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Quantification of the Post-Translational Addition of Amino Acids to Proteins by MALDI-TOF Mass Spectrometry

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Aminoacyl-tRNA protein transferases catalyze the post-translational addition of amino acids to proteins. The eubacterial leucyl/phenylalanyl-tRNA-protein transferase (L/F transferase) catalyzes the transfer of leucine or phenylalanine from their respective aminoacylated tRNAs to the N-termini of substrate proteins possessing an N-terminal lysine or arginine amino acid. Conventional assays to quantify L/F transferase activity involve measuring radioactive amino acid incorporation into substrate proteins. We have developed a quantitative matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry procedure to measure the enzymatic activity of L/F transferase. The procedure utilizes stable isotope labeled substrate and internal standard peptides. The method is used to determine the kinetic parameters of k_{cat} and K_m for the enzymatic transfer of phenylalanine and three unnatural amino acid derivatives from an aminoacyl-tRNA to a peptide substrate.

The post-translational N-terminal addition of amino acids is critical in several biological processes such as heart development[1] and apoptosis.[2] In addition to their biological functions, the enzymes that catalyze these reactions are of research interest in the development of tools for protein engineering, as they are capable of adding unnatural amino acids with diverse chemical functionalities to the N-termini of substrate proteins.[3−6]

Aminoacyl-tRNA protein transferases are peptidyl transferases which catalyze the transfer of an esterified amino acid from an aminoacyl-tRNA (aa-tRNA) substrate to the N-terminus of a substrate protein. Two types of aminoacyl-tRNA protein transferases have been identified, differing in the identities of their substrate tRNAs and N-termini of target proteins. Prokaryotic aminoacyl-tRNA transferases catalyze the transfer of an esterified amino acid from $tRNA^{Leu}$ or $tRNA^{Phe}$ to polypeptides with an N-terminal lysine or arginine amino acid[7] (see Figure 1). Selective utilization of these two tRNA substrates has resulted in this prokaryotic enzyme being named leucyl/phenylalanyl-tRNA-protein transferase (EC 2.3.2.6, L/F transferase). Eukaryotic aminoacyl-tRNA transferases selectively transfer arginine from $tRNA^{Arg}$ (EC 2.3.2.8, Arg transferase) to the N-terminus of proteins with acidic N-termini.[8,9] This definition of prokaryotic L/F transferases and eukaryotic Arg transferases is not an absolute division as transferases identified in the human pathogens *Plasmodium falciparum* and *Vibrio vulnificus* exhibit hybrid specificities.[10]

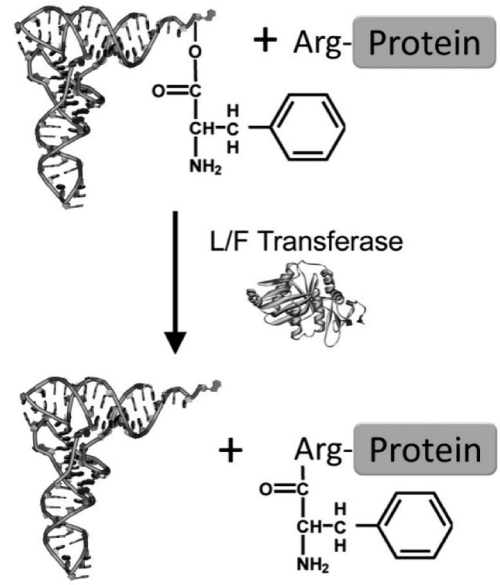

Figure 1. tRNA dependent peptide bond formation by L/F transferase. L/F transferases catalyzes the transfer of the esterified amino acid from $tRNA^{Leu}$ or $tRNA^{Phe}$ (shown) to the N-terminus of a protein with a N-terminal lysine or arginine (shown) residue.

A molecular outcome of enzymatic addition of leucine or phenylalanine to the N-terminus of a protein is the degradation of the modified protein via the N-end rule pathway.[11,12] Nonetheless, the biological implications of this are unclear as there are

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no known in vivo substrates for the bacterial L/F transferase. Presently, several X-ray crystal structures of L/F transferase have been reported with and without various substrate analogues and inhibitors.[13−15] Through structural investigations and biochemical studies of L/F transferase mutants, a catalytic mechanism for the enzyme has been proposed that is analogous to the reverse of the acylation step during peptide hydrolysis by serine proteases.[15]

Beyond the enigmatic role in biology, L/F transferase has been a recent focus for the development of novel tools for protein engineering. L/F transferase has been used for N-terminal labeling of proteins with radioactive[16] and unnatural amino acids.[3−6] Protein engineering with unnatural amino acids introduces novel chemical functionalities which can be applied to a variety of applications described in several reviews[17,18] including protein visualization,[19] protein isolation,[20] and protein function studies.[21,22]

