Title: “Ribopepzymes” are probably a link from ribozymes to protein enzymes

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The evolutionary relationship between RNA- and protein-based biocatalysts was key to the evolution of living systems\(^1\). This relationship is thought to have depended upon the transfer of both genetic information and catalytic function in living systems\(^2\). We investigated whether ribozymes could transfer genetic information and catalytic function at the chemical level. We identified a family of peptides encoded by ribozymes: 13-residue peptide encoded by the hammerhead ribozyme, a 19-residue peptide encoded by the genomic hepatitis delta virus (HDV\(^+\)) ribozyme, a 25-residue peptide encoded by the antigenomic HDV (HDV-) ribozyme, a 15-residue peptide encoded by the smallest trans-acting genomic HDV (SHDV) ribozyme, and a 22-residue peptide encoded by an open reading frame (ORF) of RNase P. We show that all these peptides possess the ability to cleave single-stranded RNA substrates. Furthermore, we expressed a 56-residue peptide encoded by an intact ORF (616-783) of the VS ribozyme, and found that it has single-stranded RNA cleavage activity as well. Because these catalytic peptides arise from a ribozyme-based code and their catalytic activity is ribonuclease-like, we have designated these peptides as “ribopepzymes”. Ribopepzymes could be a link from ribozymes to protein enzymes in the early origin and evolution of enzymes. They also may provide a basis for designed, divergent evolution of enzyme function.
According to the “RNA world” hypothesis, RNA once served both as genetic material and as primordial biocatalysts. It seems possible that naturally occurring ribozymes transitioned to protein enzymes via transferring both genetic information and catalytic function. Previously, we employed a DNAzyme-RNAzyme combination strategy to construct a 10-23 deoxyribozyme- hammerhead ribozyme combination that recognizes and cleaves different sites of the β-lactamase mRNA. The combinator gene was cloned into the phagemid vector pBlue-script II KS (+). The single-stranded recombination phagemid vector exhibited 10-23 deoxyribozyme activity in vitro, and the linear transcript also displayed hammerhead ribozyme activity. Subsequently, we chemically synthesized a 25-residue peptide encoded by the combinator gene and found that the peptide possesses single-stranded RNA cleavage activity. To further explore the catalytic mechanism of this peptide, we analyzed the RNA cleavage activities of chemically synthesized 12- and 13-residue peptides that were encoded by the 10-23 deoxyribozyme and the hammerhead ribozyme, respectively. Results showed that the 13-residue peptide exhibits single-stranded RNA cleavage activity (Fig. 1a), while the 12-residue peptide does not. Because the 13-residue peptide arises from a ribozyme-based code and exhibits catalytic activity similar to that of ribonucleases, we have designated the catalytic peptide “ribopezyme R” (RPZ\textsubscript{R}) (Table 1).

Given that there are no naturally occurring DNAzymes, and that no RNA cleavage activity was detected from the peptide encoded by the 10-23 deoxyribozyme, we focused our analysis on the activities of peptides encoded from naturally occurring ribozymes. The peptides encoded by the hammerhead ribozyme had single-stranded RNA cleavage activity. We wanted to know if this holds true for peptides encoded by other naturally occurring ribozymes. The hammerhead ribozyme, the hairpin ribozyme, and the hepatitis delta virus (HDV) ribozyme all show an extreme degree of convergence in sequence, structure, and reaction mechanism. We thus analyzed the peptides encoded by the genomic hepatitis delta virus (HDV+) ribozyme, the antigenomic HDV (HDV-) ribozyme, and the hairpin ribozyme. Their nucleotide sequences are shown in Table 1. Applying the same approach described above, we
produced a 19-residue peptide and a 25-residue peptide from the HDV (+) and HDV (-) ribozymes, respectively. Both demonstrated single-stranded RNA cleavage activities (RPZ_{HDV(+)}, RPZ_{HDV(-)}, Table 1 and Fig. 1b). The smallest trans-acting sequence of genomic HDV (SHDV) ribozyme contains a conserved nucleotide sequence and demonstrated the same cleavage activity as in wild-type HDV ribozyme. A 15-residue peptide encoded by this small sequence also exhibited single-stranded RNA cleavage activity (RPZ_{SHDV}, Table 1 and Fig. 1c). However, perhaps owing to a loss of functional information, a 17-residue peptide encoded by the hairpin ribozyme exhibited no single-stranded RNA cleavage activity (P_{HPRZ}, Table 1 and Fig. 1d).

