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Aminoacyl-tRNA Synthesis by a Resin-Immobilized Ribozyme

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Ribozymes are RNA enzymes that are capable of catalyzing chemical reactions. Naturally occurring ribozymes are limited in their catalytic abilities toward the chemistry of phosphodiester bonds;[1] however, in vitro selection of ribozymes from a combinatorial pool of molecules has extended the catalytic ability of RNA to include a wide spectrum of chemical reactions.[2−4] Most importantly, these ribozymes are user-made de novo catalysts, which can be tailored for unique and specific catalytic properties. By means of in vitro selection, we have successfully generated ribozymes with tRNA aminoacylation activity.[5−8] Such ribozymes have potential applications for in vitro translation, and in particular, they could be useful for preparing tRNAs that are charged with nonnatural amino acids.[9−11] The in vitro synthesis of aminoacyl-tRNAs currently relies on chemical aminoacylation of a dinucleotide followed by enzymatic ligation to an engineered tRNA fragment. These steps are unfortunately time-consuming and laborious.[12] Here, we report a new method for the aminoacylation of tRNA, using a resin-immobilized ribozyme and the cyanomethyl ester (CME) of an amino acid substrate.

The ribozyme, called r24, was originally isolated as a cis-acting ribozyme, which possesses a catalytic 5′-leader sequence capable of aminoacylating the 3′-terminal hydroxyl group of a covalently attached orthogonal suppresser tRNA (otRNA).[6,7] Upon further examination, it was observed that this 5′-leader sequence can act alone as a catalyst and aminoacylate the 3′-OH group of the tRNA. Extensive studies have revealed that the 89-nucelotide (nt) r24 can be truncated into a minimal 57-nt ribozyme, referred to hereafter as r24mini.[8] Moreover, r24 was shown to preferentially aminoacylate tRNA with α-*N*-biotinyl-L-phenylalanine (α-Biotin-Phe, Figure 1B, **1**) and α-Biotin-Tyr, while discriminating against other natural amino acids.[8] In addition, r24 shows higher activity toward an engineered otRNA, referred to as v1-tRNA, as compared to otRNA.[6] Although this ribozyme is an effective single-turnover catalyst for tRNA aminoacylation, its multiple-turnover ability is inherently limited due to slow dissociation of the ribozyme from v1-tRNA. To capitalize on this property, we have developed a resin-immobilized form of r24mini that aminoacylates tRNA and allows affinity purification of the aminoacylated product. This new system simplifies the isolation of aminoacyl-tRNA.

Periodate oxidation of the 3′-cis-diol on the ribose of RNA is well-known chemistry, which yields the corresponding dialdehyde. This new functional group allows us to immobilize the RNA on an inexpensive hydrazide resin, and reductive amination makes the interaction an irreversible linkage (Figure 1A).[13] To avoid compromising the ribozyme's integrity and activity due to perturbation of the requisite catalytic fold by the resin surface, the r24mini was engineered to bear an additional 20-nt adenosine linker at its 3′-end (r24mini-A20, Figure 1A). We confirmed that this modification did not affect on the catalytic ability in solution (Figure 2A, lane 4). Thus, this engineered r24mini ribozyme was readily immobilized on the hydrazide resin at the designated site of the ribozyme.

