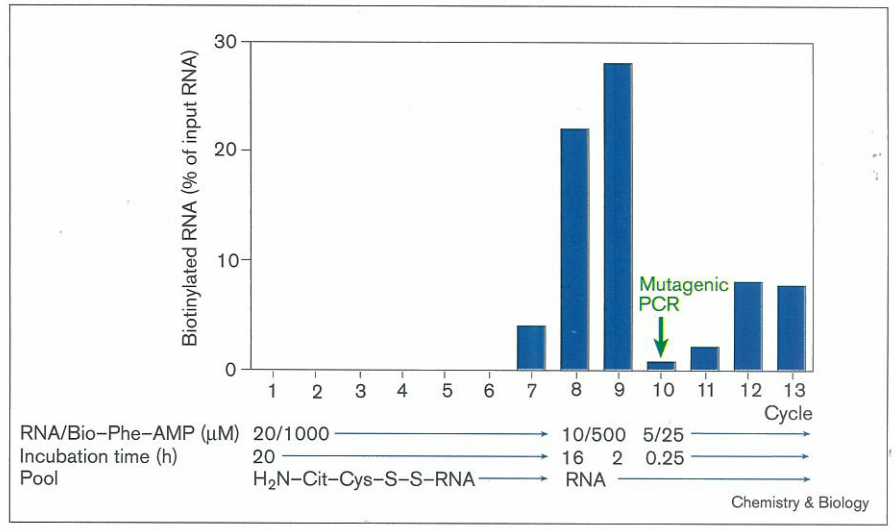
Figure 2



(a) Generation of the random RNA library 'tagged' with the citrulline–cysteine dipeptide. **(b)** Details of compounds **2** and **3**. Compound **3** was used to test the stability of the disulfide linkage used in the construction of the tagged RNA library.

Figure 3

Enrichment of biotinylated RNA at each round of selection. The ratio of RNA to substrate as well as incubation times are shown at the bottom. In the first six rounds the amount of biotinylated RNA was below 0.01%. In rounds 1–7, a 5′-amino-functionalized RNA pool (H_2N–Cys–Cit–S–S–RNA) was used. Starting from cycle 8 the selection was performed without the dipeptide coupled to the RNA. Mutagenic PCR was performed in cycle 10 to allow the evolution of more active variants.



K-Mops, pH 7.4) for up to 98 h at room temperature and then analyzed using high performance liquid chromatography (HPLC). After 26 h under the selection conditions 75% of **3** remained unchanged. In the presence of 2-mercaptoethanol, instantaneous reduction of the disulfide group was observed. To ensure that most of the sequences would still carry the dipeptide after a 20 h incubation, we used several copies of each of the pool RNAs in the initial cycle.

Selection

In the first six cycles of *in vitro* selection, 1 mM of the Bio–Phe–AMP substrate **1** was incubated with 20 μM of the radiolabeled H_2N–Cit–Cys–S–S–RNA library for 20 h in selection buffer described above. Biotinylated RNA molecules were then immobilized on streptavidin–agarose, and nonbiotinylated RNAs were removed by extensive washing with denaturing buffers to avoid the selection of matrix binders. Bound RNAs were eluted from the streptavidin resin by washing with a buffer containing 0.2 M 2-mercaptoethanol, reverse-transcribed, and amplified using the polymerase chain reaction (PCR). *In vitro* transcription of the resulting DNA yielded an enriched RNA pool which was used as the input for the next round of selection. Quantification of the eluted RNAs revealed no detectable enrichment of activity in the first six rounds. After the seventh cycle, however, the amount of streptavidin-immobilized RNA rose to 4% of the input RNA (Figure 3). Unexpectedly, when incubated with elution buffer only a small portion of the bound RNA could be washed off the streptavidin resin. A considerably larger fraction of the immobilized RNA was eluted when the 2-mercaptoethanol concentration was raised to 2 M. This behaviour of the selected pool was inconsistent with RNA molecules linked to the streptavidin resin through a disulfide bond, as a low 2-mercaptoethanol concentration would be expected to cleave the disulfide bond and quantitatively yield the immobilized RNA [23]. Furthermore, in a series of pilot experiments using GMPS-primed RNA bound to thiopropyl agarose we were able to collect all of the RNA in a single elution step with 0.1 M 2-mercaptoethanol. We therefore tested whether the 5′-dipeptide group was actually required for the self-biotinylation reaction. Round 7 DNA was transcribed without GMPS and incubated with Biotin–Phe–AMP for 20 h. Exactly the same amount of RNA became self-biotinylated and attached to streptavidin, indicating that we had quantitatively enriched for a catalytic activity other than one catalysing peptide-bond formation. At this point we decided to investigate what kind of activity had been selected, so we removed the GMPS-priming and dipeptide-coupling steps in further rounds of selection. Moreover, to increase the stringency in subsequent cycles, the concentrations of both the substrate Bio–Phe–AMP and the RNA library, as well as the incubation time, were gradually reduced (Figure 3). At cycle 10, the PCR amplification was carried out under mutagenic conditions, to allow the evolution of even more active variants with improved activity [24]. The selection was then continued for three more rounds using standard PCR amplification. After cycle 13, the selected RNAs were cloned and sequenced.

Selected sequences

Sequences from 27 clones were obtained, revealing three major classes of selected sequences (Figure 4). Class I contained 11 sequences, class II had 10, and class III had five members. In each class, individual members differ only by single-base point mutations, probably as a result of additional diversity introduced during the mutagenic PCR step in cycle 10, whereas no obvious conservation of sequence or sequence elements could be identified when comparing sequences from different classes. One unique

