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Chemoenzymatic Transfer of Fluorescent Non-natural Amino Acids to the N Terminus of a Protein/Peptide

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Leucyl/phenylalanyl-tRNA–protein transferase (L/F transferase) from *E. coli* is known to catalyze the transfer of hydrophobic amino acids from an aminoacyl-tRNA to the N terminus of proteins that possess lysine or arginine as the N-terminal residue.[1–3] We have reported that substrates of the enzymatic reaction can be expanded to include non-natural amino acids by using a $tRNA^{Phe}$ aminoacylated with various types of non-natural amino acids.[4,5] This should become a new and versatile tool for N-terminal-specific incorporation of specialty amino acids into proteins and peptides. However, it is difficult to transfer large non-natural amino acids to the N terminus by using wild-type L/F transferase.[4] Here, we engineered L/F transferase to allow the incorporation of large fluorescent non-natural amino acids. Another possible problem with this technique was the tedious and costly transcription process of a tRNA that lacks the 3′-terminal CpA unit. To solve this problem, we minimized the tRNA structure as substrate for the transferase and found that even micro-RNAs can mediate aminoacyl transfer.

Recently, the crystal structure of wild-type L/F transferase complexed with an aminoacyl-tRNA analogue (puromycin) was reported (PDB ID: 2DPT).[6] Judging from the crystal structure, we postulated that there are steric conflicts between large amino acids on a bound aminoacyl-tRNA and hydrophobic residues in the binding pocket of the wild-type transferase. To expand the substrate specificity so as to accommodate non-natural amino acids with large side groups, we designed and expressed four different mutant transferases (M144A, F173A, F177A, I185A)[6] in which hydrophobic amino acids in the binding pockets were replaced by alanines to minimize the steric conflicts with amino acid side groups bound to aminoacyl-tRNAs. By using these mutant transferases, successful transfers were observed for fluorescent non-natural amino acids that possessed acridonyl and benzoacridonyl groups (Figure 1A and B).[7] Acrydonylalanine (acdAla; **1**) and benzoacrydonylalanine (badAla; **2**) are known to be highly fluorescent and photodurable when excited with a blue-laser at the wavelength of about 405 nm or with a visible laser at approximately 450–500 nm, respectively.[7] Thus the attachment of these fluorescent amino acids should enable the visualization of peptides and proteins.

tRNAs charged with these fluorescent amino acids at the 3′ ends were prepared according to the chemical aminoacylation method developed by Hecht and co-workers.[8] Key intermediates for chemical aminoacylation (aminoacyl pdCpAs) were synthesized as described before.[7] The resulting aminoacyl-tRNA was added to a reaction mixture that contained a target peptide (H-KRRPPGFSPFR-OH=Lys-bradykinin)[5] and each mutant transferase.[6] Products of the aminoacyl transfer to the peptide were identified by MALDI-TOF mass spectroscopy. As described in the Supporting Information, all mutant transferases successfully transferred the large fluorescent amino acids to Lys-bradykinin. Aminoacyl transfer to a target protein (Lys-SoCBM13)[3–5] was also successful. Fluorescence images of SDS-PAGE experiments are shown in Figure 1. The images indicate that the fluorescent amino acids were transferred to Lys-SoCBM13 when the reaction was performed with some of the mutant transferases. The product of the aminoacyl transfer to the Lys-SoCBM13 protein was also identified by MALDI-TOF mass spectroscopy. The average mass of the substrate Lys-SoCBM13 (*m/z* found 17000) was found to be shifted by 313 (*m/z* found 17313). This shift corresponds to the mass of a single badAla unit (314). Interestingly, amino acid preferences were somewhat different for different mutant enzymes: the F173A mutant preferred badAla rather than acdAla (lane 3), whereas acdAla was favored by I185A in preference to badAla (lane 5). After the enzymatic reaction, N-terminal sequences of resultant proteins were analyzed by Edman degradation by using an amino-acid sequencer.[5] The efficiency of aminoacyl transfer of acdAla with I185A was 22%, and that of badAla with F173A was 41%, under optimized conditions.[4,5] Changing the molar ratio of the reagents or increasing the reaction time did not enhance the efficiency. Favorable mutation positions seem to depend on the side-chain structures of amino-acid substrates. For example, *p*-aminophenylalanine was more efficiently introduced to Lys-bradykinin by the I185A mutant than either by the F173A mutant or wild-type transferase. In the case of 6-methoxy-2-naphthylalanine, successful introduction was observed with the F173A mutant, but not with the I185A mutant or wild-type transferase.