The hammerhead, hairpin, and HDV ribozymes all have relatively simple structures and their catalytic mechanisms are clear. We examined two other naturally occurring ribozymes, RNase P and the VS ribozyme. *E. coli* RNase P consists of a small protein and a catalytic RNA containing an ORF (125–193). The 22-residue peptide encoded by its ORF demonstrated single-stranded RNA cleavage activity (RPZ_{P}, Table 1 and Fig. 1e). The VS ribozyme is an 881 nt self-cleaving RNA found in the mitochondria of *Neurospora*, and is transcribed from the Varkud satellite. The conserved sequence of the VS ribozyme comprises an intact ORF (616–783). We expressed the 56-residue peptide encoded by its ORF and analyzed the peptide activity. The peptide exhibited single-stranded RNA cleavage activity (RPZ_{VS}, Table 1 and Fig. 1f). Using this approach, we obtained a family of novel ribopepzymes: RPZ_{R}, RPZ_{HDV(+)}, RPZ_{HDV(-)}, RPZ_{SHDV}, RPZ_{P}, and RPZ_{VS}.

Compared with other synthesized catalytic peptides, these ribozyme-encoded ribopepzymes are extraordinary. They are almost all arginine- and proline-rich petides (Table 1). Arg, Trp, and Gly were common to all the ribopepzymes. Of these, Pro, Trp, and Gly were favorable to the stretching of the peptide chain. In contrast to ribozymes, ribopepzymes do not require divalent metal ions for catalysis, as Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Ba^{2+}, Cu^{2+} and Co^{2+} inhibited the catalytic activity of ribopepzymes to different degrees (Fig. 2). According to the searching results from BLAST, members of the family of ribopepzymes we generated do not have homology with other known peptides or proteins. Structural prediction results from PEPstr
robetta indicate that ribopepzymes primarily consist of a random coil. Circular dichroism (CD) analysis demonstrated that ribopepzymes exist mainly in an unfolded state in aqueous solution (data not shown).

Because the ribopepzymes we generated all appear to have similar structures and functions, we used RPZ$_R$ as a representative to conduct a more detailed characterization of their catalytic mechanisms. Since ribopepzymes are arginine-rich, and an arginine residue is present in many active sites of enzymes$^{16}$, we examined the possibility that arginine is required for catalytic activity. Peptides exhibited no catalytic RNA cleavage activity when they contained fewer than three arginine residues. Likewise, cleavage activity was present only when peptides contained 3 or more arginine residues (Table 1). However, no relationship between cleavage activity and the position of the arginine residues could be identified. To confirm the relationship between RNA cleavage activity and the number of arginine residues, we produced a mutant of RPZ$_R$ in which three arginine residues were replaced by alanine residues (RPZ$_{RM1}$, Table 1). As expected, this mutant exhibited no single-stranded RNA cleavage activity. Further experiments showed that any mutation of arginine residues resulted in a loss of ribopepzyme activity (RPZ$_{RM2}$–RPZ$_{RM4}$, Table 1). However, substituting other, non-arginine residues with alanine still left the ribopepzyme’s RNA cleavage activity, although activity was weakened, likely from disrupting the original ribopepzyme scaffold (RPZ$_{RM5}$, Table 1). The above results indicate that three arginine residues are involved in RPZ$_R$ catalysis.