Figure 4

Class I

Clone	Sequence	k_{obs} (h^{-1})
3	CGCGCTTAGTGGCGAACGTCCGATATCGTTTTATTGTGAGCTCGCAGGGTCATAAACCGGATGAGTATCCAATGGGAGTCAATGAGTGTTGTTGTTTCCCATGTCATCGCGGGAATCTTC	
6	CGCGCTTTGTGGCGAACGTCCTATATATTTTTATTGTGAGCTCGCAGGGTCATAAACCGGATGAGCATCCAATGGGAGTCAATGAGTGTTGTTGTTTCCCATGTCATCGCGGGAATCTGG	
9	CGCGCTTTGTGGCGAACGTCCAATATCGTTTTATTGTGAGCTCGCAGGGTCATAAACCGGATGAGTGTCCAATGGGAGTCAATGAGTGTTGTTGTTTCCCATGTCATCGCGGGAATCTTC	
10	CGCGCTTCGTGGCGAACGTCCAATATCGTTTTATTGTGAGCTCGCAGGGTCATAAACCGGAAGAGCATCCAATGGGAGTCAATGAGTGTTGTTGTTTCCCATGTCATCGCGGGAATCTTC	
17	CGCGCTTTGTGGCGAACGTCC_ATATCGTTTTATTGTGAGCTCGCAGGGTCATAAACCGGATGAGTGTCCAATGGGAGTCAATGAGTGTTGTTGTTTCCCATGTCATCGCGGGAATCTTC	2.2 ± 0.3
18	CGCGCTTCGTGGCGAACGTCC_ATATCGTTTTATTGTGAGCTCGCAGGGTCATAAACCGGATGAGTGTCCAATGGGAGTCAATGAGTGTTGTTGTTTCCCATGTCATCGCGGGAATCTGG	
23	CGCGCTTTGTGGCGAACGTCCAATATCGTTTTATTGTGAGCTCGCAGGGTCATAAACCGGATGAGTGTCCAATGGGAGTCAATGAGTGTTGTTGTTTCCCATGTCATCGCGGGAATCTTC	
25	CGCGCTCTGTGGCGAACGTCCAATATCGTTTTATTGCGAGCTCGCAGGGTCATAAACCGGATGAGTGTCCAATGTGAGTCAATGAGTGTTGTTGTCTCCCATGTCATCGCGGGAATCTTC	
28	CGCGCTTTGTGGCGAACGTCC_ATATCGTTTTATTGTGAGCTCGCAGGATCATAAACCGGATGAGTGTACAATGGGTGTCAATGAGTGTTGTTGTTTACCATGTCATCGCGGGAATCTTC	
29	CGACCTTTGTGGCGAACGTCCAATATCGTTTTATTGTGAGCTCGCAGGGTCATAAACCGGATGAGTGTCCAATGGGAGTCAATGAGTGTTGTTGTTTCCCATGTCATCGCGGGAATCTTC	1.9 ± 0.3
36	CGCGCTTTGTGGCGAACGTCCTATATCTTTTTATAGTGAGCTCGCAGGGTCATAAACCGGATGAGTGTCCAATGGGAGTCAATGAGTGTTGTTGTTTCCCATGTCATCGCGGGAATCTTC	

Class II

Clone	Sequence	
4	GGGGCGACGTTCTATGGAGCGCCTCATGATGTCCGTCATGTCGCACGACGGGCACGTGTTCGTCTTAGCGCGCATTTTGAGAGAGTGTTACTCATTATGGTTGCGAGCGCGTGTATCAGAG	
7	GGGGCGACGTTCTATGGAGCGCCTCATGATGTCCGTCATGTCGCACGACGGGCACGTGTTCATCTTAGCGCGCATTTTGAGAGAGGGTTACTCATTATGGTTGCGAGCGCGTGTATCAGAG	
15	GGGGCGACGTTCTATGGAGCGCCTCATGATGTCCGTCATGTCGCACGACGGGCACGTGTTCGTCTTAGCGCGCATTTTGAGAGAGTGTTACTCATTATGGTTGCGAGCGCGTGTATCAGAG	
16	GGGGCGACGTTCTATGGAGCGCCTCATGATGTCCGTCATGTCGCACGACGGGCACGTGTTCATCTTAGCGCGCATTTTGAGAGAGGGTCACTCATTATGGTTGCGAGCGCGTGTATCAGAG	
19	GGGGCGACGTTCTATGGAGCGCCTCATGATGTCCGTCATGTCGCACGACGGGCACGCGTTCGACTTAGCGCGCATTTTGAGAGAGGGTTACTCATTATGGTTGCGAGCGCGTGTATCAGAG	
26	GGGGCGACGTTCTATGGAGCGCCTTATGATGTCCGTCATGTCGCACGACGGGCACGTGTTCGTCTTAGCGCGCATTTTGAGAGAGGGTTACTCATTATGGTTGCGAGCGCGTGTATCAGAG	2.1 ± 0.3
27	GGGGCGACGTTCTATGGAGCGCCTCATGATGTCCGTCATGTCGCACGACGGGCACGTGTTCATCTTAGCGCGCATTTTGAGAGAGGGTTACTCATTATGGTTGCGAGCGCGTGTATCAGAG	
30	GGGGCGACGTTCTATGGAGCGCCTCATGATGTCCGTCATGTCGCACGACGGGCACGTGTTCGTCCTAGCGCGCATTTTGAGAGAGGGTTACTCATTATGGTAGCGAGCGCGTGTATCAGAG	2.9 ± 0.2
33	GGGGCGACGTTCTATGGAGCGCCTCATGATGTCCGTCATGTCGCACGACGGGCACGTGTTCGTCTTAGCACGCATTTTGAGAGAGGGTTACTCATTATGGTTGCGAGCGCGTGTATCTGAG	
35	GGGGCGACGTTCTATGGAGCGCCTCATGATGTCCGTCAAGTTGCACGACGGGCACGTGTTCGACTTAGCACGCATTTTGAGAGAGGGTTACTCATTATGGTTGCGAGCGCGTGTATCTGAG	

Class III

Clone	Sequence	
5	CTATTGTGTCCTTTGCCGGCGAGCGCAGTATGATCGTCACTCTGCGCCCTTCGTGTTAAGGGAGTATTCATTTTCCAGTCGTGACGGGGAGAGTAGCTGTTACGATTGTTACTCCCGAGTCGGG	
11	CTATTGTGTCCTTTGCCGGCGAGCGCAGTACGATCGTCACACTGCGCCCTTCGTGTTAAGGGAGTATTCATTTTCCAGTCGTGACGGGGAGAGTAGCTGTTACGATTGTTACTCCCGAGTCGGG	1.8 ± 0.4
24	CTATTGTGTCCTTTGCTGGCGAGCGCAGTACGATCGTCACACTGCGCCCTTCGTGTTAAGGGAGTATTCATTTTCCAGTCGTGACGGGGAGAGTAGCTGTTACGATTGTTACTCCCGAGTCGGG	
31	CTATTGTGTCCTTTGCCGGCGAGCGCAGTACGATCGTCACACTGCGCCCTTCGTGTTAAGGGAGTATTCTTTTTCCAGTCGTGACGGGGAGAGTAGCTGTTGCGATTGTTACTCCCGAGTCGGG	2.3 ± 0.2
32	CTATTGTGTCCTTTGCTGGCGAGCGCAGTACGATCGTCACACTGCGCCCTTCGTGTTAAGGGAGTATTCATTTTCCAGTCGTGACGGGGAGAGTAGCTGTTACGATTGTTACTCCCGAGTCGGG	

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Sequences of the selected clones. Only sequences from randomized regions are given. Mutations within individual clones are shown in red. For comparison, the values of k_{obs} of pool 9 = 0.8 ± 0.2 h^{-1}, pool 13 = 2.2 ± 0.6 h^{-1}.

sequence, not shown in Figure 4, was isolated that does not fall into any of the other three classes.

Clone 11, a member from class III, was investigated in further detail as class III showed slightly increased elution behaviour than the other two classes when incubated with 2-mercaptoethanol.

Deletion analysis and Mg^{2+} dependence of the clone 11 ribozyme

An extensive deletion analysis of clone 11 was carried out by constructing a series of truncated versions of the ribozyme, to examine the minimal sequence requirements for catalytic activity. This was achieved by nested PCR amplification of the DNA using a series of appropriate primers, and subsequent *in vitro* transcription. Table 1 summarizes the sequences and activity of the various constructs tested. Figure 5 shows the minimal 117 nucleotide sequence that is necessary for self-biotinylation.