For L/F transferase to label a protein with an unnatural amino acid, a substrate tRNA aminoacylated with the unnatural amino acid must be obtained. Taki and colleagues have chemically synthesized aminoacyl-tRNAs or aminoacyl-RNA mini helices with unnatural amino acids and successfully labeled proteins using L/F transferase with a variety of unnatural amino acids.[3−5] As aminoacyl-tRNAs possess half-lives of less than an hour at neutral pH,[23,24] their chemical synthesis and handling can be challenging. An alternative to synthesizing unstable tRNAs aminoacylated with unnatural amino acids is enzymatic aminoacylation with the appropriate engineered aminoacyl-tRNA synthetase, the enzymes responsible for tRNA aminoacylation in vivo. Datta and colleagues demonstrated that expanding the amino acid binding pocket of *Escherichia coli* phenylalanyl-tRNA synthetase (PheRS) by introducing two point mutations (A294G; T251G) into the α-subunit of the enzyme enables aminoacylation of *E. coli* $tRNA^{Phe}$ with p-acetyl phenylalanine.[25] More recently Connor and colleagues coupled the enzymatic activity of mutant PheRS with the L/F transferase reaction[6] to label a protein with a range of unnatural amino acids, including an amino acid with an alkyne functionality that was subsequently derivatized with an azide biotin probe using the increasingly popular "click" chemistry procedures.[26]

Conventionally, L/F transferase activity has been observed by the incorporation of radiolabeled ([^{3}H] or [^{14}C]) amino acids into substrate proteins (i.e., casein). Incorporation is determined either by TCA precipitation[27,28] or gel electrophoresis followed by autoradiography.[16] These procedures can be slow, generate high backgrounds, and are not applicable to measurements with unnatural amino acid substrates where the isotopically labeled form is not available. A recent report described a novel fluorescent L/F transferase substrate that enables product detection and quantification by HPLC analysis.[6] This HPLC method is applicable to analysis with unnatural amino acid substrates. However, all current procedures for measuring L/F transferase activity require significant amounts of material and are quite labor intensive and time-consuming such that their applications have mostly been limited to qualitative analysis.

In this manuscript we report a novel method to quantify L/F transferase activity by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). This method requires a minimal amount of material and minimal sample handling. MALDI-TOF analysis has been used by others in the past to verify product formation of L/F transferase reactions,[3−5] but this was never developed into a quantitative process using internal standards. To demonstrate the ability of the MS based method, we determined the apparent rate constants of the transfer of phenylalanine from aminoacyl-$tRNA^{Phe}$ to the N-terminus of a synthetic peptide substrate. Additionally, the kinetic parameters of the transfer of three different unnatural amino acid substrates were determined, one of which has been demonstrated qualitatively to be impaired in transfer efficiency.[4,6]

EXPERIMENTAL SECTION

Materials. Unless stated otherwise, all chemicals and peptides were purchased from Sigma-Aldrich. α-Cyano-4-hydroxycinnamic acid (CHCA), triethylamine (TEA), and bromoethane were purchased from Acros Organics (Belgium).

Unnatural amino acid, *p*-bromo-DL-phenylalanine (BrPhe) was purchased from Sigma-Aldrich. The D/L mixture was used since the specificity of the synthetase (PheRS) does not require a chirally pure amino acid. *H*-Phe(4-azido)-OH (azidoPhe) was purchased from Chem-Impex International, Inc. Boc-L-Phe(4-$C(O)CH_3$) (acePhe) was purchased from RSP Amino Acids LLC, as tBOC protected amino acid. Deprotection and final preparation of the amino acid is described in the Supporting Information.

The substrate peptide of a sequence REPGLCTWQSLR (MW 1445.6 g/mol) was purchased from Sigma-Genosys. Stock solutions of the peptide were made and the absolute concentration was determined by amino acid analysis by the Institute for Biomolecular Design (University of Alberta, Canada). Purified *E. coli* $tRNA^{Phe}$ was purchased from Chemical Block (Russia).

A clone of *E. coli* L/F transferase with an N-terminal 6× His tag in a pCA24N vector was obtained from the ASKA (−) strain collection maintained at the National Institute of Genetics (Ja-

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pan).[29] Cloned 6× His tagged nucleotidyl transferase (CCA adding enzyme) in a pET22b expression plasmid was a generous gift from Allen Weiner (University of Washington).[30] A pET28a expression plasmid containing 6× His-tagged PheRS was also a gift from Jack Szostak (Harvard Medical School). The mutation A294G and the double mutation A294G:T251G were introduced into PheRS using a Stratagene QuickChange kit. The DNA oligos for site directed mutagenesis were purchased from IDT, and the procedures used for expression and purification of all recombinant proteins are described in the Supporting Information.

