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A flexizyme that selectively charges amino acids activated by a water-friendly leaving group

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ABSTRACT

We have developed a new flexizyme (a flexible de novo tRNA acylation ribozyme) system, a pair of amino-derivatized benzyl thioester (ABT) and amino flexizyme (aFx). ABT bearing the ammonium ion was designed to render the acyl-donor substrates better water solubility. Although the previously reported flexizymes (eFx and dFx) did not show acylation activity for the ABT derivatives, a new flexizyme variant aFx, generated by in vitro selection against an amino acid activated ABT, exhibits high selectivity toward those activated ABT. The flexizymes system including aFx, eFx, and dFx enables us to prepare a wide variety of acyl-tRNAs charged with non-proteinogenic amino acids.

Engineering of the universal genetic code has given us a new opportunity to express peptides or proteins bearing non-proteinogenic amino acids.[1–4] Recent developments in methodologies of the genetic code reprogramming have enabled us to reassign multiple codons to non-proteinogenic amino acids.[5–21] By means of such methodologies, libraries of non-standard peptides can be prepared in the mRNA-encoding manner.[14] We have engaged the development of such a methodology integrated with flexizymes.[10,22–24] Flexizymes are flexible tRNA acylation ribozymes and facilitate the preparation of various non-proteinogenic aminoacyl-tRNAs, which are otherwise complex and technically difficult processes. Two flexizymes, referred to as eFx and dFx, have been devised (Fig. 1); the eFx acylates tRNA with amino or hydroxy acids upon activation with cyanomethyl ester (CME) group or *p*-chlorobenzyl thioester (CBT), where its activity relies on the recognition of aromatic sidechain or the CBT group in the substrate; on the other hand, dFx acylates tRNA with those activated with 3,5-dinitrobenzyl ester (DBE) group, where its activity fully relied on the recognition of the DBE group. Since both flexizymes bind the 3′-end sequence of tRNA via the 3-base pairing interaction (RCCA-3′, R = G, A and U are the preferable discriminator base at position 73, but even C is acceptable under prolonged incubation), any tRNAs can be the acyl-acceptor for the flexizymes. Thus, by the combination of these two flexizymes, virtually any desired acyl-tRNAs can be readily prepared.

However, a shortcoming of the current flexizyme system is that since the CBT or DBE-derived the acyl-donor substrates make them more hydrophobic, some derivatives, particularly those with hydrophobic sidechains, are occasionally difficult to dissolve in the aqueous reaction buffer. Notably, eFx and dFx are able to retain their catalytic activity in the buffer containing up to 20% and 40% DMSO, respectively, but the addition of DMSO to the reaction buffer

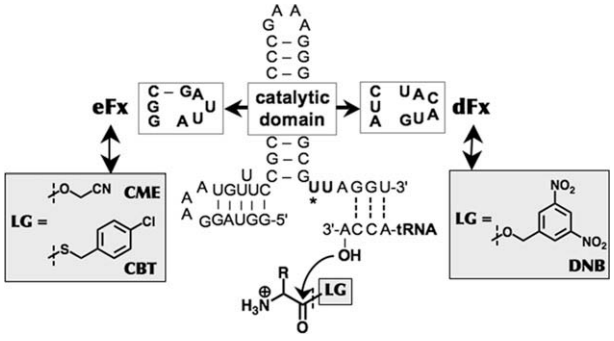

Figure 1. Flexizymes and their cognate leaving groups in substrates. Chemical structures of a benzyl ester and thioester leaving group. R represents amino acid sidechains including non-proteinogenic ones. Each flexizyme recognizes the specific leaving group and charges the acyl group onto the 3′-hydroxyl group at the tRNA 3′-end. *Abbreviations*: LG, leaving group; CME, cyanomethyl ester; CBT, *p*-chlorobenzyl thioester; eFx, enhanced flexizyme; DNB, 3,5-dinitrobenzyl ester; dFx, dinitro flexizyme. U with asterisk denotes the absolutely conserved U-turn base. Bold bases orchestrate to constitute the catalytic domain of each flexizyme.

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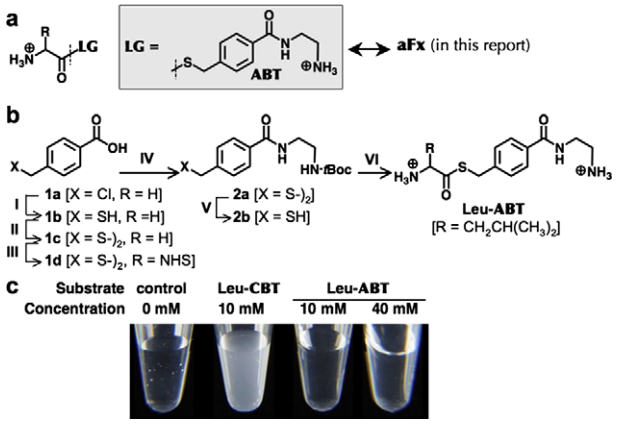