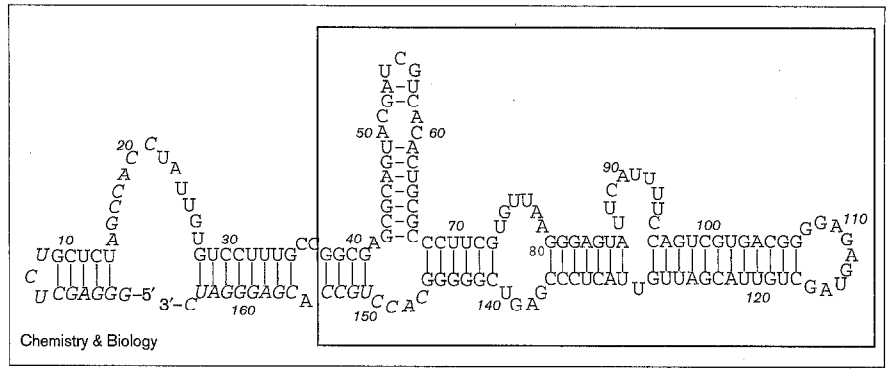
Deletion of the first 28 nucleotides of clone 11, which includes the entire 5′-primer-binding site, results in even more activity in self-biotinylation compared to the full-length clone, whereas deletion of only the four 3′-terminal bases resulted in complete loss of activity. The ten 3′-terminal bases can, however, be deleted if, at the same time, the first 37 bases at the 5′-end are removed as well. Among the various constructs with truncations from both termini, sequence 38–154 was the only one that retained any activity (Figure 5, framed sequence). Attempts to reduce this minimal motif further failed: we deleted parts of the hairpin helix 96–128 in the minimal construct 38–154 and found that this construct, designated 38–154_{small}, is inactive (Table 1).

It is also revealing to compare constructs 35–164 and 38–164. The longer one, 35–164, is virtually inactive catalytically, but the construct which lacks three more nucleotides at the 5′ end, 38–164, is nearly as active as the full-length clone. We assume that the presence of the three 5′-terminal nucleotides in 35–164 leads to an entrapment of the catalyst in an inactive conformation, as has been discussed in more general terms for RNA folding [25,26]. Along the same lines, the results obtained with the full-length clone 1–164 compared to the deletion construct 1–160 provide an interesting example of how arbitrary sequence (in this case, four additional bases at the 3′ end) can influence the catalytic efficiency of a ribozyme.

The catalytic activity of the selected RNAs strongly depends on the presence of Mg^{2+} (Figure 6). In the absence of Mg^{2+}, clone 11 lacks detectable enzymatic

Figure 5

Predicted secondary structure of clone 11 [51] and minimal sequence motif of the ribozyme active in intramolecular aminoacylation. Deletion analysis revealed that only nucleotides within the boxed region are necessary for catalysis.



activity, and the optimal Mg^{2+} concentration for clone 11 is around 12.5 mM. Whether the divalent metal ions are essential for RNA folding or are required for catalysis (or both), remains to be investigated.

Determination of the reaction site

Next, we sought to determine the reactive site within the clone 11 ribozyme. RNA radiolabeled at its 5′ or 3′ ends was incubated with substrate **1** and loaded onto a streptavidin column. After removal of the nonbiotinylated molecules, the immobilized RNAs were partially digested at guanine residues with RNase T1. The nonbiotinylated RNA fragments resulting from this treatment were then collected by washing with a buffer, whereas the remaining biotinylated fragments were eluted with buffer containing 2 M 2-mercaptoethanol and collected separately. Both samples were analyzed using denaturing polyacrylamide gel electrophoresis (data not shown). This analysis revealed that the reaction site has to be located within the 3′-constant region, or in close proximity to it, because fragments starting from position 137 did contain the biotin tag.

In order to facilitate analysis of the exact position that became modified and to investigate whether or not the clone 11 ribozyme could be turned into an intermolecular catalyst, we constructed an oligonucleotide substrate and a catalytic portion of the originally selected intramolecular ribozyme. The catalytic portion, designated 'ribozyme 28–136', ranges from position 28 to position 136, whereas the 'substrate' corresponds to the 3′ terminus of clone 11, residues 137–164 (Figure 7a). When incubated with ribozyme 28–136 and Bio–Phe–AMP **1**, a significant portion of the 28-mer oligonucleotide substrate could be immobilized on streptavidin, revealing that ribozyme 28–136 catalyzes the biotinylation of the 28-mer oligonucleotide substrate.

To determine which nucleotide position had reacted, we sequenced the 5′-radiolabeled RNA in the biotinylated 28-mer oligonucleotide substrate by digesting it with a number of different base-specific RNases. The result is shown in Figure 7b. The sequencing pattern for the biotinylated and the nonbiotinylated RNAs is the same from the 3′ end up to position C147. The unreacted 28-mer shows the expected band at C147 in the U/C lane (Figure 7b, blue circle) but no band is visible for the biotinylated 28-mer oligonucleotide (Figure 7b, red frame), indicating that biotinylation results in nuclease resistance

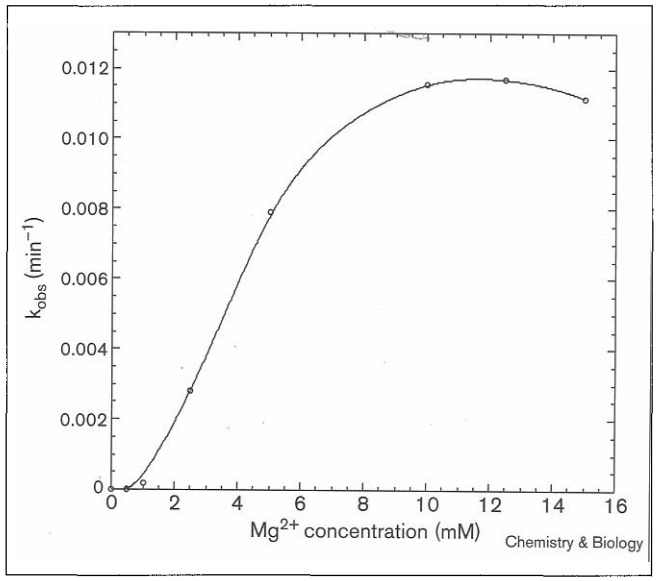
Table 1

Comparison of the intramolecular aminoacylation rates of truncated clone 11 variants.

Clone 11 construct (nucleotide position)	k_{obs} (h^{-1})*
1–164	1.8 ± 0.4
16–164	2.7 ± 0.2
28–164	5.6 ± 0.1
35–164†	nd‡
38–164†	3.6 ± 0.3
43–164	0
48–164	0
1–160	0
1–154	0
1–145	0
38–160	0
38–154	1.0 ± 0.4
38–145	0
38–164small§	0

*Reaction conditions: ribozyme (5 μM) was incubated with **1** (50 μM) at room temperature in selection buffer containing 12.5 mM $MgCl_2$. Aliquots were taken at different timepoints and coupled on streptavidin agarose. First order rate constants were obtained from the data by fitting the equation $[P]_t = [RNA] - [RNA]\cdot\exp(-k_{obs}\cdot t)$, where $[P]_t$ is the concentration of aminoacylated RNA at time t and [RNA] is the concentration of clone 11. †These constructs were assayed in triplicate. The activity of all other constructs was measured in duplicate. The length of the RNA was confirmed by PAGE. ‡The reaction was too slow to calculate k_{obs} (nd, not determined). §In this construct the five terminal base pairs and apical GGAGAGUAG loop of the hairpin helix (96–128) were deleted. Helix 38–41/150–154 was closed by a GUGA loop. The sequence of this construct is: 5′-GGG AUU GUU ACU CCC GAG UCG GGG GCA CCU GCC CGU GAG GGC GAG CGC AGU ACG AUC GUC ACA CUG CGC CCU UCG UGU UAA GGG AGU AUU CAU UUU CCA GUC CC-3′.