Next, we made an attempt to minimize the RNA structures as carriers of the amino acid. A previous report has suggested that the tRNA acceptor stem region is important for substrate recognition.[9] We isolated the acceptor stem region of *E. coli* $tRNA^{Phe}$ and aminoacylated the resulting microhelix with 1-naphthylalanine (napAla). The RNA was chemically synthesized (Japan Bioservice, Saitama, Japan) and charged with napAla at

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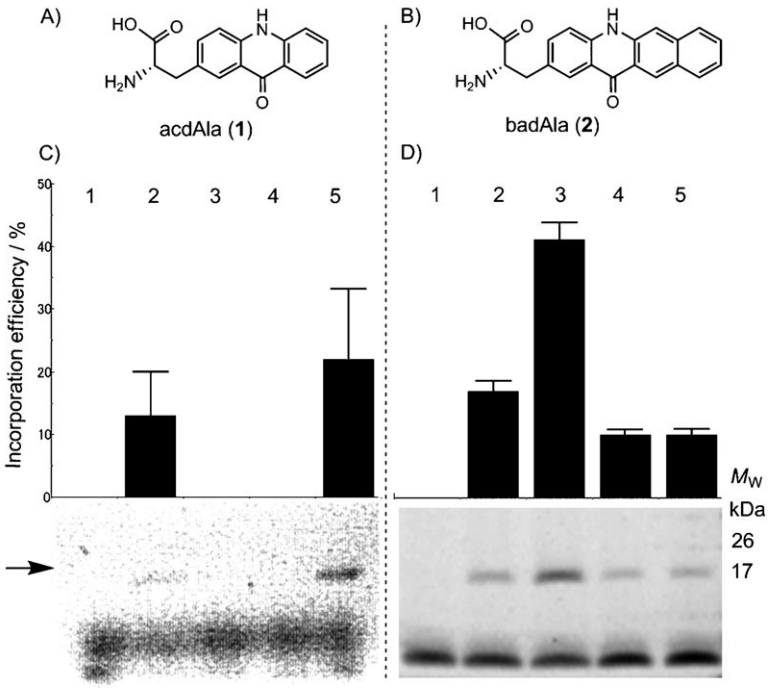

Figure 1. Chemical structures of the fluorescent non-natural amino acids A) acdAla, and B) badAla. Fluorescence imaging of the products of aminoacyl transfer to Lys-SoCMB13 protein for C) acdAla, visualized under excitation at 365 nm; and D) badAla, visualized under excitation at 488 nm. The same amounts of protein were subjected to SDS-PAGE (15% gels). Lanes 1: wild-type L/F transferase, lanes 2–5: M144A, F173A, F177A, I185A mutant transferases, respectively. The optimized reaction conditions were: aminoacyl-tRNA (400 pmol), target protein (Lys-SoCBM13; 180 pmol), and L/F transferase (13 pmol) in a 2.5 μL reaction mixture incubated at 37 °C for 0.5 h.[4,5] The horizontal arrow indicates the position of Lys-SoCBM13, and the bands at the void volume were tentatively assigned as free (hydrolyzed) amino acids. Incorporation efficiency was evaluated by Edman degradation.

its 3′ end by chemical aminoacylation.[8] Transfer of a single napAla unit to Lys-bradykinin was observed in the presence of aminoacylated microhelix **3** and transferase (Figure 2). Judging from the results of the Edman degradation, the efficiency of the aminoacyl transfer of napAla was 36%. Aminoacyl transfer was not detected either in the absence of the microhelix or transferase.

We deleted further nucleotide units from the acceptor stem to find the minimum sequence that could act as amino-acid carrier. All of the microhelices **4**–**6** that carried a napAla unit at the 3′ end were successfully recognized by the wild-type transferase (Figure 3A). Results from TOF-MS analysis indicated that the target peptide (Lys-bradykinin) disappeared and the desired product (napAla-Lys-bradykinin) appeared exclusively when we used microhelix **5**; napAla was almost quantitatively transferred to the peptide under the optimized reaction conditions (see footnote of Figure 1). The product of the aminoacyl transfer from microhelix **5** to Lys-SoCBM13 protein was also identified by MALDI-TOF-MS; the average mass of the substrate Lys-SoCBM13 shifted by 194. This shift corresponds to the mass of a single napAla (197) unit. The TOF-MS data clearly show that a single napAla was covalently attached to the substrate protein. Shorter microhelices **3**, **4**, and **6** gave smaller yields. Most surprisingly, an aminoacylated dinucleotide (napAla, **7**) also worked as substrate for the transferase (Figure 3B), although Leibowitz and Soffer[1] have reported unsuccessful transfer for **8** when it was aminoacylated with phenylalanine. Of course, aminoacyl transfer did not occur when the transferase was absent.