Figure 2. A new substrate design, synthesis, and solubility in the reaction buffer. (a) Chemical structures of acyl-donor substrates. R represents amino acid sidechains including non-proteinogenic ones. Abbreviations are as follows: LG, leaving group; ABT, amino-modified benzyl thioester; aFx, amino flexizyme. (b) Synthesis of aminoacyl-ABT. An example of Leu derivative is shown. Reagents and conditions: (I) thiourea, H_2O, 1 h, reflux, then 10% NaOH, 1 h, reflux, quant.; (II) I_2, EtOH, 0 °C, 1.5 h, quant.; (III) *N*-hydroxysuccinimide (NHS), EDC·HCl, 1,4-dioxane, CH_2Cl_2, rt, 1 h, quant.; (IV) mono-*t*Boc-ethylendiamine, H_2O, 1,4-dioxane, 0 °C, 2 h, 91%; (V) DTT, DMF, 95 °C, 1 h, 50%; (VI) *t*Boc-L-leucine, EDC·HCl, DMAP, CH_2Cl_2, rt, 1 h, then HCl, AcOEt, CH_2Cl_2, rt, 0.5 h, 30%. (c) Solubility of Leu-CBT and Leu-ABT. Each compound was dissolved in 0.1 M Hepes-K (pH 7.5), 20% DMSO at the concentration as shown.

hampers the intrinsic ability of the flexizymes. Therefore we have a dilemma between poor solubility of the substrates and loss of flexizyme activity by the addition of DMSO. Here we report a new pair of flexizyme and substrate activated by a benzyl thioester in which *p*-position is derived to an amino group that is protonated in the reaction buffer to give the ammonium salt. By using this pair, we have demonstrated aminoacylation of tRNA with amino acids bearing hydrophobic sidechains or the α-*N*-octanoyl modification on the sidechain.

In order to make the leaving group more water-soluble, we derived a commercially available 4-chlorobenzoic acid to have a primary amino group and a thiol group for activation of the acyl group (Fig. 2a). Synthesis of this leaving group and its derivatization of amino acid was straightforward as shown in Figure 2b. We referred this amino-derivatized benzyl thioester aminoacyl-donor to as aminoacyl-ABT (Fig. 2a).

First, we compared the solubility of 10 mM Leu-CBT and Leu-ABT in the flexizyme reaction buffer containing 20% (v/v) of DMSO. Despite the solution of Leu-CBT with a cloudy suspension, Leu-ABT gave a completely clear solution. The solution remained clear even with 40 mM Leu-ABT (Fig. 2c). Thus, the ABT leaving group significantly improved the water solubility of the Leu substrate. However, the alteration of the leaving group turned out to be detrimental for the acylation activity of the available flexizymes, dFx and eFx, yielding less than 1% leucylation onto a microhelix RNA (Fig. S1; regarding this analytical method see below). This observation imposed us to perform in vitro selection of a new flexizyme capable of reacting the ABT-derived substrate.

The method of in vitro selection and the flexizyme pool containing three random regions (Fig. S2a) were the same as our previous report[10] except that the active sequences for self-aminoacylation were enriched by the reaction with Leu-ABT followed by the selective biotinylation on the α-amino group of the charged Leu. After six rounds of selection of active sequences by capturing on a streptavidin-agarose resin (Fig. S2b), we observed an enrichment of the pool. The 3′-terminal specific activity of the pool round 6 was confirmed by the activity loss upon the periodate oxidation (Fig. S2c). 23 clones isolated from the pool were sequenced, and their alignment revealed that 19 clones fell in a single class (Fig. S2d). We

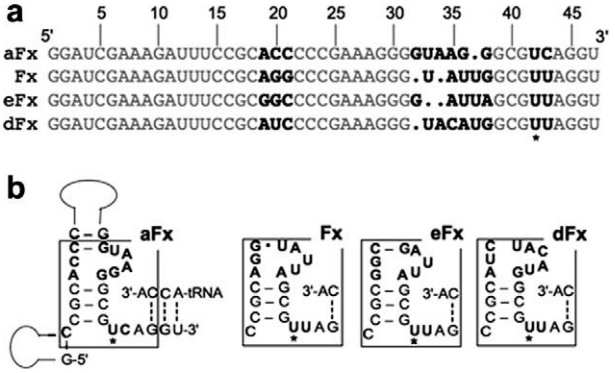

Figure 3. Sequence alignment and secondary structures of aFx and other flexzymes. (a) Sequence alignment. Bases originating from the random regions were shown in bold, and the conserved bass (U42) was shown with asterisk. (b) The secondary structure of aFx compared with the catalytic domain in other flexizymes.

picked 5 representative clones and tested for self-aminoacylation; and found that all are active toward Leu-ABT. Among them, clone 11 exhibited the desired 3′-terminal specific activity in the highest efficiency (Fig. S3). Therefore, we focused on this clone for further studies. We referred this new flexizyme to as aFx as oppose to eFx and dFx.