Heavy and Light Standard and Substrate Peptides. Alkylation reactions of bromoethane or deuterated bromoethane with the cysteine containing peptide was carried out to generate light and heavy substrate and standard peptides as described by Hale et al.[31] Specifically, 1 μL of 100 mM tris(2-carboxyethyl) phosphine (TCEP) was added to 30 μL of 250 μM peptide solution, and the sample was incubated at 37 °C for 10 min. Following this incubation, 100 μL of acetonitrile, 1 μL of triethylamine, and 5 μL of bromoethane (or bromoethane-d_5) were added and the solution was again incubated at 37 °C for 30 min. The sample was dried under vacuum and suspended in 100 μL of distilled water and dried again (to remove residual reagents). The final heavy or light peptide product was resuspended in distilled water, and quantitative conversion was observed using MALDI-TOF analysis.

Peptide Bond Formation Assay. The 100 μL reaction contained reaction buffer (final concentrations: 50 mM HEPES pH 7.5, 50 mM KCl, 15 mM $MgCl_2$, 2 mM ATP, 0.2 mM CTP, 2 mM β-mercaptoethanol), 2 mM Phe (or one of the unnatural amino acids), 2 μM $tRNA^{Phe}$ (unless stated otherwise), 0.59 μM to 5.91 μM "light" peptide, 0.5 μL of 2.5 mg/mL nucleotidyl transferase enzyme, 1 μL of 1 mg/mL Phe-tRNA synthetase (wild type or mutants). The reaction mixture was incubated at 37 °C for 7 min for aminoacylation of all tRNA with natural and non-natural amino acids. With the addition of 1 μL of 2.95 mg/mL L/F transferase, the time course was started and incubation continued at 37 °C. The 5 μL aliquots were withdrawn and quenched at selected time points with an equal volume of quench solution (10 μL of 1 mg/mL BSA, 10 μL of acetonitrile, and 2.5% TFA to a final volume of 100 μL) containing equimolar amounts of "heavy" peptide. To the mixture of reaction and quench solutions, 10 μL of matrix (saturated CHCA in 50% acetonitrile and 0.2% TFA) was added and ~0.7 μL spotted in duplicate on a stainless steel MALDI-TOF MS sample plate. Once dried, the spots were washed with 5 μL of 4% formic acid and air-dried prior to MS analysis. The spectra were collected on MALDI-TOF instruments in the Institute for Biomolecular Design (University of Alberta, Canada). MALDI-TOF instruments used for analysis included a Bruker Daltonics Ultraflex and an Applied Biosystems Voyager DE-Pro.

RESULTS

L/F Transferase Substrates. L/F transferase catalyzes a bimolecular reaction, the transfer of an esterified amino acid from an aminoacylated tRNA to the N-terminus of a polypeptide as shown in Figure 1.[32,33] As aminoacylated tRNAs are very labile,[23] Phe-$tRNA^{Phe}$ substrate was generated in situ with a coupled enzymatic system. CTP, ATP, and nucleotidyl transferase were added to the reaction mixture to regenerate the tRNA's 3′ CCA terminus that may have been degraded by contaminating ribonucleases. Inclusion of $tRNA^{Phe}$, ATP, phenylalanine (free amino acid), and phenylalanyl-tRNA synthetase (PheRS) results in the continuous generation of Phe-$tRNA^{Phe}$ for the L/F transferase reaction. The aminoacylation reaction is initiated several minutes prior to initiation of L/F transferase reactions to ensure that formation of the aminoacyl-tRNA substrate is not the rate limiting step in the L/F transferase reaction. The model polypeptide substrate used for our investigations has the sequence: REPGLCTWQSLR. The peptide has the necessary N-terminal arginine amino acid for L/F transferase recognition. Additionally, this substrate peptide is highly soluble, is efficiently ionized during MS analysis, and contains an internal cysteine for stable isotope labeling.