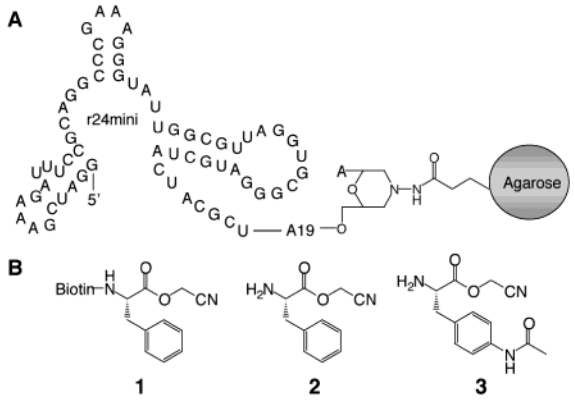

Figure 1. Ribozyme and amino acid substrates used in this study. (A) Secondary structure of the r24mini-A20 immobilized on an adipic acid dihydrazide resin. Twenty adenosines were added at the 3′-end of the wild-type r24mini. (B) Amino acid substrates: (**1**) α-*N*-Biotinyl-L-phenylalanine cyanomethyl ester (α-Biotin-Phe-CME), (**2**) L-phenylalanine cyanomethyl ester (Phe-CME), and (**3**) *p*-*N*-acetylamido-L-phenylalanine cyanomethyl ester (*p*-acetylamido-Phe-CME).

The resin derivatized with r24mini-A20 was packed in a disposable column and then incubated with v1-tRNA to form the ribozyme-tRNA complex. The reaction was initiated upon the addition of the substrate **1** (Figure 1A). After 30 min, the resin was washed with a sodium acetate buffer to remove the unreacted substrate and a trace amount of unbound v1-tRNA (Figure 2A, lanes 6 and 7). Finally, the α-Biotin-Phe-tRNA along with unreacted tRNA was eluted from the resin by using a urea buffer (lane 8). The yield of the aminoacyl-tRNA observed for the on-column reaction was 36%, which was comparable to that observed for the solution-phase reaction (Figure 2A, lane 8 vs 4; Figure 2B, first column vs C1). In contrast, the background reaction in the absence of ribozyme was negligible (Figure 2A, lane 5; Figure 2B, column C2). It should be noted that the aminoacyl-tRNA fraction was not contaminated with ribozyme that had leached from the resin, suggesting that the ribozyme-resin was still intact after the washing−elution cycle.

The above observations in the first cycle of the on-column aminoacylation reaction led us to attempt to recycle the column. After equilibration of the column with the reaction buffer, we proceeded with five additional rounds of on-column aminoacylation. We observed that the aminoacylation efficiency of the on-column reaction changed only nominally between cycles (Figure 2A, lanes 9−20). This result demonstrates unequivocally the durability and "recyclability" of the ribozyme-resin. Thus, although r24mini has limited turnover ability, this disadvantage can be overcome through the multiple use of this ribozyme-resin method. Most importantly,

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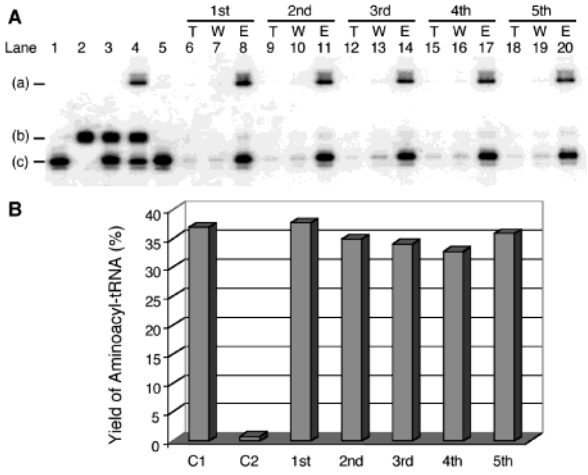

