Peptide Cyclization

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Synthesis of the Backbone Cyclic Peptide Sunflower Trypsin Inhibitor-1 Promoted by the Induced Peptidyl-tRNA Drop-off**

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Ribosomes occasionally stall on mRNA in the middle of translation and one of the mechanisms to rescue the ribosome involves the drop-off of peptidyl-tRNA from the P site of the stalled ribosome.[1] Although peptidyl-tRNA drop-off can, in principle, occur at any position during the translational elongations,[2] the slower rate of the termination step relative to the elongation step can result in the stalling of the ribosome at the last sense codon, which ultimately leads to increased rate of peptidyl-tRNA drop-off.[3] This process presents the possibility for the peptidyl-tRNA drop-off event to be artificially induced under the appropriate conditions. Buckingham and co-workers indeed showed that specific peptidyl-tRNA species could be released from the P site of ribosomes that were paused at the last codon of the A site.[4] Specifically, when an mRNA template coding for fMet-Phe-Leu (fMet = formylated methionine; fM) having a UAA stop codon was translated in an in vitro system, which lacked release factors, fMet-Phe-Leu-tRNA^{Leu} was released from the ribosome. Also the same fMet-Phe-Leu-tRNA^{Leu} drop-off event was observed when an mRNA coding for fMet-Phe-Leu-Ile was translated in an in vitro translation system lacking Ile-tRNA^{Ile}. This observation demonstrates that the peptidyl-tRNA drop-off can be artificially induced by an appropriate manipulation of the translation conditions. Although the outcome of the above strategy is the termination of the translation, we postulated that this experimental strategy could be extended by exploiting the unique ester bond in the resulting peptidyl-tRNA. In the course of our studies on reprogramming the genetic code, we have also realized that peptidyl-tRNA drop-off might be induced by the use of a specially reconstituted translation system, from which one of the proteinogenic amino acids is omitted to generate a "vacant codon". Significantly, this approach would allow designation of the site of ribosome stalling, and therefore control peptidyl-tRNA drop-off in a site-specific manner. Herein we report proof-of-concept experiments in which specific peptidyl-tRNA drop-off is brought about by creating a vacant codon. In particular, the drop-off of Pep-Cys-Pro-tRNA^{Pro} (Pep = peptide) results in a series of steps leading to the conversion of the C terminus of the peptide into a thioester structure, which is key to the formation of a backbone-cyclic peptide.

A general scheme for the preparation of backbone cyclic peptides using peptidyl-tRNA drop-off is given in Figure 1. In the typical translation system, the ribosome encounters the His CAC codon, for instance His-tRNA$^{His}_{GUG}$, and the peptide

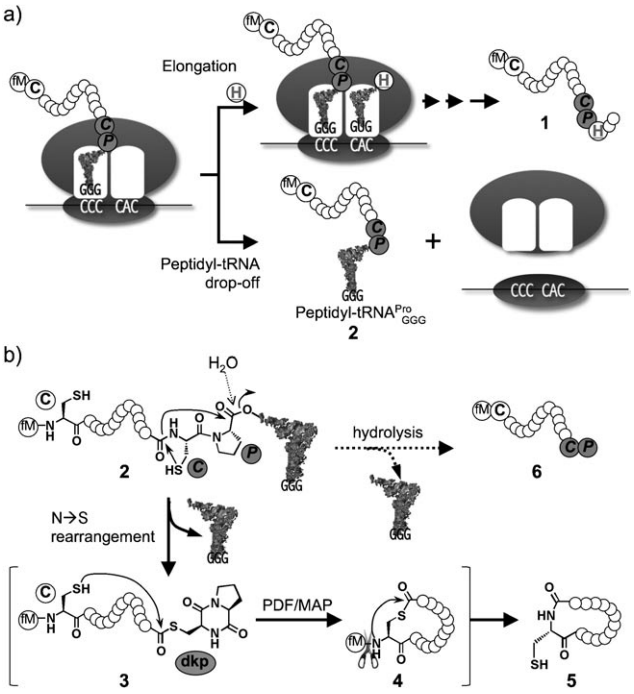

Figure 1. Ribosomal expression of backbone cyclic peptides through the programmed peptidyl-tRNA drop-off. a) Induced peptidyl-tRNA drop-off at the predetermined site. In this example a codon for His (CAC) is used as a vacant codon for the site of peptidyl-tRNA drop-off. b) Intramolecular rearrangements occurring in the released peptidyl-tRNA.

