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Kinetic analysis of the leucyl/phenylalanyl-tRNA-protein transferase with acceptor peptides possessing different N-terminal penultimate residues☆

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ABSTRACT

The introduction of non-natural amino acids at the N-terminus of peptides/proteins using leucyl/phenylalanyl-tRNA-protein transferase (L/F-transferase) is a useful technique for protein engineering. To accelerate the chemoenzymatic reaction, here we systematically optimized the N-terminal penultimate residue of the acceptor peptide. Positively charged, small, or hydrophilic amino acids at this position show positive effects for the reaction. Kinetic analysis of peptides possessing different penultimate residues suggests that the side chain of the residue affects peptide-binding affinity towards the L/F-transferase. These findings also provide biological insight into the effect of the penultimate amino acid on substrate specificity of natural proteins to be degraded via the N-end rule pathway.

1. Introduction

Introduction of non-natural amino acids into peptides/proteins is a powerful and versatile technique for protein engineering [1,2]. We have recently discovered that various non-natural amino acids can be enzymatically introduced only at the basic N-terminus of peptides/proteins by using *Escherichia coli* leucyl/phenylalanyl-tRNA-protein transferase (L/F-transferase) [3–5]. We extended this L/F-transferase-mediated functionalization of peptides/proteins in combination with aminoacyl-tRNA synthetase (ARS) mutant [6,7], namely the NEXT-A (N-terminal Extension of protein by Transferase and Aminoacyl-tRNA synthetase) reaction (Fig. 1). For the efficient introduction via the NEXT-A reaction, we have engineered α-subunit of the *E. coli* phenylalanyl-tRNA synthetase (FRS) structure, resulting in a doubly-substituted mutant (A294G and T251A) of the α-subunit that accepts various kinds of non-natural amino acids with good conversion [6,8].

We empirically noticed that the introduction rate might depend on the amino acid sequence near the N-terminus of the acceptor peptides/proteins. This motivated us to perform a systematic study of the L/F-transferase mediated transfer reaction from aminoacyl-tRNA to acceptor peptides possessing different penultimate residues.

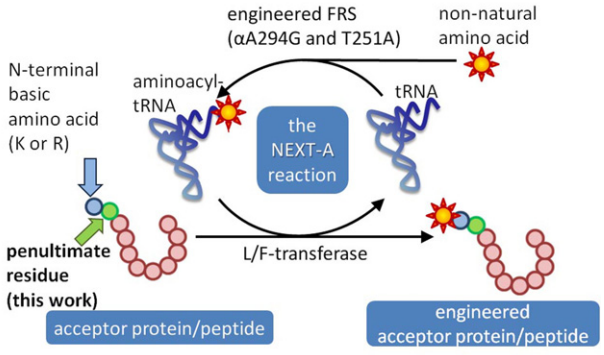

Fig. 1. Non-natural amino acid introduction toward acceptor protein/peptide by the NEXT-A reaction. L/F-transferase catalyzes the transfer of a non-natural amino acid from aminoacyl-tRNA^{Phe} to the N-terminus of an acceptor protein/peptide. A previous report has demonstrated for substrate preference that an N-terminal basic amino acid (K or R) is required for the efficient phenylalanine transfer mediated by L/F-transferase (see Ref. [14]).

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2. Materials and methods

2.1. General

For the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis, α-cyano-4-hydroxycinnamic acid was used as matrix. Before the analysis, peptides were desalted, concentrated, and purified using ZipTipC18 silica resin (Merck Millipore, Bedford, MA) according to the instruction manual. MALDI-TOF mass spectra were taken on a Voyager DE Pro instrument. L/F-transferase (wild type) and doubly-mutated *E. coli* FRS (αA294G and T251A) were overexpressed and purified according to the reported procedure [6]. *E. coli* $tRNA^{Phe}$ was obtained from Sigma; use of pure *E. coli* $tRNA^{Phe}$ (Sigma#R3143), instead of a crude tRNA mixture (Sigma #R1753 or R4251), is important to obtain consistent results. Model peptide RGPCRAFI (UTIF; Urinary Trypsin Inhibitor Fragment) was purchased from Bachem (#H-2692). Fmoc-protected fluorescent amino acids, acrydonylalanine (acdAla) was synthesized according to the reported procedures [9,10], and is currently available from Watanabe Chemical Industries (Hiroshima, Japan). A model non-natural amino acid (*O*-(2-fluoroethyl)-L-tyrosine) [11], whose radioactive isotope is potentially useful for positron emission tomography (PET) imaging, was synthesized as shown in supplementary material. Small scale quantitative analysis of peptides were carried out by using a reversed-phase semi-micro HPLC system (JASCO PU-2085 with C18 column) connected to a fluorescence detector followed by a photodiode array (PDA). The peptides were separated using a 0–100% gradient of acetonitrile containing 0.1% aq. trifluoroacetic acid (TFA) during 10 min at a flow rate of 200 μL/min.

