A Ribozyme Exclusively Aminoacylates the 3′-Hydroxyl Group of the tRNA Terminal Adenosine

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The RNA-based aminoacylation system could have played a critical role in establishing an ancient genetic code.[1−4] In this system, a set of catalytic RNA molecules (ribozymes) should have been capable of aminoacylating tRNAs (or their primitive analogue) in a manner analogous to that of modern protein aminoacyl-tRNA synthetases (ARSs).[1,4−6] We have used an in vitro evolution technique to isolate such an ARS-like ribozyme (Figure 1A) from a combinatorial pool of RNA containing 10^{15} unique sequences.[7] Here we report that this ribozyme is able to aminoacylate exclusively the 3′-hydroxyl (3′-OH) group of tRNA.

In the modern translation system, the genetic coding event is carried out by the ARSs.[8] They generally exist in 20 different forms, each catalyzing the esterification of the specific amino acid to the 3′-terminus adenosine (A76) of the cognate tRNA isoacceptor, thereby connecting each amino acid to its corresponding tRNA's anticodon triplet. All members of ARSs are structurally divided into two classes (I and II),[9,10] and the selection of the esterification site is different between these classes: The class I enzymes aminoacylate the 2′-OH of A76 on tRNA, whereas class II enzymes aminoacylate the 3′-OH.[11−14]

In the context of the RNA world hypothesis,[1,15,16] we have evolved a precursor tRNA (pre-tRNA) that specifically charges phenylalanine (Phe) to its own 3′-end.[7] This catalytic pre-tRNA is compatible with a naturally occurring endonuclease ribozyme (RNase P RNA), resulting in its fragmentation to the 5′-leader sequence and mature tRNA (otRNA,[17,18] Figure 1B). The resultant 5′-leader segment is capable of charging Phe onto otRNA, thereby behaving as a *trans*-acting ARS-like ribozyme (referred to as the 5′-leader ribozyme, Figure 1A). This ribozyme exhibits some functional similarities to protein ARSs; (1) the ribozyme can use phenylalanyl-adenylate as an aminoacyl donor in addition to the *N*-biotinylated Phe cyanomethyl ester (biotin-Phe-CME) that was originally used for the in vitro evolution, (2) it critically recognizes the CCA sequence and discriminator base present at the 3′-terminus of otRNA, and (3) it is able to aminoacylate the minihelix RNA[8,19,20] consisting of the acceptor stem and TΨC stem-loop of otRNA.

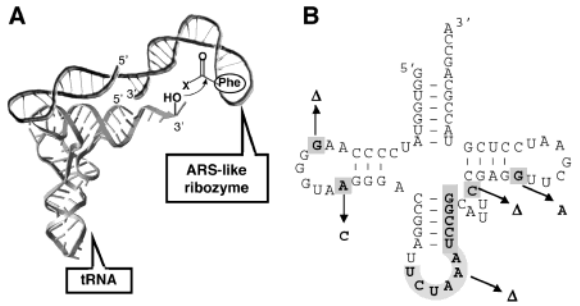

Figure 1. (A) Schematic representation of the ARS-like ribozyme (the 5′-leader ribozyme). The phenylalanine substrate (the side chain and leaving group are shown with Phe and X, respectively) binds to the 5′-leader ribozyme, and the nucleophilic attack (indicated by a curved arrow) is accelerated. (B) Secondary structure of otRNA and truncated tRNA (rtRNA). Bold letters in gray boxes highlight the mutations and deletions observed in rtRNA. Abbreviations: Δ, the deletions observed in rtRNA.

As a result of these remarkable similarities observed between the ribozyme and ARSs, we wondered whether the ribozyme aminoacylates the 2′- or 3′-OH group of the terminal A76 exclusively, as protein ARSs do. In earlier work,[7] we have shown indirect evidence for the necessity of the OH group(s) at the 3′-end, demonstrating inhibition of the ribozyme activity by periodate oxidation of the 3′-terminal diol or deletion of A76. Determination of the specific site on either OH group should also provide concrete evidence for the 3′-end aminoacylation of otRNA.

