ChemComm

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Journal:	*ChemComm*
Manuscript ID	CC-COM-05-2019-003547.R1
Article Type:	Communication

COMMUNICATION

Optimizing Aromatic Oligoamide Foldamer Side-Chains for Ribosomal Translation Initiation

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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The tolerance of ribosomal peptide translation for helical aromatic oligoamide foldamers appended as initiators has been investigated. Small cationic foldamer side chains were shown to expand the range of foldamer-peptide hybrids that can be produced by the ribosome to more rigid sequences.

Genetic code reprogramming (GCR) has allowed for the incorporation of non-natural functional groups into ribosomal peptides.[1,2] The main objective of such insertions is to endow the peptides with new chemical or biophysical properties, such as programmable chemical reactivity, tight target binding affinity and peptidase-resistance.[3-8] Among the different GCR methods available, the use of flexizymes coupled with an *in vitro* translation system (the FIT system)[9] stands out because of their compatibility with a plethora of non-natural residues.[10-14]

In a recent study, abiotic helical aromatic oligoamide foldamers comprised of quinoline (Q) and pyridine (P) amino acids have been used to initiate translation, affording the first-of-their-kind ribosomal foldamer-peptide hybrids (Fig. 1).[15] This sets an important milestone as it gives access to chimeric peptides with unusual structures and topologies. Indeed, the compact aromatic foldamer conformation was shown to convey folding information to otherwise flexible appended peptides. Foldamers spanning over 1.5 helical turns were incorporated as translation initiators, albeit in relatively low yield. Nevertheless, incorporation appeared not to proceed at all when the structural features of foldamer building blocks, *i.e.* molecular volume and conformational flexibility were not suitable to traverse the ribosome exit tunnel. For instance, a foldamer substrate with three quinoline units equipped with aspartate-like side-chains $(Q^{Asp})_3$ prevented translation (Fig. 1b). Yet conformationally more flexible versions containing aminomethyl-pyridine units were tolerated. Indeed, it has been demonstrated that the P monomer, owing to its additional rotatable bond, can abate the conformational stability of the folded structure.[16,17] An energy-minimized model of Ac-$(Q^{Asp})_3$G-OH in its folded conformation gives an estimate of its volume (718 Å^3) and width (17.4 Å) including side-chains (Fig. 2a, left). Considering that the narrowest point of the ribosome exit tunnel has an approximate diameter of 10 Å,[18] this foldamer is unlikely to pass unless it partly unfolds. Unfolding would still be required with smaller side chains but we devised that the barrier might nevertheless be reduced and thought it worth

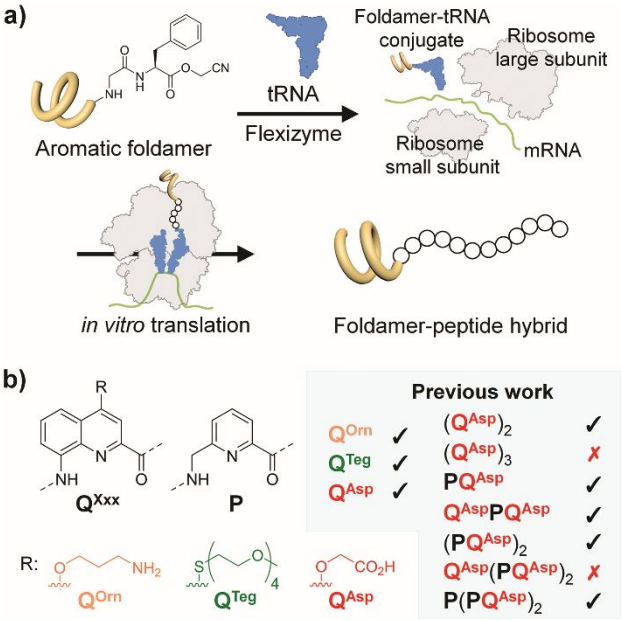

Figure 1. (a) Schematic illustration of flexizyme-assisted ribosomal translation of a helical foldamer-peptide hybrid; (b) Foldamer building blocks and sequences tested for *in vitro* translation experiments in the previous study.

