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Designer Ribozymes: Programming the tRNA Specificity into Flexizyme

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Flexizyme (Fx) is an in vitro evolved ribozyme with the ability to aminoacylate tRNA in trans.[1] The interaction of Fx with its tRNA substrate is dictated by a 3-base-pair interaction between Fx G43-U45 and tRNA A_{73}-C_{75}[2] (Figure 1A), and thus this particular construct of Fx is referred to as Fx3. Moreover, Fx3 can charge a wide range of phenylalanine derivatives onto tRNA, and thus is useful in preparing such nonnatural aminoacyl-tRNAs in vitro.[3] Although Fx3 shows modest selectivity toward cognate tRNAs based on the complementarity of U45 to the discriminator base (N_{73}),[4] it is unable to distinguish between tRNAs sharing the same N_{73}. Herein, we report our initial attempts toward generating tRNA-specific Fx by appending a sequence at its 3′-end, which can invade the acceptor stem of the tRNA by base pairing. Since sequence complementarity dictates tRNA specificity, designer ribozymes can easily be engineered, adapting to a user-specified tRNA.

The idea for the designer Fx was hinted by our recent findings on the parental ribozyme of Fx3, r24mini, which has 11 additional nucleotides at the 3′-end.[5,6] Like Fx3, r24mini interacts with its tRNA substrate via the G43-U45/A_{73}-C_{75} base pairs, and thus lacks tRNA selectivity (see Figure S1 in the Supporting Information). However, during the course of our extensive studies on r24mini, we found that insertion of the consecutive GC between U45 and the following base (G46) resulted in 5-fold enhancement of selectivity toward $tRNA^{Phe}$ in comparison to other tRNAs (Saito and Suga, unpublished results; see Figure S1). This increase in selectivity can be attributed to the increase in base pairs from three to nine upon the GC insertion, presumably caused by the invasion of the acceptor stem of $tRNA^{Phe}$ by the mutant ribozyme. In parallel to this finding, we found that the 3′-end sequence after U45 in r24mini was redundant for the aminoacylation activity and therefore could be deleted to afford the simpler Fx3. These two independent experiments prompted us to test if we could engineer tRNA specificity into Fx3 based on the concept of the acceptor stem invasion.

To build the tRNA specificity in Fx3, we appended a 7-nucleotide tRNA-specific sequence (TSS) to the 3′-end of Fx3, which is complementary to the acceptor stem of the cognate tRNA. This family of Fx can potentially form 10 base pairs with its cognate tRNA, and thus it is referred to as Fx10. To this end, we designed four pairs of Fx10 variant and *Escherichia coli* tRNA: FxPhe-$tRNA^{Phe}$, FxAsn-$tRNA^{Asn}$, FxMet-$tRNA^{Met}$, and FxfMet-$tRNA^{fMet}$ (Figure 1B).[7]

To test whether Fx10 is able to selectively aminoacylate the cognate tRNA against noncognate tRNAs, we performed four independent experiments for every Fx10 construct. Each experiment contained one ^{32}P-labeled tRNA and three other non-radiolabeled

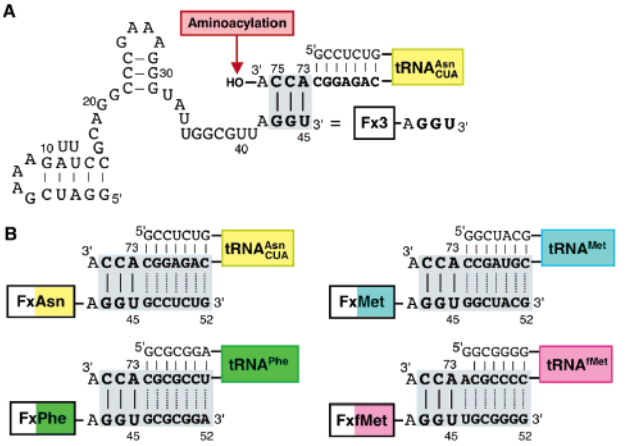

Figure 1. Strategy for programming tRNA specificity into the generic Fx3. (A) The three-base-pair interaction between the generic Fx3 (G43-U45) and $tRNA^{Asn}$ (A_{73}-C_{75}) is highlighted by the gray shaded area. (B) Schematic representation of the potential interaction of each Fx10 variant with its cognate tRNA. Each Fx10 can potentially form 10 base pairs with its cognate tRNA (bold letters in gray area). The respective Fx10 and its cognate tRNA are color-coded as shown.

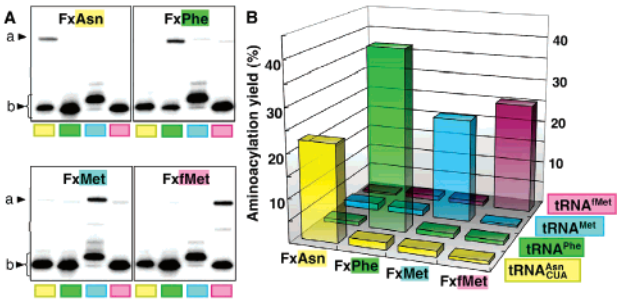

Figure 2. Aminoacylation activity of Fx10 toward its cognate and noncognate tRNAs. (A) Aminoacylation of tRNAs after a 15-min incubation. In each lane, one of the four tRNAs (represented by the colored box shown in Figure 1B) is radiolabeled: a, biotinylated aminoacyl-tRNA complexed with SAv; b, unreacted tRNA. (B) Quantitative representation of tRNA specificity of Fx10 variants. The aminoacylation yields were obtained from experiments performed in triplicate (accurate yields of aminoacylation with standard deviations are shown in Figure S2 in the Supporting Information).