To probe the specific aminoacylation site, we took an approach to prepare a set of semi-synthetic otRNAs[10,21] consisting of two pieces of otRNA fragments (Figure 2A), where the 3′-fragment RNA contains deoxy-analogues of A76, that is, 2′- or 3′-deoxyadenosine (dA). In this set, three synthetic 19-mer RNAs (**1**, **2**, and **3** in Figure 2B) were chemically synthesized, 5′-radiolabeled, and then assembled with an in vitro transcribed 56-mer otRNA fragment to construct the respective semi-synthetic otRNAs. These molecules were referred to as otRNA−**1**, otRNA−**2**, and otRNA−**3** (Figure 2A).

The semi-synthetic otRNAs were aminoacylated in the presence of the 5′-leader ribozyme and biotin-Phe-CME, and the reactions were analyzed by streptavidin-dependent mobility-gel-shift assay[22,23] (Figure 3). The initial rates of the individual reactions are shown in Table 1. The otRNA−**1** was aminoacylated effectively by the 5′-leader ribozyme (lane 1), and its initial rate was nearly the same as that of the in vitro transcribed full-length otRNA. This confirms that the semi-synthetic otRNA can act as an effective substrate for the 5′-leader ribozyme. The otRNA−**2** also functions as an aminoacyl acceptor (lane 2), and displays only a mild reduction (2.4-fold) of activity compared with otRNA−**1**. In stark contrast, the otRNA−**3** exhibited a 170-fold

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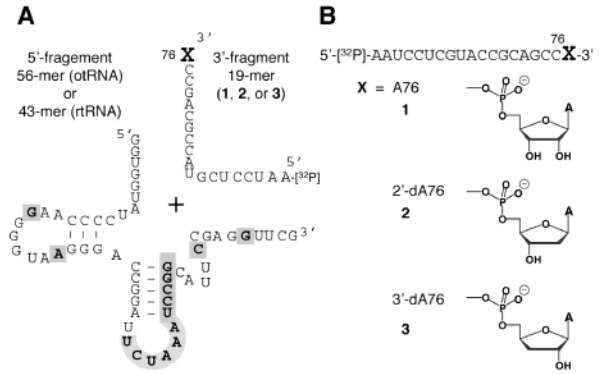

Figure 2. (A) Semi-synthetic tRNAs. Synthetic 19-mer 3′-fragment was assembled with in vitro transcribed 56-mer 5′-fragment to construct the corresponding semi-synthetic otRNA. In the case of semi-synthetic rtRNAs, bold letters in gray boxes were deleted or mutated as shown in Figure 1B. (B) Sequence and structure of 19-mer RNAs containing 2′- or 3′-deoxyadenosine at position 76.

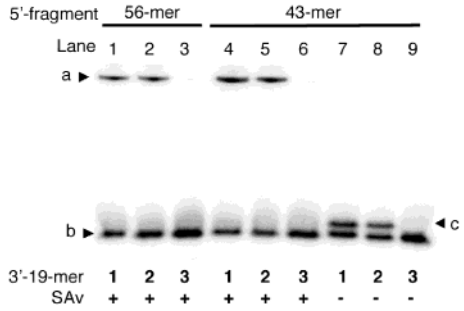

Figure 3. Trans-aminoacylation assay of semi-synthetic tRNAs. After 1 h incubation, streptavidin-dependent (lanes 1−6) or -independent (lanes 7−9) mobility-gel-shift assay was carried out to detect the specific aminoacylation site for the ARS-like ribozyme. Triangles indicate: (a) Biotin-Phe-19-mer RNAs complexed with streptavidin; (b) 19-mer RNAs; (c) Biotin-Phe-19-mer RNAs.

Table 1. Rate Constants Observed for tRNA Variants with Different 3′-Terminal Adenosine in the Presence of 2.5 mM Biotin-Phe-CME

	semi-synthetic, k_{obs} (min^{-1})		full length, k_{obs} (min^{-1})	
RNA oligo (3′-end)	otRNA ($\times 10^3$)	rtRNA ($\times 10^2$)	otRNA ($\times 10^3$)	rtRNA ($\times 10^2$)
1 (A76)	7.1	3.0	5.4	4.1
2 (2′-dA76)	3.0	1.5	4.5	3.4
3 (3′-dA76)	0.042	0.015	–	–

reduction of activity (lane 3). These results provide a strong argument for the specific aminoacylation site at the 3′-OH, as opposed to the 2′-OH group.