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Electronic Supplementary Information (ESI) available: Supporting figures, experimental procedures, and ^{1}H NMR spectra. See DOI: 10.1039/x0xx00000x

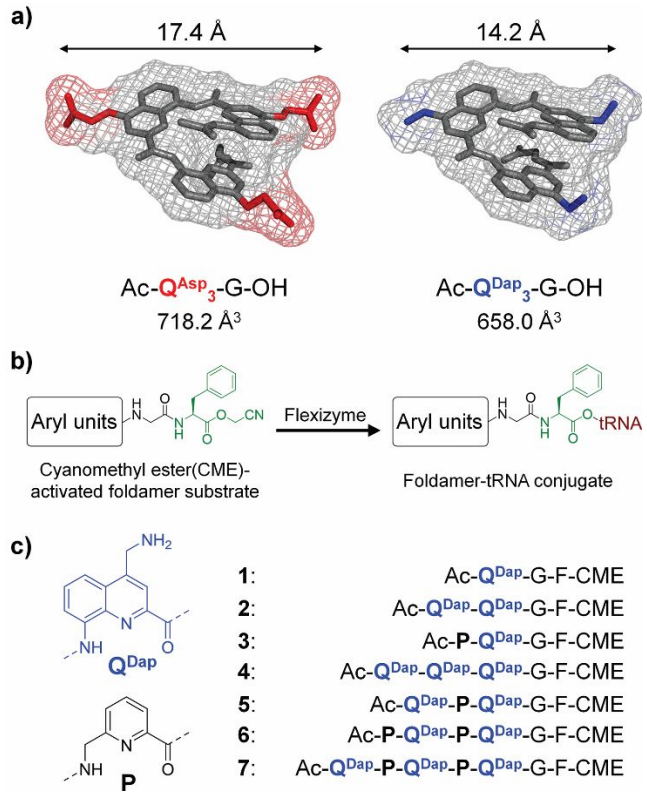

Figure 2. (a) Model structures of helically folded Ac-QQQ-G-OH with Asp- (left) and Dap-like (right) side-chains. (b) Foldamer substrates were attached to the N-terminus of a GF dipeptide. The acid terminus was activated as a cyanomethyl ester (CME). The Flexizyme recognition site is highlighted in green. (c) Sequences of aromatic oligoamide foldamer initiators tested for flexible *in vitro* translation.

exploring. In the following we report our success at extending the scope of foldamers as translation initiators through the use of a smaller and cationic side chain. We also examined the effect of elongation cofactor P (EF-P) to assess the translation limit.

A positively charged monomer (Q^{Dap}) bearing a short aminomethyl group akin to diaminopropionic acid was recently developed for the purpose of enhancing foldamer crystal growth ability in water (Fig. 2c),[19] as opposed to Q^{Orn} which bears a longer ornithine-like side-chain (Fig. 1b). In comparison with Ac-$(Q^{Asp})_3$G-OH, a model structure of Ac-$(Q^{Dap})_3$G-OH has reduced volume and width of 658 Å^3 and 14.2 Å, respectively (Fig. 2a, right). In principle, the conformational stability of such sequences is largely determined by the backbone and does not show significant side chain dependence. As a possible issue, the cationic nature of Q^{Dap} mightpromote aggregation of Q^{Dap}-containing sequences with tRNA or binding to the rRNA that lines the ribosome exit tunnel. For instance, other cationic aromatic foldamers have been shown to bind to DNA.[20-22] We nevertheless endeavoured to test this unit and set to prepare foldamer sequences **1**-**7**, based on various combinations of Q^{Dap} and P connected *via* a glycine spacer to a cyanomethyl ester (CME) activated phenylalanine. The respective sequences were assembled on solid phase on an acid labile trityl resin. Cleavage from the resin under mild conditions made it possible to keep tBoc side chain protections on. Cyanomethyl esters (CME) were then installed, before a final purification step and tBoc cleavage wich proceeded quantitatively (see supplementary information).