Figure 6



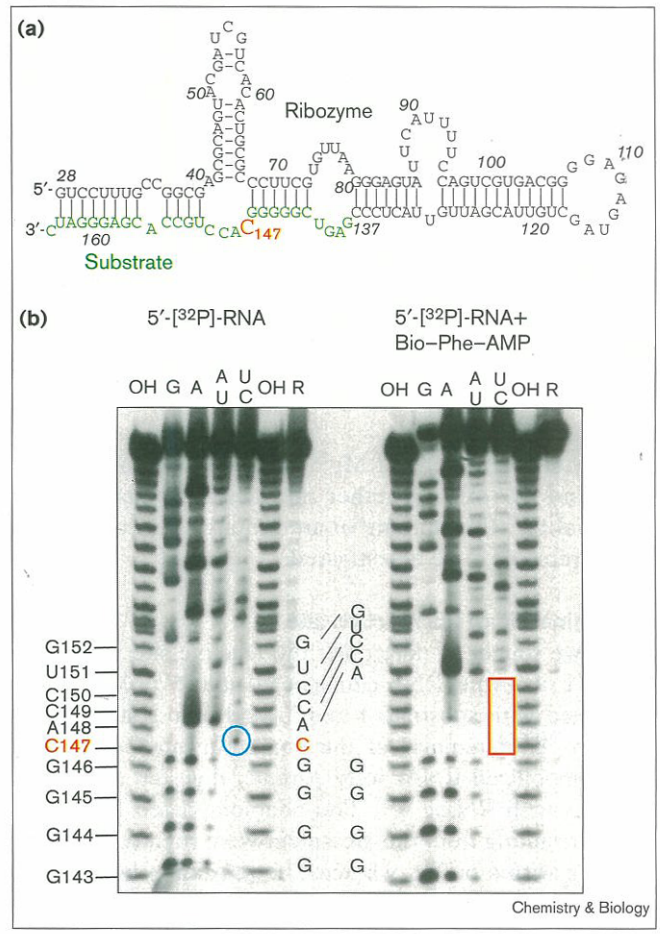
The ribozyme activity of clone 11 is dependent on Mg^{2+}. Reactions were conducted at room temperature with 5 μM of clone 11 RNA and 25 μM of **1** in a selection buffer whose Mg^{2+} concentration was adjusted to the values shown on the x axis. Aliquots were taken at six different timepoints, coupled on streptavidin and quantified as described in the Materials and methods section. RNA precipitation was observed at $MgCl_2$ concentrations above 50 mM.

at this position. Furthermore, the bands corresponding to positions 148–164 of the substrate oligomers that have reacted with **1** exhibit significantly reduced gel mobility compared with the fragments of unreacted 28-mer, probably because these fragments are covalently attached to the biotin tag. Taken together, these results strongly suggest that C147 in the 28-mer oligonucleotide substrate is the position that has reacted.

Clone 11 RNA is an ester transferase

To determine to which functional group of residue C147 the biotin tag has been attached, we studied a series of modified versions of the 28-mer oligonucleotide substrate, summarized in Table 2. When C147 is replaced by a uracil residue, the substrate is biotinylated as efficiently as the 'wild-type' 28-mer oligonucleotide substrate. This result provides strong evidence that it is not the base itself that is modified. We next analyzed whether the 2′-OH group is required for catalytic activity by testing 28-mer oligonucleotides in which the hydroxyl group of the ribose at position 147 was either methylated or absent. There was no observable activity with these variants, suggesting that the 2′-OH of ribose 147 is the group that reacted with **1**. We also analyzed a 28-mer variant in which an amino group was substituted for the 2′-OH of C147. This oligonucleotide was also inactive as a substrate for this ribozyme.

Figure 7

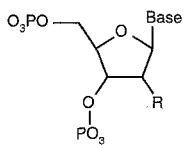


(a) Proposed secondary structure of ribozyme 28–136 and its 28-mer oligonucleotide substrate (green). The aminoacylated position C147 is shown in red. **(b)** RNase sequencing of the wild type (left) and the aminoacylated 28-mer oligonucleotide substrate (right). The difference in the pattern of the two substrates indicates that residue C147 is the position modified by ribozyme 28–136. Aminoacylated substrate was prepared as follows: 10 μM ribozyme 28–136 was incubated with traces of 5′-labeled 28-mer oligonucleotide substrate and 1 mM of **1** in selection buffer. After 120 min the RNA was precipitated and loaded onto a streptavidin column. Nonbiotinylated RNAs were removed and biotinylated oligonucleotide substrates eluted with 2 M 2-mercaptoethanol. RNase sequencing was performed according to the manufacturer's protocol (Pharmacia) using ribonuclease T1 for the lane labeled G, RNase U2 for A, RNase Phy M for A/U and RNase *B. cereus* for U/C. OH: alkaline digestion ladder; R: untreated control RNA. Fragments were separated by 20% denaturing polyacrylamide electrophoresis and visualized by autoradiography. The result was confirmed using a 3′-labeled 28-mer oligonucleotide substrate (data not shown).

Having demonstrated that the 2′-OH group of C147 is involved in biotinylation, we reasoned that the most likely chemical transformation catalyzed by the ribozyme had to be the transfer of the biotinylated amino acid from Bio–Phe–AMP to the hydroxyl functional group. To find out which portion of the Bio–Phe–AMP substrate was

Table 2

Comparison of the intermolecular aminoacylation rates using 2′-modified 28-mer oligonucleotides as substrates.



Compound	R	$Base_{147}$	k_{obs} (min^{-1})
28	OH	C	$0.019 \pm 5 \times 10^{-4}$
28-dC	H	C	0
28-OCH_3	OCH_3	C	0
28-NH_2	NH_2	C	0
28-U	OH	U	$0.023 \pm 1.6 \times 10^{-3}$

Reaction conditions: ribozyme 28–136 (5 μM) was incubated with the modified 28-mer oligonucleotide substrate (10 nM) and **1** (1 mM), at room temperature in selection buffer containing 12.5 mM $MgCl_2$.

transferred onto the 28-mer oligonucleotide substrate, we performed a matrix-assisted laser desorption/ionization–time-of-flight (MALDI–TOF) mass spectral analysis comparing the biotinylated 28-mer product and the unmodified 28-mer (Figure 8). The molecular weight of the modified 28-mer oligonucleotide substrate was determined by averaging several measurements in the presence of the unmodified 28-mer as an internal standard. This analysis confirmed that ribozyme 28–136 catalyzes the transfer of the Bio–Phe moiety from **1** to the 28-mer oligonucleotide substrate and provides strong evidence that an ester transfer reaction is catalyzed (Figure 8).

