Unexpectedly fast transfer of positron-emittable artificial substrate into N-terminus of peptide/protein mediated by wild-type L/F-tRNA-protein transferase

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Unexpectedly fast transfer of positron-emittable artificial substrate into N-terminus of peptide/protein mediated by wild-type L/F-tRNA-protein transferase

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Abstract

This article demonstrates the fastest enzymatic introduction of a positron emission tomography (PET) probe into acceptor peptides/proteins. It is site-specifically introduced at the basic N-terminus of the acceptors by using L/F-transferase in combination with aminoacyl-tRNA synthetase, namely the NEXT-A/PET reaction. Estimated from kinetic analysis, the transfer efficiency of *O*-(2-fluoromethyl)-*L*-tyrosine as an artificial amino acid PET probe mediated by the wild-type transferase is almost as good as that of the natural substrate, phenylalanine.

Keywords: L/F-transferase; non-natural amino acid (unnatural amino acid); positron emission tomography (PET) probe introduction; *O*-(2-fluoromethyl)-*L*-tyrosine (FMT); *O*-(2-fluoroethyl)-*L*-tyrosine (FET); enzyme kinetics.

Introduction

Introduction of imaging agents, such as positron emission tomography (PET) probes, into cell-targeting peptides/proteins is a versatile technology especially for specific detection of cancer at early stages (Gray and Brown 2014). Usually, PET probes are introduced to the peptides/proteins via the conventional chemical labeling methods (Okarvi 2001). The introduction chemistry of the PET probes via chemical labeling is quite limited, because the reaction and purification should be completed within their short radioactive lifetime (e.g. 110 min for ^{18}F). Moreover, because of their poor regioselectivity, heterogeneous species are usually obtained. They are not only unfavorable for medical uses, but often lose the biological properties such as cell-targeting activity (Dolle et al. 2008). Instead of chemical modification, use of enzymes for the PET probe introduction to any kind of peptides/proteins would be an ideal strategy because enzymatic reactions are usually site-specific and give a single product. However, the substrate specificity of enzymes is usually narrow and such enzymes cannot transform artificial substrates such as PET probes with practical rates.

Meanwhile, we have discovered that various non-natural amino acids as artificial substrates can be enzymatically introduced only at the basic N-terminus of any kind of acceptor peptides/proteins by using *E. coli* leucyl/phenylalanyl-tRNA-protein transferase (L/F-transferase) without any engineering of its catalytic pocket (Taki et al. 2006; Taki and Sisido 2007). We have extended this L/F-transferase-mediated functionalization of peptides/proteins in combination with aminoacyl-tRNA synthetase (ARS) mutant (Ebisu et al. 2009; Hamamoto et al. 2011), namely the NEXT-A (N-terminal Extension of protein by Transferase and Aminoacyl-tRNA synthetase) reaction (Fig. S1). The

difference of the N-terminal basic amino acid (Arg or Lys) of the acceptor would give little effect for the reaction rate (Kawaguchi et al. 2013). We empirically noticed that the transfer rate of non-natural phenylalanine derivatives mediated by the wild-type L/F-transferase is extraordinary fast (Kawaguchi et al. 2013), and the NEXT-A reaction would be potentially useful for introduction of PET probes possessing short lifetimes. Among the derivatives, *O*-(2-fluoromethyl)-*L*-tyrosine (FMT) (Iwata et al. 2003) and *O*-(2-fluoroethyl)-*L*-tyrosine (FET) (Wester et al. 1999) are well-known PET probes, and the latter is currently used for phase II clinical trials of tumor imaging (Piroth et al. 2012). To ensure these artificial probes can be enzymatically introduced to the target peptides/proteins with practical rates, here we estimated their steady-state kinetic parameters and compared with that of the natural substrate, phenylalanine (Kawaguchi et al. 2013).

Results and discussion

1. Steady-state kinetic analysis of PET probe transfer mediated by L/F-transferase

We performed the kinetic analysis of FET and its downsized analog FMT (Fig. S2) transfer to the N-terminal Arg of a model peptide (UTIF) mediated by L/F-transferase (Fig. S3, 4). The peptide contained an internal cysteine for stable isotope labeling, which allowed quantitative MALDI-TOF-MS analysis (Ebhardt et al. 2009; Kawaguchi et al. 2013). The transfer reaction was approximated as pseudo-first order and characterized in terms of the Michaelis-Menten kinetic scheme; the parameters, k_{cat} and K_m, were obtained by Lineweaver-Burk plot (Fig. 1) (Kawaguchi et al. 2013). The extension of the para-position of phenylalanine with oxy-2-fluoromethylene moiety hardly changed the affinity of the aminoacyl-tRNA for L/F-transferase, by comparison of K_m values. In contrast, the extension with oxy-2-fluoroethylene slightly disturbed appropriate binding of FET to the catalytic core of L/F-transferase; insertion of a single methylene spacer to FMT caused about 10-fold weakening of the affinity. In contrast to the K_m trend, turnover number per minute (k_{cat}) hardly changed among the three aromatic amino acids; once the aminoacyl-tRNA was bound appropriately to the catalytic core, the transfer reaction proceeded independently of the substituent. Consequently, the specificity constant (k_{cat}/K_m) of the artificial FMT transfer reaction was almost as good as that of the natural phenylalanine transfer. To the best of our knowledge, this is the fastest enzymatic introduction of a PET probe, even though the wild-type transferase is *not* engineered at all. Indeed, the constant of the *artificial* FMT transfer is 3.2-fold higher than the average k_{cat}/K_m value of several thousands of prokaryotic or eukaryotic *natural* substrate/enzyme pairs (Bar-Even et al. 2011).