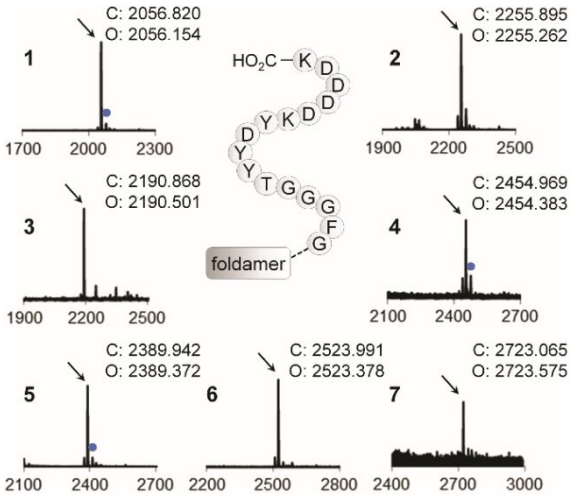

Figure 3. Mass spectra of the translated foldamer-peptide hybrid sequences using aromatic foldamer initiators **1**-**7**. The desired $[M+H]^+$ peaks are pointed by an arrow. The blue dots correspond to $[M+Na]^+$. Calculated (C) and observed (O) masses are indicated in g.mol^{-1}.

Using phenylalanine-CME as a flexizyme recognition site, the installation of the foldamers **1**-**7** was firstly assessed *via* an established protocol onto a tRNA analogue, the microhelix RNA (Fig. S1-S4).[23] Based on this assessment, the respective foldamers were charged on $tRNA^{fMet}_{CAU}$ (Fig. 2b). Each foldamer-tRNA conjugate was then supplemented to a Met-free custom-made FIT system, and the expression of the desired foldamer-peptide hybrid was monitored by MALDI-TOF mass spectrometry. Owing to the cationic nature of Q^{Dap} and taking into consideration reported ribosomal limitations stemming from the translation of multiple positively charged residues,[24] we replaced the previously used mRNA template encoding for foldamer-GF-KKKFDYKDDDDK (underlined residues constitute the so-called FLAG tag), which bore three consecutive lysine residues near the N-terminus,[15] by a new one encoding for foldamer-GF-GGGTYYDYKDDDDK. The Flag octapeptide DYKDDDDK at the C-terminus helps improving overall solubility, enables anti-FLAG antibody-mediated purification and facilitates ionization during mass spectrometric analysis. Fig. 3 shows that the translated foldamer-peptide hybrid products were detected for all foldamer initiators. This outcome is remarkable in particular for rigid initiator **4** which bears consecutive quinoline rings and for the longest initiator **7**, the equivalents of which with Q^{Asp} units failed to initiate translation.[15]

The presence of five aspartate residues in the translated peptides allowed us to carry out radioisotope quantification of product formation using [^{14}C]Asp appended in the FLAG tag region (Fig. 4), and to compare the yields of Q^{Dap}-containing sequences with those of equivalent sequences having Q^{Asp} units (Table 1). Thus, quantification was carried out using foldamer-GF-$tRNA^{fMet}_{CAU}$ and [^{14}C]Asp in a translation system from which Met and Asp have been omitted. The reaction was subsequently

separated by tricine SDS-PAGE, followed by autoradiographic quantification. In order to avoid erroneous conclusions originating from differences in the expression level of the different templates, comparisons were made using the percentage of foldamer-initiated with respect to the fMet-initiated peptide.

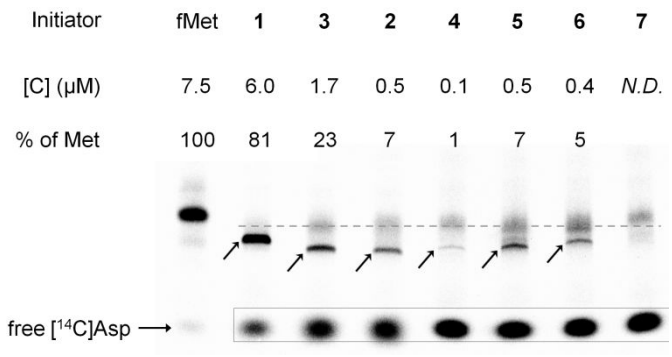

Figure 4. Quantification of foldamer incorporation. The desired bands are indicated by the arrowheads. The translation reaction initiated with fMet is shown. The broad fade band crossed with a dashed line corresponds to initiation of translation from the second amino acid (H_2N-GGGTYYDYKDDDDK).