As one would expect for an ester group at the 2′-OH position, the esterified 28-mer oligonucleotide exhibits moderate stability when incubated under mild alkaline conditions. Incubation in 100 mM $NaHCO_3$ (pH = 9.0) and heating to 90°C for 5 min, however, resulted in quantitative loss of the biotinyl-phenylalanine ester group as observed by polyacrylamide gel electrophoresis. This result is illustrated in Figure 7b. The Bio–Phe–AMP-reacted bands which correspond to positions 148–152 exhibit reduced gel mobility when partially digested with various ribonucleases, whereas the same bands do not have altered gel mobility when partially alkaline-digested. This result is consistent with preferential hydrolysis of the labile carboxylic ester group under these conditions.

Kinetic analysis of the clone 11 ester transferase ribozyme

Similar activities were identified when several clones from all three classes (Figure 4) were tested for intramolecular aminoacylation. Kinetic analysis of the *in cis* reaction catalyzed by the clone 11 ribozyme revealed a k_{cat} of $0.04 \pm 9 \cdot 10^{-4}$ min^{-1}, a K_m of 119 ± 9 μM, and a k_{cat}/K_m of $3.5 \times 10^2 M^{-1}$ min^{-1} (Figure 9). The uncatalyzed reaction was undetectable in our system, so we could not calculate the rate enhancement achieved by the ribozyme. The yield of the 2′-aminoacylated clone 11 does not exceed 40% even at the highest concentrations of **1** used (2.0 mM), indicating that the reaction catalyzed by the ribozyme reaches equilibrium because of a relatively high rate of the reverse reaction — that is the aminoacylation of AMP by the 2′-aminoacylated ribozyme. This was further investigated in the *in trans* reaction, using the 28-mer oligonucleotide substrate.

First, we determined at what concentration of ribozyme 28–136 a further enhancement of the ribozyme concentration did not result in a change of the observed rate constant (k_{obs}). The kinetics of the intermolecular reaction under single turnover conditions at various concentrations of **1** or AMP, respectively, were then performed at enzyme saturation with 28-mer (forward) and 28-mer–Phe–Bio

Figure 8

A representative MALDI–TOF analysis of the reaction product generated by intermolecular transfer of biotinylated phenylalanine onto the 28-mer oligonucleotide substrate. Ribozyme 28–136 (5 μM) was incubated with 28-mer oligonucleotide substrate (1 μM) and **1** (1 mM) in selection buffer for 120 min. The reaction was quenched by precipitation with ethanol and the mixture injected into a Dynamo mass spectrometer. The molecular weight of the modified 28-mer oligonucleotide substrate was determined as the average of several measurements in the presence of unmodified 28-mer as an internal standard. For the difference between the unreacted and reacted material a $\Delta_{measured}$ of 370.5 Da was calculated. The theoretical value for a Bio–Phe–C(O) esterified with an OH on the oligo is $\Delta_{theoretical}$ of 372.5 Da. The difference of 2 Da between reacted and unreacted material is within the error range of the expected value.

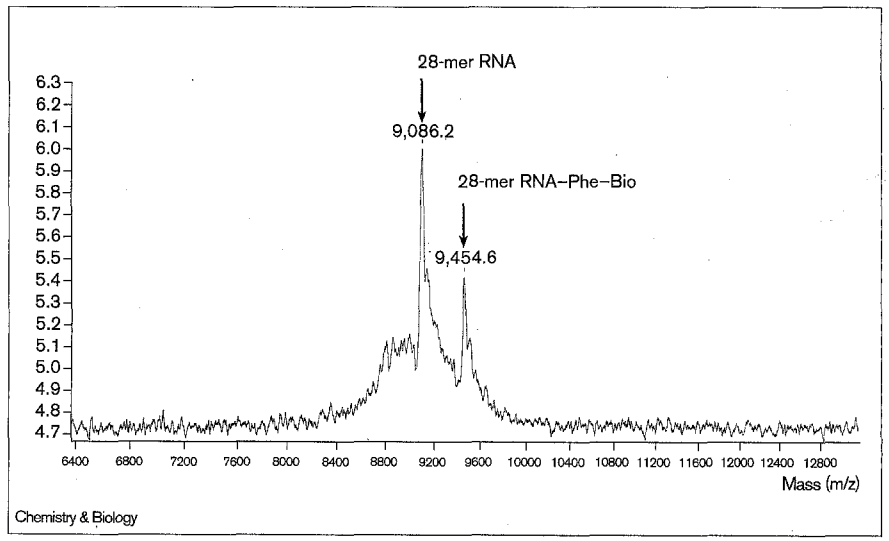
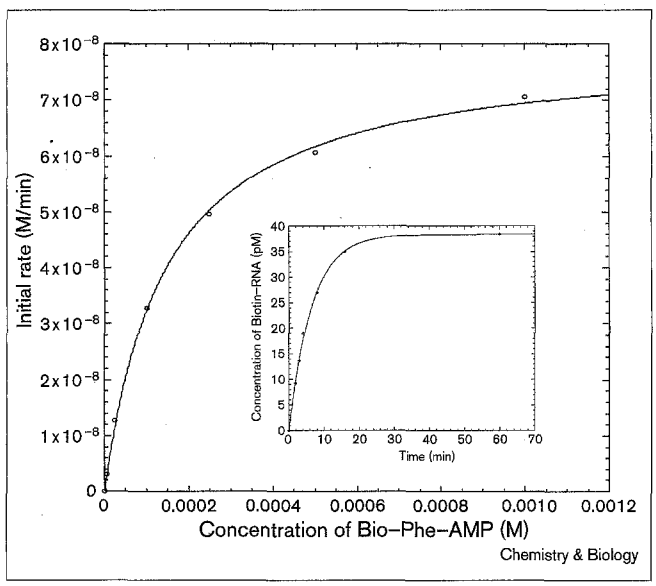


Figure 9



Characterization of the intramolecular reaction catalyzed by clone 11. Initial rates v_0 are plotted against Bio–Phe–AMP concentrations [1] ranging from 100 μM to 1 mM. Values of the Michaelis–Menten parameters K_m and k_{cat} were obtained from the data by fitting the equation $v_0 = [RNA]_0 \cdot [1] \cdot k_{cat} / K_m + [1]$ using KaleidaGraph (Abelbeck Software). Inset: Curve fitting of the aminoacylation reaction using 5 μM clone 11 RNA and 500 μM Bio–Phe–AMP **1**. Rate constants k_{obs} were calculated from the equation $[P]_t = [RNA] - [RNA] \cdot \exp(-k_{obs} \cdot t)$, where $[P]_t$ is the concentration of aminoacylated RNA at time t and [RNA] is the concentration of clone 11 RNA.