The sequence alignment and drawing the individual secondary structure of aFx, eFx, dFx and the parental Fx revealed the difference in the composition of catalytic domain (Fig. 3a and b). The only conserved base is U42; this base is known to form a unique U-turn structure and plays a critical role in presenting the catalytic domain to the 3′-hydroxyl group at the tRNA 3′-end.[24] Because of lacking our knowledge of the 3-dimensional structure of the catalytic domain in the individual flexizyme except for Fx, it is yet unclear how the sequence variation is able to adapt to the respective leaving group (Fig. 3b). However, even for Fx we have shown that the catalytic domain is susceptible to mutagenesis,[24] it seems that the catalytic domain can be readily adapted to various benzyl leaving groups by the sequence alterations. Nonetheless, the present result indicates that the selection from the doped pool of flexizyme against substrates with different characteristic leaving groups readily generates new flexizyme variants.

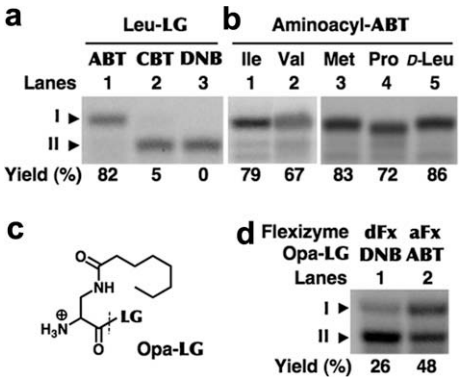

Figure 4. Aminoacylation ability of aFx. (a) Selectivity of aFx to cognate Leu-ABT against non-cognate substrates. Bands of I and II indicate aminoacyl-microhelix RNA and microhelix RNA, respectively. Conditions: 0.1 M Hepes-K (pH 7.5), 50 mM $MgCl_2$, 20% DMSO, 20 μM microhelix RNA, 20 μM aFx, and 5 mM substrate on ice for 2 h; (b) Flexibility of aFx toward sidechain in the aminoacyl-ABT structures. Conditions for Ile-ABT (lane 1): 0.1 M Hepes-K (pH 7.5) and 10 mM substrate on ice for 24 h. Conditions for Val-ABT (lane 2): 0.1 M Bicine-K (pH 9.0) and 10 mM Val-ABT on ice for 6 h. Reactions in lanes 3–5 were carried out by the same conditions described in Figure 4a. (c) Chemical structure of Opa-LG. Opa, 2-amino-3-(octanamido)propanoic acid; LG, leaving group. (d) Aminoaylation of Opa onto a microhelix RNA using aFx or dFx against the cognate Opa-LG. The reaction conditions were the same as those described in Figure 4a, lane 1.

To verify the ability of aFx for trans-activity, we prepared the aFx domain alone by in vitro transcription from the corresponding DNA template, and examined the aminoacylation onto a microhelix RNA using denaturing acid PAGE (poly-acrylamide gel electrophoresis), a previously reported method for the conventional and reliable activity assay for trans-acting flexizymes.[10] The trans-acting aFx was able to charge Leu using Leu-ABT onto the microhelix RNA in 82% yield (Fig. 4a, lane 1). Interestingly, it was virtually inactive toward Leu-CBT and Leu-DNB (Fig. 4a, lanes 2 and 3), indicating that aFx exhibited high specificity to the ABT leaving group.

To investigate the tolerance of aFx toward amino acid sidechains and α-chirality, we synthesized five additional amino acids, Ile, Val, Met, Pro, and D-Leu, activated by ABT (Fig. 4b). The CBT or DNB derivatives of these amino acids were known to have modest or poor solubility at 5 or 10 mM in the reaction buffer unless including above 20% (v/v) DMSO. The ABT derivatives of these amino acids were, however, efficiently charged onto the microhelix RNA in high yields (Fig. 4b). This result shows that aFx is able to charge the ABT-derived amino acids independent from their sidechain structure and α-chirality.

Lastly, we performed aminoacylation using a non-proteinogenic amino acid bearing a fatty acid, 2-amino-3-(octanamido)propanoic acid (Opa, Fig. 4c). We found that eFx was able to charge Opa-CBT onto the microhelix RNA in 26% yield (Fig. 4d, lane 1), whereas aFx was able to charge Opa-ABT in 48% yield (Fig. 4d, lane 2). Clearly, improving the water-solubility of the acyl-donor substrate offered a better yield.

In conclusion, we have devised a new pair of flexizyme and substrate activating group for tRNA aminoacylation, referred to as aFx and ABT. aFx selectively charges ABT-activated amino acids onto tRNA independent from the sidechain kinds. Importantly, the ABT leaving group makes hydrophobic amino acid substrates more water-friendly than CBT and DBE. We now have three pairs of flexizyme and acyl-donor, allowing us to choose the most appropriate pair of flexizyme and acyl-donor depending upon the substrate properties for the genetic code reprogramming.

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Supplementary data

Supplementary data (synthetic procedures, a method of in vitro selection, and analysis for aminoacylation were reported in supplementary data) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.114.

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