tRNAs, and hence only the radiolabeled tRNA is visible during the mobility shift assays. By comparing the yields of the cognate and noncognate aminoacyl-tRNAs under such competitive conditions, the tRNA selectivity of each construct of Fx10 can be determined. As clearly shown in Figure 2A,B, each designer Fx10 is able to aminoacylate its cognate tRNA with a high degree of specificity. The difference in activity between cognate and non-cognate tRNAs lies in the range of 20- to 40-fold. These results

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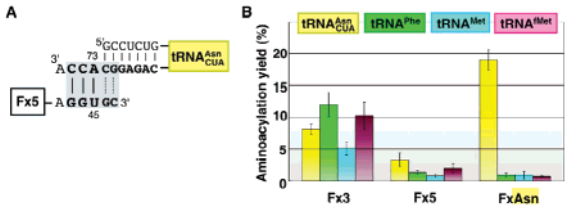

Figure 3. Comparison of aminoacylation activities of Fx3, Fx5, and FxAsn. (A) Fx5 potentially forms five base pairs with tRNA^{Asn}, as highlighted by the gray shaded area. (B) Aminoacylation efficiency of Fx3, Fx5, and FxAsn. Each experiment was performed in triplicate (a representative raw data on gel is shown in Figure S3 in the Supporting Information).

indicate that tRNA specificity can be readily programmed into Fx10 by designing an appropriate 3′-end sequence.

All tRNAs used in these experiments shared the same discriminator base (A_{73}), and hence it could be expected that Fx10 would charge noncognate tRNAs with the same level of activity as Fx3. However, the observed activities in the noncognate pairs seemed lower than expected. In fact, Fx3 was able to aminoacylate all four tRNAs in the range of 5−15% yield[8] at the time point of 15 min (Figure 3B, Fx3), which is approximately 10-fold higher than that observed for the noncognate pairs of FxAsn-tRNA (Figure 3B, FxAsn).

One possible explanation for the above observation is that the TSS of Fx10 plays a negative role in the aminoacylation activity of the noncognate pairs. Most likely, because the nonpairing sequence cannot invade the tRNA acceptor stem, it hinders the binding of Fx10 to noncognate tRNA, resulting in an increase in the K_M. Although the ideal approach to verify this assumption is to determine K_M values for the noncognate pairs, their low activity has made it difficult to assess reliable K_M values. However, if our hypothesis is correct, we can predict that the presence of a few bases after U45 has a negative impact on the aminoacylation activity for not only the noncognate pairs but also the cognate pair; because such a short stretch of nucleotides lacks the ability to invade the tRNA acceptor stem efficiently, it could act as a negative factor for binding. We thus constructed Fx5, which has G46 and C47 complementary to tRNA^{Asn} C_{72} and G_{71}, respectively (Figure 3A). As predicted, the activities of both cognate and noncognate pairs were lost by approximately 2-fold and 5−8-fold, respectively, compared to those of Fx3-tRNA pairs (Figure 3B, Fx5). Interestingly, the activities in the noncognate pairs of FxAsn-tRNA (Figure 3B, FxAsn) were still lower than those in noncognate pairs of Fx5-tRNA, indicating that the additional five 3′-end bases in FxAsn further inhibit the interaction with the noncognate tRNAs. We thus conclude that the TSS in the designer Fx10 has dual roles: it enhances charging activity onto the cognate tRNA and also suppresses mischarging activity onto the noncognate tRNAs.

For Fx10 to form the complex with its cognate tRNA, its TSS must compete with the complementary arm of tRNA acceptor stem and eventually denature the acceptor stem by the invasion. We wondered whether the observed aminoacylation rate involves the invasion rate. Two single-turnover experiments were examined to answer this question. To exclude the rate of invasion from the observed rate, in the first experiment the independently folded FxPhe and tRNA^{Phe} were mixed together for 30 min, prior to the initiation of the reaction by the addition of the amino acid substrate. To include the invasion rate in the observed rate, in the second experiment the folded tRNA^{Phe} was incubated with the amino acid substrate, followed by the addition of the folded FxPhe to initiate reaction. We observed that, in the latter experiment, there was a 2−3-min time lag in attaining aminoacylation rates similar to that of the former experiment (Figure S4 in the Supporting Information). After this threshold point, however, the observed rates for both the cases were nearly the same. We thus conclude that, while the invasion rate is involved in the observed aminoacylation rate, it is faster than the aminoacylation rate.

In conclusion, we have demonstrated that a generic ribozyme, Fx3, can easily be converted into a designer Fx10 by extending its 3′-end with a tRNA-specific sequence. In addition to the amino acid selectivity toward aromatic amino acids inherited from the parental Fx3, Fx10 can also exhibit a high degree of tRNA selectivity. Moreover, Fx10 discriminates the cognate tRNA against others by using both positive and negative determinants, similar to the protein aminoacyl-tRNA synthetases (ARSs).[9] We have thus generated a functional mimic of ARSs, stacking up evidence in favor of RNA as a versatile catalyst prior to the advent of protein catalysts.[10−12] Most importantly, the programmability of tRNA specificity in Fx10 and its ability to recognize various phenylalanine derivatives will allow us to generate user-specified aminoacylation catalysts that can be used for nonnatural amino acid mutagenesis of proteins[13−15] in vitro with great ease.[1] Such studies are under way in our laboratory.

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Supporting Information Available: Experimental procedures for the preparation of the Fx10 family, the tRNAs, the aminoacylation and kinetic studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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