(reverse). From the data obtained for the intermolecular forward reaction:

$$\text{28-mer+Bio–Phe–AMP}\,\mathbf{1} \xrightarrow[28-136]{\text{Ribozyme}} \text{28-mer–Phe–Bio+AMP}$$

the decadic logarithm of the equilibrium constant was determined as the average value of five experiments to be $\log K = -6.0 \pm 0.3$. A similar analysis performed for the reverse reaction led to a $\log K = -5.2 \pm 0.7$. This value for K indicates that the equilibrium of the reaction lies so strongly on the side of the educts that, even at the highest concentration of **1** (5.0 mM; which is a 250,000-fold excess over the 28-mer) the formation of aminoacylated 28-mer oligonucleotide substrate does not exceed 40%. On the other hand, the reverse reaction:

$$\text{28-mer–Phe–Bio+AMP} \xrightarrow[28-136]{\text{Ribozyme}} \text{28-mer+Bio–Phe–AMP}\,\mathbf{1}$$

conducted under similar conditions almost runs to completion even at an AMP concentration of 100 μM. Figure 10 shows this analysis representatively for an AMP concentration of 2.0 mM.

The 28-mer oligonucleotide substrate base-pairs over a relatively extended region with the enzyme. We therefore think that dissociation of the modified oligonucleotide substrate becomes rate-limiting for the forward reaction. We are currently trying to engineer the substrate oligonucleotide to have an increased rate of dissociation for the ribozyme/oligonucleotide substrate complex, to facilitate the determination of k_{cat}/k_M under multiple turnover conditions.

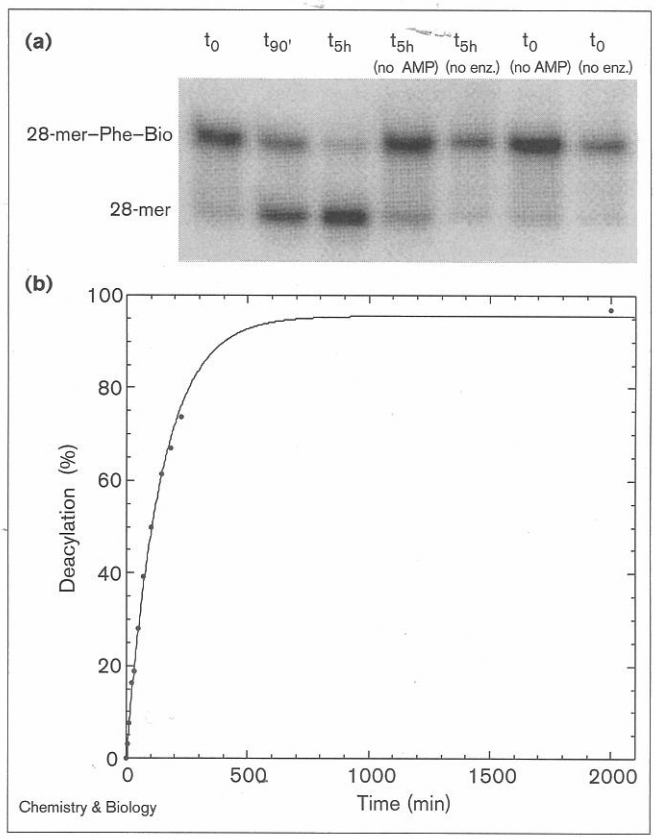
Clone 11 can be specifically inhibited

It is likely that there is a specific binding site for the Bio–Phe–AMP substrate **1** in the ribozyme, which positions the substrate appropriately for the ester transfer to occur. The substrate contains two residues, biotin and AMP, for which specific binding RNA motifs (aptamers) have been described previously [27–30]. The ester transferase ribozyme isolated here does not appear to contain any motifs related to either of these, however. To investigate whether ribozyme catalysis can be inhibited by residues contained in substrate **1**, we performed inhibition studies of the *in cis* forward reaction using increasing concentrations of AMP and biotin. With a fixed concentration of 45 μM of **1** and 11 μM of clone 11 RNA, increasing concentrations of AMP led to a concentration-dependent inhibition of the ester-transfer reaction. From these data, an apparent inhibition constant (K_i) of $1.9 \pm 0.5\ mM^{-1}$ for AMP was calculated. The effect is specific for AMP; with a 130-fold excess of guanosine monophosphate over substrate **1** no inhibition of the reaction was detectable. In comparison, the presence of a 100-fold excess of AMP over substrate **1** led to a significant decrease in aminoacylation activity of the ribozyme ($k_{obs(uninhibited)}/k_{obs(100\cdot AMP)} = 6.25$). The inhibition of the aminoacyl transfer reaction is presumably composed of two overlapping effects: a shift in equilibrium by enforcing the reverse reaction as the concentration of AMP increases, and binding of AMP, instead of **1**, to the enzyme. Indeed, increasing concentrations of AMP not only led to a decrease of V_{max}, due to a shift in equilibrium, but also to a decline of k_{obs}, as a result of competitive occupation of the substrate-binding site with AMP (data not shown). Biotin was also found to act as a competitive inhibitor, albeit with a significantly lower K_i (4.7 ± 0.7 mM). In contrast to AMP, biotin did not affect V_{max}, as no decrease in the plateau of the saturation curves was found. This is not surprising, because biotin does not participate in the reverse reaction.

Discussion

Using a selection scheme designed for isolating a ribozyme capable of peptide-bond formation we identified a novel class of catalytic RNAs that catalyze the transfer of an amino acid ester from a small substrate (**1**; Figure 1) onto an internal 2′-OH group of the ribozyme. The way these ribozymes were selected is indeed remarkable and unexpected. How was it possible to select ester transferase ribozymes using the selection scheme described here? One

Figure 10



Analysis of the reverse reaction catalyzed by the ribozyme 28–136. The biotinylated 28-mer–Phe–Bio was radiolabeled with ^{32}P at the 5′-end, purified on streptavidin agarose, eluted with 2 M 2-mercaptoethanol, ethanol-precipitated and redissolved. **(a)** A representative time course for the reverse reaction and negative controls at 2.0 mM. Time points of 0, 90 min and 5 h show the gradual decrease of the 28-mer–Phe–Bio substrate oligonucleotide and increase of the 28-mer substrate oligonucleotide. In the absence of AMP no decrease of 28-mer–Phe–Bio was observed after 5 h – t_{5h} (no AMP) and t_0 (no AMP). In the absence of enzyme (no enz.) no change in the amount of 28-mer–Phe–Bio is observed after 5 h – t_{5h} and t_0. **(b)** Time course for the reverse reaction at 2 mM AMP, 5 μM ribozyme 28–136, and 5 nM 28-mer–Phe–Bio.