In order to further confirm the catalytic mechanism, we next analyzed the cleavage sites of the RPZ$_R$. RPZ$_R$ cleaved substrate RNA predominantly at G, U and A residues (RPZ$_R$, Fig. 3). Similarly, RNase A mainly hydrolyzes RNA at C and U residues$^{17}$ and RNase T1 mainly hydrolyzes RNA at G residues$^{18}$. Interestingly, we also found that rRNAsin (Promega) inhibited the activities of the RPZ$_R$ (Fig. 4a). Taken together, these results indicate that RPZ$_R$ employs a ribonuclease-like catalytic mechanism in which three arginine residues could be involved in hydrolyzing phosphodiester bonds. However, the active sites of RNase A and RPZ$_R$ are chemically different: RNase A consists of histidine and lysine residues$^{19}$, while RPZ$_R$ has arginine, and no histidine
residues. We replaced the arginine residues of RPZ\textsubscript{R} with the active site amino acids from RNase A. The mutant (RPZ\textsubscript{RA}) displayed single-stranded RNA cleavage activity (Table 1, Fig. 4b). The pH profile curve for RPZ\textsubscript{R} activity was bell-shaped, with an optimal cleavage rate at pH 7.5 (data not shown). Thus, RPZ\textsubscript{R} activity could be characteristic of concerted general acid-base catalysis. Both the low activity and low substrate specificity of ribopepzymes are probably due to the peptide’s flexibility. Because of their relative simplicity and small size, it is difficult to form efficiently catalytic and binding domains. Induced fit did not occur easily between the ribopepzyme and the substrate.

During early life on earth, RNAs were capable of both encoding genetic information and performing catalytic functions. At some point, peptides became the repository for both. One possible explanation is that both genetic information and catalytic function were directly transferred to a primordial enzyme. We propose that the relationship between ribozymes and ribopepzymes might represent such a transfer. Thus, ribopepzymes may represent the transition state in a possible evolutionary process (ribozymes $\rightarrow$ ribopepzymes $\rightarrow$ protein enzymes), and as such could be a possible ancestor of the modern ribonucleases. Another possibility is that RNA-based catalytic function was lost or diverged along with the loss or divergence of genetic information as part of the process of evolution. Owing to the loss or divergence of genetic information, peptides based on the hairpin ribozyme possessed no single-stranded RNA cleavage activity. Divergent evolution could cause ribopepzymes based on one ancestor to evolve diversified catalytic functions\textsuperscript{20-23}.

Based on the theories of molecular evolution and our experimental observations, we proposed an approach for the design of RNA-based biocatalysts. We successfully obtained a family of novel ribopepzymes. These results suggest that: (1) at the chemical level, both genetic information and catalytic function could be transferred from some ribozymes to protein enzymes; (2) the ribopepzyme could be a link between RNA- and protein-based biocatalysts in the early origin and evolution of enzymes; (3) the emergence of the ribopepzymes could drive biocatalyst evolution, and provide a basis for designed, divergent evolution of enzymatic function\textsuperscript{23-26}. The
developed process might even be extended to other ribozymes and create a larger variety of catalytic lineages.\

METHODS

Ribopepzyme activity was tested using a similar method in reference. A 16-µM solution of each ribopepzyme (or peptide), mixed with 0.4 µM 5′-32P-labeled substrate RNA, in 10 µl of buffer solution [50 mM Tris-HCl, pH 7.5, 0.1% diethylpyrocarbonate (DEPC)] was incubated for 18 hours at 30°C. The reaction was quenched by adding 5 µl of stop mix (9 M urea, 50 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue, and 0.1% DEPC). Substrates and products were separated on a 16% polyacrylamide-7M urea denaturing gel and visualized by autoradiography. The extent of cleavage was determined from measurements of radioactivity in both the substrate and 5′ products using a Bio-Rad PhosphorImager.