2.2. Solid-phase synthesis of acceptor peptides

Syntheses of 19 different acceptor peptides (KXC-acdAla possessing different penultimate residue, X, excluding cysteine) were carried out on the solid phase by using a multiple reaction device (PetiSyzer from HiPep Laboratories). Fmoc amino acids including fluorescent ones (Watanabe Chemicals) were used as the monomers. Side reactions seldom took place during peptide synthesis. The solid phase synthesis was performed on an Fmoc-NH-SAL-PEG resin (super acid-labile polyethyleneglycol resin from Watanabe Chemicals). Chain elongation was achieved by using 1-hydroxy-7-azabenzotriazole (HOAt) and *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(7-azabenzo-triazol-1-yl)uronium hexafluorophosphate (HATU) as the coupling agent in the presence of diisopropylethylamine (DIPEA)/*N*,*N*-dimethylformamide (DMF)/N-methylpyrrolidone (NMP). The coupling time was set to 40 °C for 12 and 3 h when fluorescent non-natural and other amino acids were elongated, respectively. The coupling efficiency at each elongation step was almost quantitative, as estimated from UV absorption of the fulvene-adduct formed upon removal of the N_δ-Fmoc protecting group with 20% piperidine/DMF for 7 min at room temperature ($\varepsilon_{290} = 4950\ M^{-1}\ cm^{-1}$). The resin-bound peptide was cleaved off from the resin with TFA/water/1,2-ethandithiol (EDT)/triisopropylsilane (TIS) (94/2.5/2.5/1 v/v/v/v) for 1 h at room temperature. Each obtained peptide was purified and analyzed by reversed-phase HPLC and only a single peak was found. Each was also identified by MALDI-TOF-MS.

2.3. Initial reaction rate/kinetic analysis of L/F-transferase-mediated amino acid transfer from aminoacyl-tRNA to acceptor peptides

Amino-acid transfer was carried out in a 50 μL reaction mixture containing 50 mM HEPES-KOH (pH 7.6), 20 mM KCl, 1 mM spermidine, 2.5 mM ATP, 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), each amino acid (50 nmol; 1.0 mM), target acceptor peptide (370–46 pmol; 7.3–0.91 μM), $tRNA^{Phe}$ (0.41 nmol; 8.2 μM), mutant FRS (33 pmol; 0.66 μM), and wild-type L/F-transferase (0.19 pmol; 3.8 nM and 1.9 pmol; 38 nM for the L-phenylalanine and *O*-(2-fluoroethyl)-L-tyrosine transfer, respectively). All reagents except L/F-transferase were pre-incubated at 37 °C for 10 min to promote all tRNA molecules to be aminoacylated. After the addition of L/F-transferase, the mixture was incubated in the dark at 37 °C for 0–60 min. During the transfer reaction, molar concentration of the aminoacyl-tRNA was kept constant at every time period (see supplementary material). At different time points, every small portion (5 μL) of the reaction mixture was separated and the enzymatic reaction was quenched by addition of trifluoroacetic acid to a final concentration of 0.1%. In case of MALDI-TOF-MS analysis (described below), the mixture was purified using ZipTipC18.

Initial reaction rate/kinetic measurements of the L/F-transferase-mediated amino acid transfer were performed by using a densitometric HPLC analysis, or a quantitative MALDI-TOF-MS analysis elegantly reported by Ebhardt et al. [12]. In the latter case, chemically synthesized acceptor peptides (KX**C**-acdAla, or RGP**C**RAFI) were alkylated at the internal cysteine (underlined) with either bromoethane or deuterated [$^2H_5$]bromoethane to generate "light" and "heavy" pairs of the peptides according to the reported procedure [13]. As the light and heavy peptide pair is identical in their physical and chemical properties, they co-crystallize with matrix and ionize identically during TOF-MS analysis. Amino-acid transfer of the light peptide mediated by L/F-transferase gives a light product peptide. Prior to TOF-MS analysis, a known amount of corresponding heavy peptide was added to the sample as an internal standard for quantification. The amount of the light peptide to which amino acid is 'not' transferred was quantified by measuring the relative ratio of the ion intensity of the light peptide to that of the heavy one [12]. The measurement was repeated three times independently to confirm reproducibility.