2. Generality of the NEXT-A/PET reaction

To demonstrate the generality of the PET probe introduction to other peptides/protein, we attempted the FMT introduction toward Lys-bradykinin (1.2 kDa synthetic inflammatory mediator peptide), Lys-Ala-7-amino-4-methylcoumarin (0.37 kDa fluorophore-fused synthetic model peptide), and Lys-SoCBM13 (17 kDa recombinant model protein) under the standard conditions. In the former case, bradykinin receptor is overexpressed in many human cancers and PET imaging of the receptor would be potentially useful for detection of the cancers (Lin et al. 2015).

For the Lys-bradykinin experiment, we also examined temperature dependence (Fig. S5). Quantitative FMT transfer was achieved within 5 minutes under the optimized temperature (37 ˚C) even when the concentration of FMT was on the order of micro-molar. Even at 4 ˚C, more than 90% peptide was successfully labeled by the PET probe with the same reaction time/concentration. It seems practical to introduce the PET probe to labile peptides/proteins at lower temperatures within the short radioactive lifetime.

For the Lys-Ala-7-amino-4-methylcoumarin, about 90% peptide was labeled by FMT within 5 minutes at 37 ˚C (Fig. S6). This slight decrease of FMT introduction efficiency compared to the same condition of Lys-bradykinin may due to a negative effect of the penultimate residue of the acceptor peptide (i.e. Ala) (Kawaguchi et al. 2013) and/or non-natural 7-amino-4-methylcoumarin moiety.

For the Lys-SoCBM13 experiment, N-terminal sequences as well as the introduction efficiencies were also evaluated by quantitative analysis of Edman

degradation with the aid of an amino acid sequencer (Taki et al. 2006; Taki and Sisido 2007). The N-terminal sequence of Lys-SoCBM13 after the coupling was found to be FMT-Lys- and the quantitative introduction was achieved within 20 minutes (Fig. 2A). The FMT-labeled Lys-SoCBM13 was also identified by MALDI-TOF mass spectroscopy (Taki et al. 2006; Taki and Sisido 2007) (Fig. 2B). The average mass of the substrate Lys-SoCBM13 [(*m* / *z*) found 17026.4, calculated for $[M+H]^+$: 17025.6] was shifted by 196, which corresponds to the mass of an FMT unit (195). These data suggest that a single FMT was exclusively linked to the N-terminal lysine and that the other five internal lysine residues were not modified by FMT.

In conclusion, we have shown a general enzymatic PET probe introduction system to N-terminal Arg or Lys of peptides/proteins. The N-terminal specific introduction proceeds under mild conditions within a very short period, so that the resulting peptide/protein would retain both biological activity and radioactivity. Indeed, our preliminary experiment demonstrated the successful introduction of radioactive [^{18}F]-FET to the model peptide, Lys-Ala-7-amino-4-methylcoumarin (Tokuda et al. 2013). This means that the micro-molar order of k_{cat}/K_m value of FET transfer deserves introduction of a limited amount of synthesized [^{18}F]-FET whose intermediate half-life is 110 minutes (Okarvi 2001). [^{18}F]-FMT with superior k_{cat}/K_m value would meet more severe reaction conditions such as when only a lower concentration of the acceptor peptide/protein is available.

More than ten different N-terminal specific functionalization methods of peptides/proteins, such as native chemical ligation and transglutaminase-mediated

enzymatic modification, are compared with our L/F-transferase-mediated system in a review (Gauthier and Klok 2008). Every method excluding ours requires harsh reaction conditions and/or elongated reaction times, which may yield heterogeneous and/or non-radioactive reaction products. To prove our N-terminal specific functionalization system more practical, molecular imaging of cancer by using a radioactive cancer-binding peptide generated by the NEXT-A/PET reaction is currently underway in the laboratory.

Conflict of interest

The authors have declared no conflict of interest.