Figure 2. On-resin reaction. (A) Autoradiogram of the fractions isolated by the on-resin reaction. Both v1-tRNA and r24mini were internally radiolabeled with ^{32}P. The aminoacylated v1-tRNAs were resolved by streptavidin-dependent gel mobility-shift assay. Reactions were carried out in the presence of 10 μM v1tRNA and 5 mM α-Biotin-Phe-CME (**1**) for 30 min at 37 °C. Lane 1, v1-tRNA; lane 2, r24mini-A20; lane 3, reaction in solution in the absence of streptavidin; lane 4, the fraction in lane 3 in the presence streptavidin; lane 5, background reaction without r24mini; lanes 6−20; on-column reaction. T, fraction of wash through with the reaction buffer; W, fraction of wash through with a buffer containing 0.1 M NaOAc, 300 mM NaCl; E, fraction of elution with a 0.1 M NaOAc, 300 mM NaCl, 7.5 M urea buffer. (a) α-Biotin-Phe-tRNA complexed with streptavidin, (b) r24mini-A20, and (c) tRNA. (B) Aminoacylation efficiency determined by the streptavidin-dependent gel mobility-shift assay of the E fraction of each round. C1 and C2 indicate the yields of aminoacyl-tRNA in solution in the presence and absence of ribozyme, respectively.

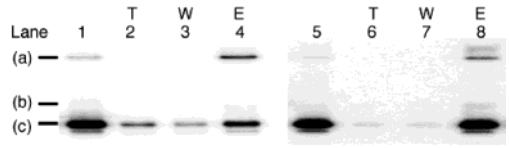

Figure 3. On-column reaction of v1-tRNA with **2** (left panel) and **3** (right panel). Reactions were carried out in the presence of 20 mM amino acid substrate for 5 min at 37 °C. Lanes 1 and 5, background reaction without r24mini-A20 in solution; lanes 2−4 and 6−7, fractions isolated by the on-column reaction. T, W, E, and (a)−(c) are the same as those in Figure 2A. In these assays, post-biotinylation[14] was used to generate the corresponding Biotin-aminoacyl-tRNA, and the product was analyzed by streptavidin-dependent gel mobility-shift assay.

this feature afforded us a method for the rapid and easy isolation of aminoacyl-tRNA from the amino acid substrate and ribozyme.

We have previously shown that Phe-CME (Figure 1B, **2**) with the free α-amino group is also a good substrate with the ribozyme. The r24mini-immobilized resin was also tested with this substrate.[14] As expected, aminoacylation of v1-tRNA with **2** took place on the resin after only a 5 min incubation (Figure 3, lane 4). Again, a 28% yield observed for this on-column aminoacylation is comparable to that for the solution phase (data not shown) and much greater than that observed for the background (Figure 3, lane 1). We further examined the on-column aminoacylation reaction using a phenylalanine analogue, *p*-acetylamido-Phe-CME, as a nonnatural amino acid (Figure 1B, **3**). The on-column aminoacylation reaction with this nonnatural amino acid is shown to proceed, but with a yield lower than those observed for the other substrates tested in this study. This modest yield is due to the insufficient activity of r24mini-A20 toward **3**, which was confirmed by the solution-phase reaction. However, this is an expected outcome since this ribozyme was evolved with **1**, and it shows remarkable specificity toward the natural side chain of Phe.

Our results here clearly demonstrate our concept that tRNA aminoacylation can be attained by means of a resin-immobilized ribozyme. The procedures, including immobilization of the ribozyme, setting up of the reaction, and the isolation of aminoacyl-tRNA by the wash and elution protocol, are remarkably simple. The resin can be recycled multiple times by simple equilibration of the ribozyme-resin with the reaction buffer. Thus, the synthesis of aminoacyl-tRNAs, potentially with nonnatural amino acids, can be significantly facilitated by this on-column aminoacylation technique. However, some issues still remain to be improved. Among them is the limited activity of the r24mini ribozyme toward Phe analog(s), which is readily addressed though the use of in vitro evolution.[15−18] On-column purification of the aminoacyl-tRNA from unreacted tRNA is also preferable. These improvements are currently under way in our laboratory. Nonetheless, our ribozyme-based aminoacylation system has significant potential to be a powerful and practical technique for supplying various nonnatural aminoacyl-tRNAs for a highly efficient in vitro translation system.[19,20]

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Supporting Information Available: Details of the preparation of ribozyme, tRNA, the ribozyme-resin, and the substrates and the procedure of the on-column aminoacylation (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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