Table 1 Quantitative effect of side-chains on translation efficiency

	Side-chain of Q monomer	
Initiator	$-CH_2NH_2$ (Q^{Dap})	$-OCH_2COOH$ [a] (Q^{Asp})
1, Ac-Q-GF	81%	29%
2, Ac-QQ-GF	10%	11%
3, Ac-PQ-GF	22%	5%
4, Ac-QQQ-GF	1%	No incorporation
5, Ac-QPQ-GF	6%	3%
6, Ac-PQPQ-GF	5%	3%
7, Ac-QPQPQ-GF	*N.D.* [b]	No incorporation

[a] from reference [15].

[b] No distinct band was observed in the radioisotope experiment and thus any approximation would be imprecise.

Subsequent quantification showed the translation efficiency of **1** to be 3-fold higher than the literature value for Q^{Asp} (81% *vs.* 29%). We advanced our investigation by adding either a Q or a P monomer to the N-terminus of **1**. Initiation with **2** and **3** yielded the desired compounds but the addition of the second monomer, in both cases, took its toll on the translation efficiency as manifested by the quantification results (Table 1). The observed translation efficiencies are in concordance with monomer size. The smaller PQ initiator **3** was incorporated two times more efficiently than **2**. Comparing these values with those obtained with Q^{Asp}, we again found that the Q^{Dap} side chain brings an advantage for the PQ initiator (22% *vs.* 5%, respectively). In the case of the QQ initiator and only in this case, the yields were similar regardless of the side chain.

An additional Q^{Dap} unit on dimer initiators **2** and **3** afforded trimers **4** and **5**, respectively (Fig. 2a), both spanning over one helix turn. Translation of the foldamer-peptide hybrid was anticipated in the case of **5**, since it had previously been successful for the Q^{Asp} related sequence. Detection by MALDI-TOF MS (Fig. 2b) followed by translation quantification demonstrated that side-chain modification led to a 2-fold increase of the expression level. In contrast, initiation had not been detected with sequences containing $(Q^{Asp})_3$. It is thus remarkable that initiator **4**, which contains $(Q^{Dap})_3$, allows translation to proceed not just at a detectable level but at a quantifiable level. As emphasized above, with three consecutive Q units, foldamer segment **4** exhibits a rigid helical secondary structure.

Motivated by the outcome of this investigation, we further extended substrate **5** by an additional P monomer, yielding tetramer **6**. Peptide expression was again found to proceed more efficiently than with Q^{Asp} monomers by a factor two. Finally, we attempted the incorporation of a QPQPQ pentamer using $tRNA^{fMet}_{CAU}$-**7** as the initiator and were able to identify the desired product by MALDI-TOF-MS analysis. Yet this time the rigidity and overall size (~ 1kDa) of the initiator impaired the quantification of the desired product. Aromatic foldamer fragment **7** is actually the largest single unnatural block that has ever been incorporated in a peptide sequence by the ribosome.