When the initial reaction rate was estimated in Table 1, the molar concentration of the target peptide was kept constant (7.3 μM). For the kinetic analysis in Table 2, the molar concentration was varied and Michaelis–Menten parameters (k_{cat} and K_m) were determined by Lineweaver–Burk plots. For details, see supplementary material.

Table 1
Initial reaction rate of *O*-(2-fluoroethyl)-L-tyrosine transfer from aminoacyl-tRNA to the 19 different acceptor peptides mediated by L/F-transferase. The sequences of the acceptor peptides are KXC*-acdAla, where the penultimate residue (X) is shown in the table. Cysteine (C*) was alkylated with bromoethane for quantitative MALDI-TOF-MS analysis.

Penultimate residue (X)	Initial reaction rate (μM/min)
S	0.16
R	0.12
A	0.089
T	0.083
K, N	0.079
I	0.073
G	0.070
L	0.068
Y	0.064
H	0.058
Q	0.057
E, V	0.051
F	0.042
D	0.041
W	0.037
M	0.035
P[a]	0.034

[a] The difference of the N-terminal basic amino acid (K or R) would give little effect for the reaction; the reaction rate of RPC-acdAla was as same as that of KPC-acdAla.

Table 2
Kinetic parameters for phenylalanine transfer from phenylalanyl-tRNA to each acceptor peptide catalyzed by L/F-transferase.

Sequence of acceptor peptide	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
KAC-acdAla	15	1.2	13
KQC-acdAla	30	5.1	5.8
KPC-acdAla	48	21	2.4
RGPC*RAFI	60	1.1	55

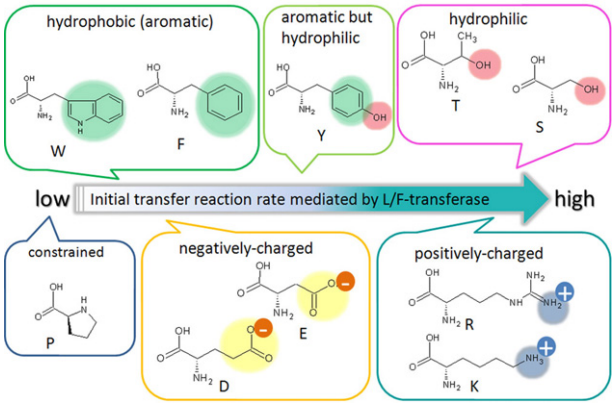

Fig. 2. Effect of penultimate residue for non-natural amino acid transfer mediated by L/F-transferase.

3. Results

3.1. Effect of penultimate residue of acceptor peptide for amino-acid transfer mediated by L/F-transferase

We synthesized 19 acceptor peptides possessing different penultimate amino acids (Table 1). Each peptide contained an internal cysteine for stable isotope labeling, which allowed quantitative MALDI-TOF-MS analysis [12]. It also contained a small fluorescent amino acid (acrydonylalanine; acdAla), which allowed quantitative HPLC analysis in a semi-micro scale. The initial reaction rate of a non-natural amino acid transfer from aminoacyl-tRNA to each acceptor peptide under the same conditions mediated by L/F-transferase was estimated and summarized in Table 1. It was once suggested that the catalytic pocket of L/F-transferase recognizes the penultimate residue of acceptor peptides in a sequence independent manner [14,15], if it has an N-terminal basic amino acid (i.e., positively-charged R or K). However, the reaction rate clearly depended on the penultimate residue. Positively-charged, small, or hydrophilic amino acids showed a higher reaction rate. In contrast, negatively charged, bulky, or hydrophilic/aromatic amino acids showed lower rates. Aromatic but positively-charged amino acids (H), or with a hydrophilic hydroxyl group (Y) showed moderate rates. Proline had the worst reaction rate of all. The effect of the penultimate residue against the initial reaction rate was schematically summarized in Fig. 2.