The original clone of the *cis*-acting catalytic pre-tRNA contained a truncated otRNA, referred to as rtRNA (Figure 1B), which shows 7.6-fold greater aminoacyl-acceptor activity *in trans* compared with otRNA (Table 1). We therefore examined whether the aminoacylation efficiency for the 19-mer fragment could be enhanced by replacing the 56-mer with a 43-mer that corresponds to the rtRNA 5′-fragment (Figure 2A, and for the initial rates see Table 1). As predicted, both **1** and **2** assembled with 43-mer rtRNA fragment (rtRNA-**1** and rtRNA-**2**) showed 4.2- and 5.0-fold enhanced activities (lanes 4 and 5), compared with those observed for the semi-synthetic otRNAs. The rtRNA-**3** showed a 200-fold poorer activity compared with rtRNA-**1** (lane 6), confirming the essential role of the 3′-OH in catalysis. In the case of the semi-synthetic rtRNA-**1** and rtRNA-**2**, the phenylalanylated 19-mers could also be visualized as a retarded band in the absence of streptavidin[24] (lanes 7 and 8), again in stark contrast to no observation of such band for rtRNA-**3** (lane 9).

The above experiments using the semi-synthetic otRNAs and rtRNAs explicitly demonstrate that the specific aminoacylation occurs on the 3′-OH group. To further confirm the unimportance of the 2′-OH for catalysis, we determined the rate constant of the full-length otRNA and rtRNA containing 2′-dA76. The in vitro transcript of the A76-deleted otRNA (otRNA−ΔA76, see Supporting Information) was 3′-end-labeled with α-[^{32}P]-2′-dATP[25] to yield a radioactive full-length otRNA (referred to as otRNA−2′-dA76). The rtRNA−2′-dA76 was also synthesized in the same manner. Both otRNA−2′-dA76 and rtRNA−2′-dA76 display nearly identical activity to their wild-types (Table 1), again confirming that the 3′-OH group of A76 is the aminoacylation site. These results clearly show that the atomic deletion of the 2′-OH group at A76 has no significant effect on the catalytic rate.

The pK_a's of both 2′- and 3′-OH groups of ribonucleotides are approximately 12.5.[26,27] Thus, nonenzymatic chemical acylation should take place nonspecifically on 2′- or 3′-OH group.[28] The ARS-like ribozyme, however, phenylalanylates on the 3′-OH of A76 exclusively. Moreover, it is active toward the otRNA−2′-dA76 with comparable rates to the wild-type otRNA (this is also true with rtRNA derivatives). Since the pK_a value of 3′-OH group of 2′-deoxyribonucleotides is at least two units higher than that of ribonucleotides, our results suggest that the specific activation of the 3′-OH group likely occurs in the ribozyme active site. In the protein ARS system, the site specificity is determined by precise positioning of aminoacyl-adenylate next to either 2′- or 3′-OH group of A76. The ARS-like ribozyme may also use a similar mechanism to control the aminoacylation event.

In conclusion, we have demonstrated that the ARS-like ribozyme can exclusively aminoacylate the 3′-OH group of the terminal adenosine of tRNA. The discrimination between the 2′- and 3′-OH groups at A76 is a characteristic function of natural protein ARSs. Thus, our demonstration represents the first example of a ribozyme that displays site-specific aminoacylation activity remarkably similar to protein ARSs. This strengthens the view that an RNA-based aminoacylation system could have played a critical role in establishing a proto-translation apparatus before the advent of protein ARSs.

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Supporting Information Available: Experimental details of the synthesis of RNA molecules, their radiolabeling methods, and kinetics (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(24) Due to the low molecular weight of the 19-mer RNA, the molecular weight change by the aminoacylation is significant enough to retard the band of biotin-Phe-19-mer RNA on denaturing PAGE. This was not observed in the case of the full-length rtRNA because of its higher molecular weight.

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(28) It has been reported in the literature[26,27] that the pK_a of 2′-OH is a little lower than that of 3′-OH. Therefore, nonenzymatic acylation may occur on 2′-OH group slightly more than 3′-OH.