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chain is elongated by incorporating His to yield the full-length peptide **1** (Figure 1a). We postulated that if His were omitted from the translation components, the ribosome would stall at this vacant His codon and induce the peptidyl-tRNA drop-off (Figure 1a). If this vacant codon was placed right after the Cys-Pro sequence, the ribosome would stall at the Pro codon of the A site, thus resulting in the release of Pep-Cys-Pro-tRNA$^{Pro}_{GGG}$ (**2**, Figure 1). Importantly, it can be envisioned that the ester bond in Pro-(3′-O)-adenosyl76 of Pep-Cys-Pro-tRNA$^{Pro}_{GGG}$ is analogous to that in Pro-glycolic acid (^{HO}G) that was generated by the codon reassignment of CAC to ^{HO}G, as we reported recently.[5] Therefore we can expect an intramolecular rearrangement in which an non-enzymatic acyl shift from the nitrogen atom to the sulfur atom affords the diketopiperadine thioester (dkp thioester; **3**) with the concomitant release of tRNA$^{Pro}_{GGG}$ (Figure 1b). This transformation would be driven by the formation of the ring in dkp and would compete with the hydrolysis of the Pro-tRNA$^{Pro}_{GGG}$ ester bond to give **6** (Figure 1b). The dkp thioester formation could promote an additional intramolecular rearrangement involving the thiol group of another Cys to afford the macrothiolactone **4** (Figure 1b). Moreover, if the free N terminus of the amino group of this Cys was generated by the enzymatic removal of fMet, an acyl shift from the sulfur atom to the nitrogen atom would generate the backbone cyclic peptide **5** (Figure 1b). Notably, this sequence recycles tRNA$^{Pro}_{GGG}$ instead of sequestering it as peptidyl-tRNA, and synthesizes backbone cyclic peptides without the use of a nonproteinogenic ^{HO}G.

To experimentally prove the above concept, we designed an mRNA template that encoded a 20-mer peptide sequence, in which the 14-mer peptide sequence of sunflower trypsin inhibitor-1 (SFTI-1)[6] was embedded from position 2–15 (Figure 2a). Codons encoding Cys16-Pro17, His18 (a vacant codon), and Lys19-Lys20 were placed after those encoding SFTI-1. We designed and carried out four reactions to prove the mechanism proposed in Figure 1. When the DNA template was subjected to sequential transcription and translation in the typical translation system, the full-length peptide fMet1-Lys20 (**7**) was expressed (Figure 2b and c). When the same DNA template was decoded under reducing conditions and in the absence of His a new single peak appeared (Figure 2d). The *m*/*z* value obtained for a singly charged peptide species was consistent with the calculated value of fMet1–Arg15 minus a water molecule, suggesting that the peak corresponds to macrothiolactone **8** (Figures 2b and d). Importantly, we observed a very minor peak for the hydrolyzed side-product **9** (Figure 2d), suggesting that the rearrangement of **10** into **8** via the corresponding dkp thioester was rapid enough to out compete the ester hydrolysis. To establish whether the macrothiolactonization was derived from the released Pep(1–15)-Cys16-Pro17-tRNA$^{Pro}_{GGG}$ (**10**; Figure 2b), we attempted to enzymatically hydrolyze the ester bond between Pro17 and tRNA$^{Pro}_{GGG}$ in **10** by using peptidyl-tRNA hydrolase (PTH) in the translation system.[7] When PTH was present in the translation mixture, a new single peak corresponding to the molecular mass of the linear peptide fMet1-Pro17 (**9**) was observed (Figures 2b and e). This data supports the idea that the peptidyl-tRNA drop-off

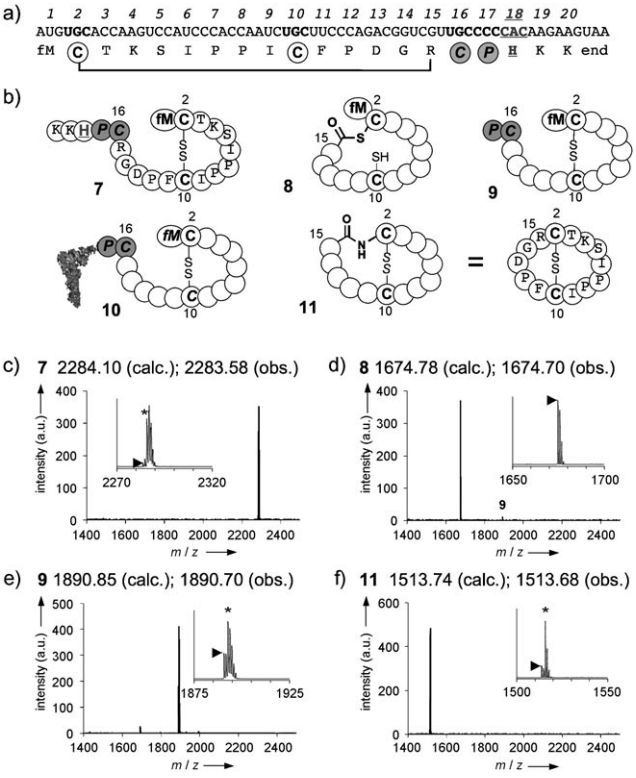

Figure 2. A proof-of-concept study expressing sunflower trypsin inhibitor 1 (SFTI-1) by the induced peptidyl-tRNA drop-off. a) The sequence of mRNA used for the synthesis of SFTI-1. The two internal Cys residues shown in blank circles form the disulfide bond in SFTI-1, whereas Cys and Pro residues in gray circles form the dkp thioester. b) Structure of the key peptide intermediate or the product expressed in this study. c–f) MALDI/TOF analysis of the peptides. For **7**, **9**, and **11** the molecular masses were calculated based on the structure containing a disulfide bond, and the arrow head indicates a set of peaks corresponding to the respective peptide with the *m*/*z* value shown in each spectrum. The peaks representing peptides with the reduced form of the disulfide bond, that is, the two free thiols of the Cys2 and Cys10 residues are indicated by ⋆.