explanation is that the elution of immobilized active RNAs with 2-mercaptoethanol (that is, specific cleavage of the disulfide linker) was not selective enough to ensure enrichment of only the desired peptidyl transferase activity. Indeed, the interaction between biotinylated oligonucleotides and streptavidin can be disrupted to a considerable extent by the presence of 2-mercaptoethanol at concentrations as low as 0.2 M used in the selection (these data are available as Supplementary material). Consequently, almost every RNA capable of attaching the biotin anchor onto itself had the potential to become selected. We believe that the observed aminoacylation reaction might have been preferred over other possible chemical transformations. The finding that only ribozymes with ester transferase activity were selected suggests that there are more sequence solutions for the catalysis of an ester transferase than for peptide-bond formation. There may be a simple statistical reason for this, as there are many more potentially nucleophilic 2′-oxo-anions in the RNA molecules, compared to the single nucleophilic amino group at the peptide coupled to the 5′ end; potential catalysts for peptide-bond formation might therefore be outcompeted by the ester transferases during iterative rounds of selection. Although a peptidyl transferase activity is likely to be present in the starting pool, we think that a much greater population of ester transferases might have overwhelmed that functionality under our selection conditions. On the other hand, Zhang and Cech [18], who used a highly similar selection scheme in parallel to our study, did isolate the desired peptidyl transferase activity. It is possible that an activity as complex as peptide-bond formation requires a relatively long RNA sequence. Indeed, RNA library members were significantly longer than ours (196 versus 158 nucleotides). Of the two classes of active sequence isolated in 19 cycles of selection, one was characterized in detail and shown to require the full-length ribozyme for catalysis [18]. The relatively long sequence requirement for isolating a particular ribozyme activity has also been shown in the case of the class I ligase ribozyme [31].

Biotinylation and elution with 2-mercaptoethanol alone are probably not sufficiently stringent requirements to allow the enrichment of the ester transferase ribozyme described here. During amplification, active RNAs have to be reverse-transcribed and PCR-amplified. Modification at an internal 2′-OH group usually leads to a stop or pause of the reverse transcriptase [32]. So, it is likely that the ribozyme described here was selected because it had the 2′-OH-modification located within the 3′-primer annealing site and was thus amplified more easily. Consequently, this ribozyme had an advantage over other trans-esterases which might have been present in the initial pool but would have became extinct because they could not be amplified.

Our selected ribozyme is, to the best of our knowledge, the second example of an ester transferase ribozyme isolated by *in vitro* selection. Lohse and Szostak [16] recently described a ribozyme that catalyzes the transfer of a biotinylated methionine residue from an oligonucleotide substrate to the 5′-OH of a library RNA molecule. The design of their acyl donor molecule involved linkage of the amino acid to the 3′ end of a short 6-mer oligonucleotide capable of hybridizing to the ribozyme. The ribozyme presented in our study does not utilize a substrate that is templated by Watson–Crick base-pairing with the enzyme but most probably had developed a specific binding pocket for the substrate **1**. Inhibition studies performed with AMP and biotin provide evidence for this, and suggest that the substrate as a whole is recognized by the ribozyme.

The ester transferase isolated by Lohse and Szostak [16] was also engineered to perform a corresponding transfer of the amino acid to the 5′-NH_2-modified ribozyme, resulting in the first example of an RNA that catalyzes formation of an amide bond. Interestingly, in our case substitution of the 2′-OH for a 2′-NH_2 group did not result in amide-bond formation. A possible (and likely) explanation for this result could be the altered geometry of the ribose in the active site resulting from the 2′-OH→2′-NH_2 substitution. Sterically, pyrimidine ribonucleotides prefer the C3′-endo mode at the ribose whereas deoxyribonucleotides favor the C2′-endo puckering [33], presumably because of the reduced electronegativity of the 2′-OH→H substitution [34]. In case of the 2′-NH_2 substitution, however, NMR studies [35] revealed that this substitution results in a strongly favored C2′-endo conformation, even more than the deoxyribose residue itself [35], possibly because of an intramolecular N2′–H···O3′ hydrogen bond not observed in other derivatives [36].

Like our ribozyme, the hammerhead ribozyme also loses activity when the attacking 2′-OH group is replaced by a 2′-NH_2 group [37–39]. Substitution of the 2′-OH for 2′-NH_2 groups in pyrimidine residues in RNA oligonucleotides also results in resistance to ribonuclease cleavage [40,41]. In addition, model studies with UpU derivatives showed that the nature of the leaving group is critical for UpU cleavage when the 2′-NH_2 group is the attacking nucleophile, indicating that nucleophilicity is not the only determinant of cleavage rate [42]. The 5′-oxo anion leaving group is much more resistant to cleavage by an attacking 2′-NH_2 group than when the leaving group is the 5′-thio-anion. The fact that raising the pH to 8.0, 8.5, 9.0 or 9.5 does not lead to an active 2′-NH_2 modified substrate can be attributed to a pK of 6.2 for the 2′-NH_2 group in the ribose [43].

Aminoacylated RNAs and aminoacyl adenylates play a central role in translation processes, suggesting that acyl transfer reactions catalyzed by RNA might have contributed to the formation and optimization of the translation apparatus during early evolution. As Hager *et al.* [4] pointed out recently, ribozymes which transfer aminoacyl groups between aminoacyl donor oligonucleotides (which, as we show here, can be as primitive as a monomeric adenosine) and acceptor oligonucleotides would have allowed a self-aminoacylating RNA to hand off its amino acid to the RNA catalysts that would be required for more specialized metabolic transformations. Such activities would have greatly facilitated the development of metabolism in the RNA world [11,44,45], by increasing its flexibility and adaptability.

Significance

We have isolated a new ribozyme which catalyzes the transfer of an amino acid ester from a biotinyl-*N*-phenylalanyl-2′(3′)-adenosine-5′-monophosphate (Bio–Phe–AMP) substrate onto a specific ribose 2′-OH group of the ribozyme. The ribozyme described here provides an example of an RNA that catalyzes a reaction at a carbon center by utilizing a low molecular weight substrate. The reaction is strongly dependent on the presence of divalent metal ions and can be inhibited by AMP, but not by GMP, indicating that there is a specific binding pocket for the Bio–Phe–AMP substrate. The transformation reaches equilibrium because of a significant level of the corresponding reverse reaction, 2′(3′)-aminoacylation of AMP. The reverse reaction was used to determine the equilibrium constant of the ester transfer reaction, which lies strongly on the side of the 2′(3′)-aminoacyl AMP educt. To the best of our knowledge, this is the first example of a selected ribozyme in which both the forward and the reverse reactions have been measured and quantified.

The central role that aminoacylated RNAs play in translation processes suggests that acyl transfer reactions catalyzed by RNA might have facilitated the development and optimization of the translation apparatus during early evolution. It has been proposed that the evolutionary precursor of rRNA might have been a ribozyme with 3′-OH→2′(3′)-OH acyl transferase activity [16]. The ribozyme described here expands the scope of RNA catalysis in this direction and shows that such transformations are — in principle — possible.