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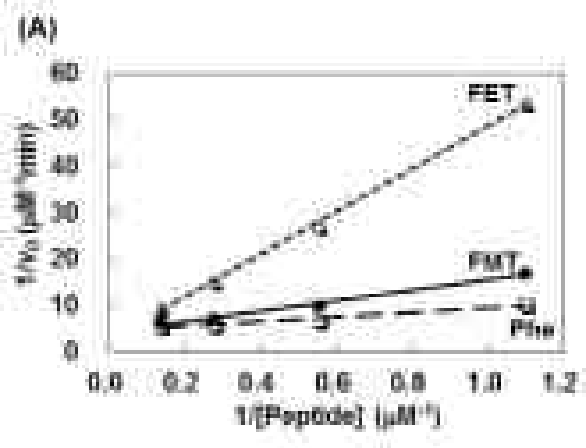
FIGURES



(B) Amino acid	Phe	FMT	FET
K_m (μM)	1.1	2.6	10
k_{cat} (min^{-1})	60	62	1.0×10^2
k_{cat}/K_m ($\mu M^{-1} min^{-1}$)	55	24	5.7

Figure 1. Kinetic parameters for amino acid (X_{aa}) transfer from X_{aa}-tRNA to the model acceptor peptide catalyzed by wild-type L/F-transferase were estimated from Lineweaver-Burk plot. A straight line is formed by plotting the inverse initial reaction rate ($1/V_0$) as a function of the inverse of the acceptor peptide concentration (1/[S]). The $1/V_{max}$ and $-1/K_m$ values were determined from y- and x-intercepts, respectively.

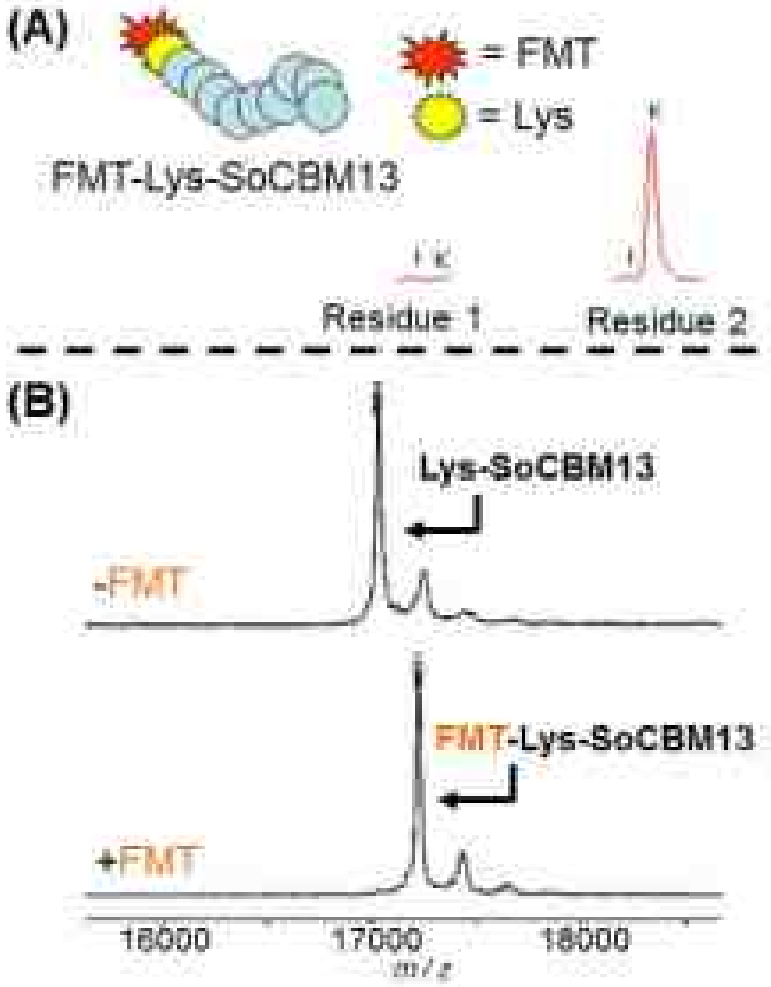


Figure 2. Efficiencies of FMT transfer to Lys-SoCBM13 under the standard reaction condition; the incubation time and temperature for the NEXT-A/PET reaction were 20 minutes and 37 ˚C, respectively. They were evaluated by quantitative analysis of both (A) Edman degradation with the aid of amino acid sequencer and (B) MALDI-TOF-MS peak intensities. In the former case, when FMT is successfully introduced to the N-terminus of Lys-SoCBM13, a Lys unit should be detected as the 2nd residue. Otherwise, it should be detected as the 1st residue. Chromatographic analysis of the phenylthiohydantoin

derivatives of the N-terminal amino acid separated from the remaining portion of the protein identifies the residues on the basis of Edman degradation.