Thus, we found that amino acid specificity could be expanded to include badAla (**2**) by the use of a mutant transferase (F173A). Further, the amino acid carrier could be minimized to microhelix **5** for efficient attachment, and even to **7** for partial attachment. Combining these results, we attempted the synthesis of a fluorescently labeled protein, badAla-Lys-SoCBM13, using microhelix **5** aminoacylated with badAla. The efficiency of aminoacyl transfer to Lys-SoCBM13 was 21% in the presence of the

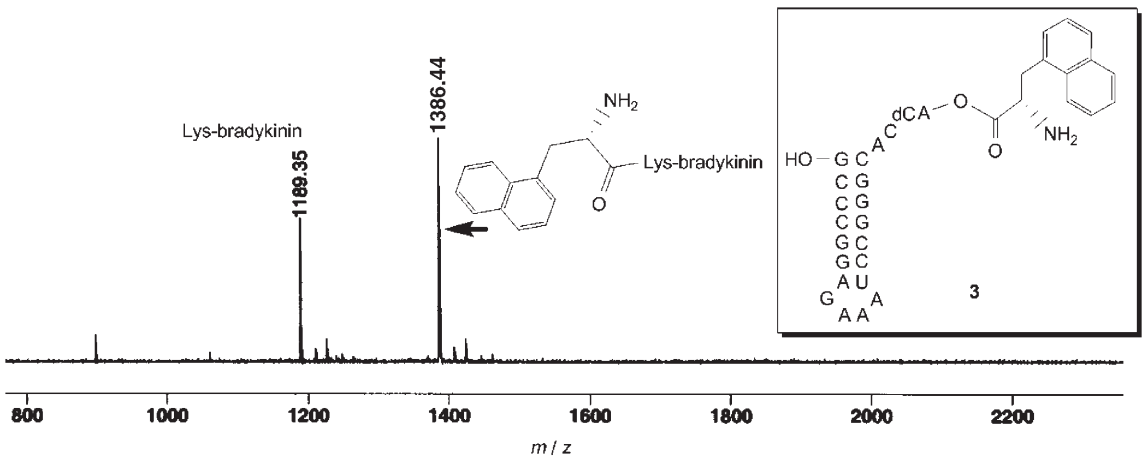

Figure 2. MALDI-TOF mass spectrum of Lys-bradykinin after transfer of the napAla unit by using aminoacylated microhelix RNA **3** with wild-type L/F transferase. The enzymatic reaction was performed at 37 °C.