was induced at the designated His codon, and that the released product **10** rearranged into **8** via the dkp thioester intermediate.

In a final reaction, two enzymes, peptide deformylase (PDF) and methionine aminopeptidase (MAP), were included in the translation system lacking a His to promote not only the rearrangement into **8** but also simultaneous removal of fMet1 to expose the N-terminal amino group on Cys2. This system should promote the rearrangement to form the bond resulting in the backbone cyclic peptide. Pleasingly, a new single peak was detected in the mass analysis, and the molecular mass of the purified peptide was in good agreement with the expected mass of the backbone cyclic SFTI-1 **11** (Figures 2b and f). Overall, peptidyl-tRNA drop-off and the subsequent series of intramolecular rearrangements took place in one pot in 1 hour to give approximately 3.3 μg mL^{-1} (2.2 nmol mL^{-1}) of the cyclic SFTI-1 **11** in the reaction solution (see Figure S1 and S2 in the Supporting Information for the quantification and the activity assay, respectively). Notably, SFTI-1 can exist as a bicyclic peptide (**11**(ox) versus

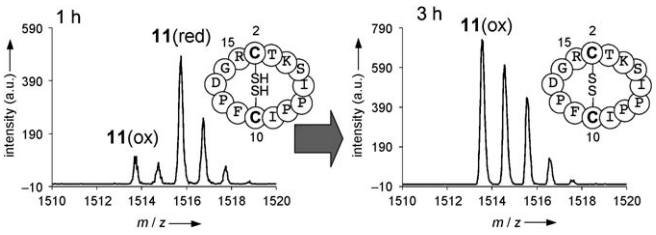

Figure 3. Formation of the disulfide bond in cyclic SFTI-1 by prolonging the translation reaction time. **11**(ox) and **11**(red) indicate oxidized and reduced cyclic SFTI-1, respectively.

11(red); see Figure 3) containing a single disulfide bond. Even though the translation reaction was carried out under reducing conditions using dithiothreitol to promote the series of intramolecular rearrangements from **10** to **11** (Figure 2b), prolonging the reaction time from 1 hour (generic conditions for expression of peptides) to 3 hours was sufficient for the air oxidation of the two Cys side chains to occur to give the disulfide bond (Figure 3). Thus, the simple induced peptidyl-tRNA drop-off methodology enabled us to express **11**(ox) in a one-pot reaction.

We next investigated whether changing the codon assigned to Pro17 or the vacant codon assigned to amino acid 18 would influence the efficiency of the peptidyl-tRNA drop-off event. We found that all four codons, CCN (N = A, U, G, C), coding for Pro gave qualitatively the same result albeit with a higher yield when using CCC rather than the other three codons (see Figure S3 in the Supporting Information). Substitution of the His codon with the Val or Trp codon as a vacant codon also yielded **11**(ox) (see Figure S4 in the Supporting Information).

We have achieved the synthesis of a cyclic peptide by the combination of peptidyl-tRNA drop-off and the spontaneous dkp thioester rearrangement. Although we have previously achieved the same outcome with a site-specific installation of ^{HO}G after the Cys-Pro sequence[5] the peptidyl-tRNA drop-off methodology is simpler and does not require substitution of an amino acid with an α-hydroxy acid. The chemical concept presented here is also applicable to other experimental setups, such as the expression of proteins using an in vitro system that lacks release factors. Studies along this line are underway in our laboratory.

Experimental Section

Experimental details are described in the Supporting Information. Generally, a 2.5 μL scale translation reaction was conducted, using the reconstituted protein synthesis system developed and described previously[5] with 40 nM of DNA template and 1 mM dithiothreitol at 37 °C for 1 hour in the presence or absence of His. In the case of the synthesis of cyclic SFTI-1, the reaction was conducted for 3 h at 37 °C with 1 μM PDF and 3 μM MAP. The peptide was purified and desalted using a C18 ZipTip (Millipore) column and eluted with a solution of acetonitrile/water (1:1) containing 0.1% of trifluoroacetic acid that was saturated with α-cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltonics). MALDI-TOF mass analysis was performed using an Autoflex TOF/TOF (Bruker Daltonics) operated in the linear positive mode or reflective positive mode (for monoisotopic analysis) with external calibration (peptide calibration standard II, Bruker Daltonics).

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