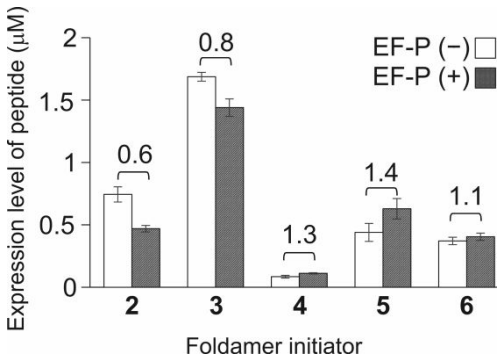

Figure 5. Expression level of peptide MGGGTYYDYKDDDDK in which foldamer-GF segments were introduced at the initiator position (M). Numbers above the bars express the relative translation yield calculated as the ratio of EF-P(+) to EF-P(-). The concentration of EF-P in the EF-P(+) reaction is 5 μM. Reaction time is 30 min. Error bars, S.D. (n = 3)

Having demonstrated beneficial effects of altering the side-chain composition on the ribosomal incorporation of foldamers, we set to investigate the impact of EF-P in the context of this work. EF-P is a bacterial translation factor that has been shown to play a role in translation initiation and elongation.[10, 13, 14, 25] In the case of the translation initiation event, it has been suggested that EF-P can accelerate the formation of the first peptide bond. We thus hypothesized that adding EF-P may benefit the foldamer-initiated translation. Note that the conventional composition of the FIT system, as used above, does not include EF-P.[9] Moreover, fine-tuning of translation factors, that is only feasible in the custom-made FIT system, has recently been shown to significantly enhance the efficiency when incorporating D- and β-amino acids. Herein, a final EF-P concentration of 5 μM was used, based on optimization experiments (data not shown) and previous reports.[13] The effect of EF-P was evaluated for foldamer segments **2** – **6** and the results are depicted in Fig. 5. Effects are moderate but reproducible and beyond experimental error. Our results suggest that in the case of dimers **2** and **3**, the presence of EF-P

slightly inhibits the expression level. Instead, in the case of trimers **4** and **5** and tetramer **6** EF-P moderately increases the expression levels of the desired hybrids. Similar to the effect of EF-P in translation elongation, no generalization can be made, and differences can be observed case-by-case. To our knowledge, this is the first demonstration of the effect EF-P in translation initiation, in the context of an *in vitro* translation system.

In conclusion, we have shown that the range of helical aromatic foldamers that can be used as initiators for the ribosomal synthesis of foldamer-peptide hybrids extends beyond the anionic sequences initially presented. Sequences bearing multiple positive charges are also tolerated. Furthermore, cationic side chains made it possible to initiate translation with more rigid foldamers than previously found. Although our initial work hypothesis was to test a smaller side chain to facilitate the passage through the ribosome tunnel, other factors than size may be involved as well, for example positive charges. We have also demonstrated that the addition of EF-P leads to a moderate enhancement of the translation efficiency of the longer foldamer substrates. Altogether, our findings suggest that there is room for optimization of translation initiation with a variety of foldamer appendages reaching almost 1 KDa in size. An expected benefit of incorporating foldamers is to contribute to the overall folding propensity of the foldamer-peptide hybrids. To exploit this effect, the next step is to achieve the identification of foldamer-peptide hybrids to bind specific protein targets using screening display technologies. Current efforts toward this goal are underway and will be reported in due course.

This work was supported by Grant-in-Aid for JSPS Postdoctoral Fellowship for Research in Japan (Standard) (P18727) to C.T; a Joint ANR-JST grant (ANR-14-JITC-2014-003 and JST-SICORP), JST CREST (JPMJCR12L2), AMED (JP18am0301001 and JP18am0101090) to H.S., and by Basic Science Research Programs through the National Research Foundation grant (NRF-2018R1C1B5086457).

Conflicts of interest

There are no conflicts to declare.

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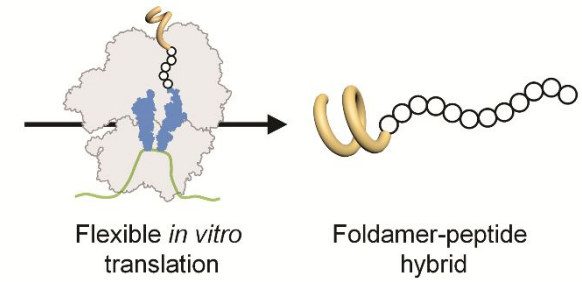

The tolerance of ribosomal peptide translation for helical foldamers appended as initiators was extended to longer and more rigid structures.