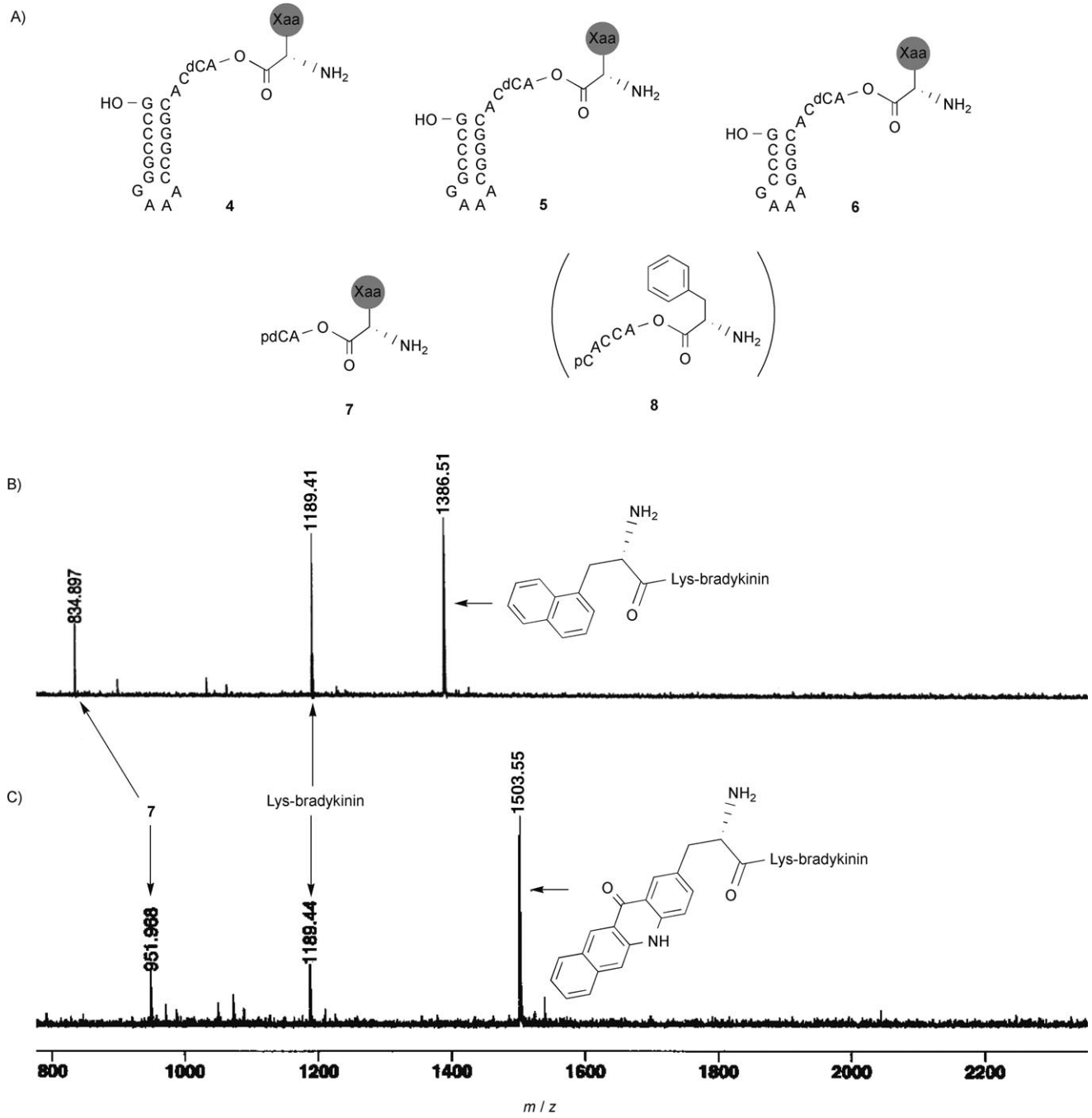

Figure 3. A) Structures of the aminoacylated oligonucleotide substrates that successfully worked as substrates of L/F transferase; **8** has been reported not to work.[1] MALDI-TOF mass spectra of Lys-bradykinin after the transfer of B) napAla, and C) badAla units by using the corresponding aminoacyl-dinucleotide **7** with wild-type and F173A-mutant L/F transferase, respectively. The enzymatic reaction was performed at 37 °C.

F173A mutant. It seems that the lack of the cloverleaf structure of tRNA had an unfavorable effect on the transfer reaction to the target protein. Most probably lack of interaction between the D stem of the aminoacyl-tRNA and transferase prevented efficient complexation between the aminoacyl-microhelix and transferase.[6] Despite this, partial aminoacyl transfers from a dinucleotide aminoacylated with badAla (**7**) to Lys-bradykinin were definitely detected in the presence of the same mutant transferase (Figure 3C). Again, transfer of the fluorescent amino acid from **7** to the peptide did not occur in the absence of transferase. We also prepared an AMP–napAla conjugate, which worked as a good substrate for wild-type transferase and was linked to Lys-bradykinin. Very recently, AMP–phenylalanine was found to be a substrate for wild-type transferase.[10] The hydrophobic amino acid itself might be sufficient for substrate recognition not only for natural phenylalanine[10] but also non-natural aromatic amino acids.

In conclusion, we have succeeded in the transfer of large fluorescent non-natural amino acids that can be excited by either visible or blue-lasers, to the N terminus of a Lys-terminated

protein or peptide by using engineered L/F transferase mutants. Also, we have found that the tRNA structures could be simplified to microhelices or even to dinucleotide (pdCpA) or mononucleotide (AMP; pA) as carriers for the non-natural amino acids. With this method we can avoid tedious and costly transcription of tRNA using chemically synthesized oligoRNAs. Aminoacylation of a microhelix might be chemically or enzymatically achieved by using an aminoacyl phosphate oligonucleotide hybridized to the 3′ end of the microhelix[11] or aminoacyl-tRNA synthetase,[12] respectively.

Experimental Section[13]

In vitro aminoacyl transfer from aminoacyl-nucleotide to the N terminus of Lys-bradykinin peptide or Lys-SoCBM13 protein: Standard in vitro aminoacyl transfer to the Lys-peptide or Lys-protein was carried out in a 2.5 μL reaction mixture containing Tris-HCl buffer (50 mM, pH 8.0), KCl (200 mM), peptide or protein (180 pmol each), aminoacylated oligonucleotide (400 pmol), and L/F transferase (13 pmol). The reaction mixture was incubated for 0.5 h at 37 °C unless otherwise noted.

To obtain the mass spectra, the reaction solution was desalted and concentrated by using ZipTipC18 silica resin (Millipore) after the enzymatic reaction, and eluted directly onto the MALDI target.

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[13] Detailed experimental protocols and results (Figures S1–S10, Table S1) are available in the Supporting Information.

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