3.2. Steady-state kinetic analysis of acceptor peptide

To determine if the reduced reaction rates arose from poor substrate binding or slower turnover by L/F-transferase, we determined steady-state kinetic parameters for several peptides including proline-containing ones (Table 2). The concentration of phenylalanyl-tRNA (8.2 μM) is about five times higher than its K_m value for L/F-transferase (1.6 μM [16]), allowing the reaction to be approximated as pseudo-first order. Thus, we characterized the reaction in terms of the Michaelis–Menten kinetic scheme; the parameters, k_{cat} and K_m, were obtained by Lineweaver–Burk plot. The penultimate residue substitution from alanine to proline caused a more than 10-fold weakening of the affinity of the peptide for L/F-transferase, by comparison of K_m values. It seems that conformational rigidity of proline at the penultimate residue prevents appropriate binding between the N-terminal site of the peptide and the catalytic core of L/F-transferase. When a proline was located at the 3rd residue from the N-terminus (RGPC*RAFI; C was alkylated), the conformational rigidity rarely seemed to affect the affinity. In contrast to the K_m trend, turnover number per minute (k_{cat}) seldom changed among all of the peptides we tested; once the peptide was bound appropriately to the catalytic core, the transfer reaction proceeded independently of the penultimate residue. About 10-times smaller k_{cat} (2.6 min^{-1}) value of the phenylalanine transfer reported in a previous paper [17] may be caused by either the substrate not being a peptide but a protein, or by fragile aminoacyl-tRNA being rapidly hydrolyzed [18] at pH 8 in the absence of FRS during the kinetic analysis. In our experiment, FRS was present and molar concentration of the aminoacyl-tRNA was kept constant at every time period during the transfer reaction.

4. Discussion

These trends about penultimate residues raised a fundamental question about why L/F-transferase possesses this peptide substrate specificity. The difference of the reaction rate may reflect the physiological function of L/F-transferase. Naturally, L/F-transferase originated from *E. coli* is known to catalyze the transfer of hydrophobic amino acids (L or F) from aminoacyl-tRNA to the N-terminal basic amino acid (K or R) of an acceptor protein [19,20]. In the cytoplasm of *E. coli*, the acceptor protein bearing leucine or phenylalanine at the N-terminus should be degraded within several minutes; the N-terminal site of the acceptor protein is recognized by an adapter protein (ClpS) [21] of a degradation pathway, also known as the N-end rule pathway [22]. However, when the N-terminal penultimate residue of the acceptor protein is an acidic amino acid (D or E), it causes a negative binding effect for ClpS [15,23,24], and such proteins avoid prompt degradation [25]. This weakening of the binding comes from electric repulsion of acidic sites between the N-terminal site of acceptor and binding site of ClpS [24]. This acceptor binding disability of ClpS seems to be complementary to that of L/F-transferase. Most probably, a negatively-charged side group of an acidic amino acid at the penultimate residue caused repulsion against the negatively charged catalytic pocket of L/F-transferase [17].

Recently, a natural L/F-transferase substrate with a physiological function, PATase, was discovered from *E. coli* for the first time [21,26]. Surprisingly, the N-terminus of the acceptor protein to be degraded was not a positively-charged amino acid (K or R) but a neutral one (M). In this case, the penultimate residue is a neutral and hydrophilic amino acid (N). Asparagine might bind favorably near the catalytic pocket of L/F-transferase, to overcome unfavorable binding of the N-terminal methionine to the catalytic core [26]. This is also in good accordance with our result of penultimate sequence dependency.

In conclusion, although the transfer reaction occurred to all acceptor peptides with a basic amino acid at the N-terminus, the transfer rate clearly depended on the penultimate residue of the acceptor. To introduce non-natural amino acids most efficiently by the NEXT-A reaction, one should use acceptor peptides/proteins with serine or arginine at the N-terminal penultimate residue. The reaction rate of phenylalanine transfer to the acceptor peptide (e.g. 0.076 μM/min for 7.3 μM KQC-acdAla) was slightly higher than that of the non-natural amino acid transfer. Nevertheless, the reaction rate of non-natural amino acid transfer (Table 1) seems practical for the prompt introduction of the probe toward various kinds of peptides. In addition to the protein engineering point of view, our results implied natural bacterial substrate proteins to be degraded may possess small, hydrophilic, or positively-charged amino acids at the N-terminal penultimate

residue. As physiologically important substrates of L/F-transferase for protein degradation, also known as "the N-end rule substrate" have been seldom found until today [15], further systematic study for finding the N-end rule substrate is needed to prove this implication.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2013.06.001.

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