To ensure that the activity of the ribopepzyme was not contaminated by ribonucleases or other types of interference, we performed the following detection: Proteinase K digestion of ribopepzymes: A 100-µl reaction containing 1% DEPC, 160 µM ribopepzyme, and 200 µg/ml proteinase K was incubated for 1 hour at 37°C. A 1-µl digest solution (16 µM ribopepzyme concentration) was used according to the method described above. The ribopepzyme mixture treated without proteinase K cleaved the RNA substrate; in contrast, the ribopepzyme mixture treated with proteinase K did not. The results showed that the RNA-cleaving activity was specific to the ribopepzyme and not from other experimental components.

The substrate cleavage site for RPZr was determined using a similar method in reference. Reaction rates (kobs) were determined as described for single-turnover kinetics.

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1. The blue letters represent the mutant bases and corresponding amino acids.
2. RPZ$_{RA}$ represents that Arg residues of RPZ$_{R}$ were replaced with amino acid residues from the active site of RNase A.
3. ND represents no detectable RNA-cleaving activity.

**Figure legends**

**Figure 1**

The autoradiography analysis of the activities of RPZ$_{R}$, RPZ$_{HDV(+)}$, RPZ$_{HDV(-)}$, RPZ$_{SHDV}$, P$_{HPRZ}$, RPZ$_{p}$ and RPZ$_{VS}$ with the hammerhead ribozyme substrate (Sa:5’
GAGCGUGGGUCUCGCGG 3'). Ctrl, the substrate without the ribopezyme or the peptide; S, the substrate (Sa); P, the products catalyzed by the corresponding ribopezyme; RPZ, ribopezyme; P_{HPRZ}, the peptide encoded from hairpin ribozyme.

**Figure 2**

The divalent metal ion-dependence on RNA cleavage rate (%) of RPZ_{R}. Cleavage reactions were performed with 16-μM RPZ_{R} and 0.4 μM substrate (Sa) under single-turnover conditions. 10 mM divalent cation (Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Ba^{2+}, Cu^{2+}, Co^{2+}) were added into the reaction systems respectively. The resulting products were separated by 16% denaturing PAGE, visualized by autoradiography, and quantified using a Bio-Rad PhosphorImager. Ctrl, substrate RNA without divalent metal ion. The percentage of RNA cleaved is equal to [total substrate - uncatalyzed substrate]/ [total substrate]. Data represent mean±SD (n=4).

**Figure 3**

Cleavage sites of RPZ_{R}. a, b, c and d showed four different substrates were cleaved. Ctrl, the substrate without RPZ_{R}; M, Marker. S represents the substrate. P represents the products catalyzed by RPZ_{R}. The red arrows indicate the cleavage sites of substrates Sa, Sb, Sc and Sd.

**Figure 4**

Inhibition and mutation for RPZ_{R}. a, RNasin inhibited the RNA-cleaving activities of RPZ_{R} and RNase A. Ctrl, the substrate (Sa) without RPZ_{R}, RNase A or RNasin; lane 1, the substrate cleaved by RPZ_{R}; lane 2, the RNA-cleaving activity of RPZ_{R} inhibited by RNasin; lane 3, the substrate cleaved by RNase A; lane 4, the RNA-cleaving activity of RNase A inhibited by RNasin. b, The autoradiography analysis of the RPZ_{R} in which Args were replaced with amino acid residues from the active site of RNase A. Ctrl, the substrate (Sa) without the ribopezyme or RNase; lane 1, the
substrate cleaved by RPZ_{RA}; lane 2, the substrate cleaved by RPZ_{R}; lane 3, the substrate cleaved by RNase A. S represents the substrate. P represents the products catalyzed by RPZ_{R}.

**Figure 1**

![Diagram of experiment results with labeled substrates and products](image)

**Figure 2**

![Bar graph showing percentage of RNA cleaved with divalent cations](image)
Figure 3

Sa 5' GAGCGUUGGGUCUGCCGG 3'
Sb 5' GCGAGUAGUUAAACUCUUG 3'
Sc 5' AGACUCUGCUACCCGAAAGCAGC 3'
Sd 5' GUGUAUGAGAUUUCAACAUUU 3'

Figure 4