Materials and methods

Materials

[γ-^{32}P]-ATP and [5′-^{32}P]-pCp were purchased from Amersham. T7 RNA polymerase was purified from the overproducing strain BL21/pAR1219, following the purification protocol provided by F.W. Studier and co-workers [46]. DNase I (RNase free) was purchased from Boehringer Mannheim, Taq Polymerase from Eurogentec, the RNAse sequencing kit was obtained from Pharmacia and T4 polynucleotide kinase, T4 RNA ligase and calf intestinal alkaline phosphatase were purchased from New England Biolabs. Ultrapure, unlabeled NTPs and dNTPs were obtained from Boehringer Mannheim. Guanosine monophosphorothioate (GMPS) was synthesized as described previously [47]. Modified oligonucleotides were purchased from Eurogentec. The 28-mer oligonucleotide substrate modified with a 2′-NH_2 group at C147 was synthesized by D. Earnshaw and R. Grenfell at the MRC Cambridge. The 2′-NH_2-2′-deoxy phosphoramidite required for this synthesis was a generous gift from NeXstar Pharmaceuticals in Boulder, Colorado. Primers and synthetic oligonucleotides used in PCR amplification reactions were synthesized on a Millipore Expedite oligonucleotide synthesizer using standard phosphoramidite chemistry. The syntheses of **1–3** will be described elsewhere.

Preparation of DNA, RNA and modified oligonucleotides

The starting pool was prepared by PCR using the synthetic oligonucleotide 5′-GGG AGC TCT GCT CTA GCC AC-N_{30}-*GAC GGT TAG GTC GCA* C-N_{30}-*GTG AAG GAG TGT* C-N_{30}-GGC ACC TGC CAC GAG GGA TC-3′, where N represents an equimolar mixture of A, C, G and T. Italicized nucleotides indicate a 30% doped citrulline aptamer motif [48]. Sequences of the primers were 5′-TCT AAT ACG ACT CAC TAT AGG GAG CTC TGC TCT AGC CAC-3′ and 5′-GAT CCC TCG TGG CAG GTG CC-3′. The RNAs used for this study were transcribed from DNA templates containing a T7 promotor. Oligonucleotides were purified as described previously [49]. To prepare the 5′-amino modified RNA library (H_2N–Cit–Cys–S–S–RNA) used in the first 7 rounds of

selection, T7 transcription was performed in the presence of an 8-fold molar excess of GMPS over each NTP [21]. The resulting 5′-GMPS primed RNA was then incubated with a 50-fold molar excess of the thiopyridyl activated dipeptide H_2N–Cit–Cys–(tpy)–COOH (**2**; Figure 2) in coupling buffer (5 mM EDTA, 25 mM $NaPO_4$, pH 7.7) for 20 min. Completion of the reaction was determined by measuring the absorption of released thiopyridone at 343 nm. The library was then ethanol precipitated and washed twice with 70% ethanol to remove unreacted H_2N–Cit–Cys–(tpy)–COOH and thiopyridone.

In vitro *selection*

In vitro selection was performed with an initial pool of approximately 5×10^{14} sequences using eight copies of each molecule in the first cycle. The RNA library was heated to 94°C for 2 min in Mg^{2+}-free selection buffer containing 200 mM NaCl, 50 mM K-MOPS at pH 7.4 to denature the RNA. The concentration of $MgCl_2$ was then adjusted to 5 mM and the reaction mixture was cooled down to room temperature for 10 min to allow proper folding of the RNA molecules. The reaction was then initiated by the addition of substrate **1** and incubated at room temperature for the time indicated in Figure 3. To remove the large excess of **1** the reaction mixture was filtered through a Sephadex G-50 column. Fractions containing RNA were then ethanol precipitated, resuspended in 500 µl of streptavidin-binding buffer (150 mM NaCl, 25 mM $NaPO_4$, pH 6.9) and incubated with 500 µl of a 50% slurry of streptavidin agarose for 30 min. The sample was transferred to a column and washed with 20 column volumes each of W1-buffer (1 M NaCl, 5 mM EDTA, 25 mM K-MOPS, pH 7.4), denaturing buffers (W2-buffer: 4 M urea, 5 mM EDTA, adjusted with K-MOPS to pH 7.4; W3-buffer: 3 M guanidinium chloride, 5 mM EDTA) and finally with water. RNAs linked to the streptavidin resin were eluted with 0.2 M 2-mercaptoethanol in 500 µl W1 (rounds 1–6) and 2 M 2-mercaptoethanol in 500 µl W1 (rounds 7–13). Eluted RNAs were ethanol precipitated, reverse transcribed, PCR amplified and T7 transcribed as described previously [48]. PCR amplification in round 10 was performed under mutagenic conditions [24]. Pool DNA obtained after round 13 was cloned and sequenced as described previously [48].

Ribozyme assays

All aminoacylation reactions were assayed at room temperature in selection buffer containing 12.5 mM $MgCl_2$. RNAs were 5′-^{32}P-end-labeled by standard protocols [50]. Intramolecular reactions were performed in 100 µl volumes at the concentrations of ribozyme and substrate **1** shown in Figure 9 and Table 1. 10–15 µl aliquots were taken at eight different time points and reactions were stopped by ethanol precipitation. Aminoacylated RNAs were coupled to streptavidin agarose by incubating the samples with 50 µl of swollen resin for 30 min. Reaction mixtures containing more than 100 µM of substrate **1** were passed over a Sephadex G-50 column prior to streptavidin coupling. The resin was then transferred to a spin filter tube and washed extensively with denaturing buffers W2 and W3. The amount of aminoacylated RNA was quantified by counting the radioactivity of flow through and streptavidin resin in a scintillation counter (Beckman). Initial rates were calculated from these data by curve fitting (see Figure 9). Intermolecular reactions were performed in 25 µl volumes at a ribozyme 28–136 concentration of 5.0 µM, a 28-mer concentration of 20 nM, and **1** concentrations of 0.2, 0.5, 1.0, 2.5, and 5.0 mM (forward reaction). For the reverse reaction, a ribozyme 28–136 concentration of 5.0 µM, a 28-mer–Phe–Bio concentration of 5 nM, and AMP concentrations of 0.1, 0.25, 0.5, 1.0, 2.0, and 10.0 mM were used. 2 µl aliquots were taken at 10 different time points and reactions were stopped by mixing the aliquots with 5 µl PAGE loading buffer each (9.0 M urea, 20 mM EDTA) and freezing to −80°C. Samples were analyzed on a denaturing 20% polyacrylamide gel and quantified on a phosphor imager (Molecular Dynamics). Values of k_{obs} were determined from these data by curve fitting (see Figures 9 and 10). The equilibrium constant K for the intermolecular reaction was calculated from the equation:

$$K = \frac{[\text{28-mer–Phe–Bio}]\times[\text{AMP}]}{[\text{28-mer}]\times[\text{Bio–Phe–AMP 1}]}$$

Inhibition reactions were performed using 0.045, 0.45, 2.25, 4.5 and 11.25 mM AMP or biotin, respectively, 11 µM ribozyme 28–164 and 45 µM substrate **1**. Aliquots were taken at five different timepoints and analyzed as described for the intramolecular reactions. Values of K_i were obtained from these data by nonlinear least square curve fitting using the equation $k_{obs} = k_0 / (1 + [I] / K_i)$, where k_{obs} is the observed rate constant, k_0 is the rate of the uninhibited reaction, [I] is the concentration of inhibitor, and K_i is the concentration that yields half-maximum inhibition.

Supplementary material

Supplementary material available with the online version of this paper includes one table (Table S1) of elution studies of streptavidin-coupled biotinylated radiolabeled oligonucleotides with 2-mercaptoethanol and a short discussion of these data.

Acknowledgements

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