MALDI-TOF mass spectrometry is not ideally suited for quantitative comparison of different compounds due to differences in ionization efficiencies, and nonhomogenous distribution of analytes in the matrix cocrystals lead to position specific signals for the different substrate molecules. Variations in signal intensities are dependent on the physical and chemical properties of the analytes, hampering reliable quantification with internal standards which are of a different chemical composition to that of the analyte. This nonequivalent ionization is readily apparent from peptide fingerprint experiments where a sample contains essentially equimolar amounts of the constituent peptides originating from a digested protein. Upon MALDI-TOF mass spectrometry analysis, many peptides originating from the protein are observed but peak intensities greatly vary.[34]

Stable isotope labeling of peptides enables quantitative measurements by mass spectrometry. A single peptide differentially labeled with heavy [^{2}H] or light [^{1}H] isotopes behaves identically with respect to ionization, crystallization with matrix, or any other handling procedure. The only distinguishing feature between peptides is their differences in molecular mass. The mass shift will depend on the number of isotopic atoms used in the labeling procedure. The addition of an isotopically light [^{1}H] labeled standard peptide of known concentration to a quenched reaction containing an isotopically heavy [^{2}H] labeled substrate enables quantification of the amount of substrate remaining in the sample. Measuring the ratio of ion intensities between the substrate standard peptides enables absolute quantification of the amount of standard peptide in a known sample. The approach of using stable isotope labeled peptides as internal standards for quantification by mass spectrometry was originally described more than 25 years ago.[35]

To enable quantification of L/F transferase kinetics by mass spectrometry, the substrate peptide, REPGLCTWQSLR, was derivatized by alkylating the cysteine with either a light or heavy labeling reagent. The alkylation reaction was carried out using a modified protocol first described by Hale and colleagues.[31] The

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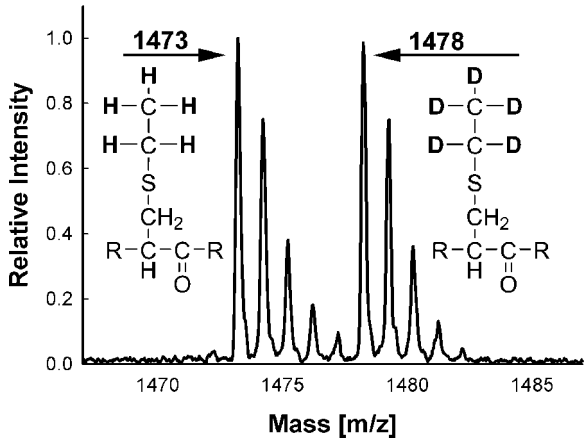

Figure 2. MALDI-TOF mass spectrum of heavy and light peptide pairs. Equal amounts of the peptide (REPGLCTWQSLR) were alkylated on the internal cysteine with either bromoethane or deuterated [d5]-bromoethane. After purification, equal amounts of peptide were combined and examined by MALDI-TOF MS. A mass shift of 5 Da was observed between the heavy and light peptide in the pair.

labeling reagents used were bromoethane or [d5]-deuterated bromoethane, which differ by the presence of either five protons or five deuterons. The peptide is quantitatively converted to one of two products (m/z 1473 and m/z 1478) depending on whether bromoethane or deuterated bromoethane was used in the labeling reaction. Figure 2 reveals the structures of the alkyated cysteines and the mass spectrum from MALDI-TOF analysis of an equimolar mixture of the two derivatized peptides. The two alkylated substrate peptides are referred to as the light (m/z 1473) and heavy peptides (m/z 1478). As seen in the spectrum, with a mass shift of 5 m/z there is negligible overlap of the isotopically resolved peaks for each peptide which arise from the natural abundance of [^{13}C] in the peptides.

To measure L/F transferase activity, the light or heavy peptide was used as a substrate in the enzymatic reactions. At selected time points after reaction initiation, aliquots are taken and added to an equal volume of reaction quench. In addition to TFA and other components to stop the reaction, the quench solution contains either the heavy or light peptide in an amount equal to the initial amount of substrate peptide in the sample. Combinations are either a light substrate peptide and a heavy standard peptide in the reaction quench or a heavy substrate peptide and the light standard peptide in the reaction quench. If no reaction occurs, the ion intensities for the light and heavy peptides during MALDI-TOF analysis will be equal. As the reaction proceeds there is correspondingly less substrate peptide remaining in the reaction. The amount of standard peptide is known and remains constant for every time point examined. Figure 3A illustrates the disappearance of heavy substrate peptide (indicated by S) over time in relation to the standard light peptide present in the sample quench (indicated by Q). The product with a mass of m/z 1625 (indicated by P) forms as a function of time as phenylalanine is transferred onto the substrate peptide. The m/z 147 mass shift corresponds to the theoretical mass for the N-terminal addition of phenylalanine. The ratio of ion intensities between the quench-peptide (Q, m/z 1473) and the substrate-peptide (S, m/z 1478) was quantified and the calculated concentration of substrate peptide plotted as a

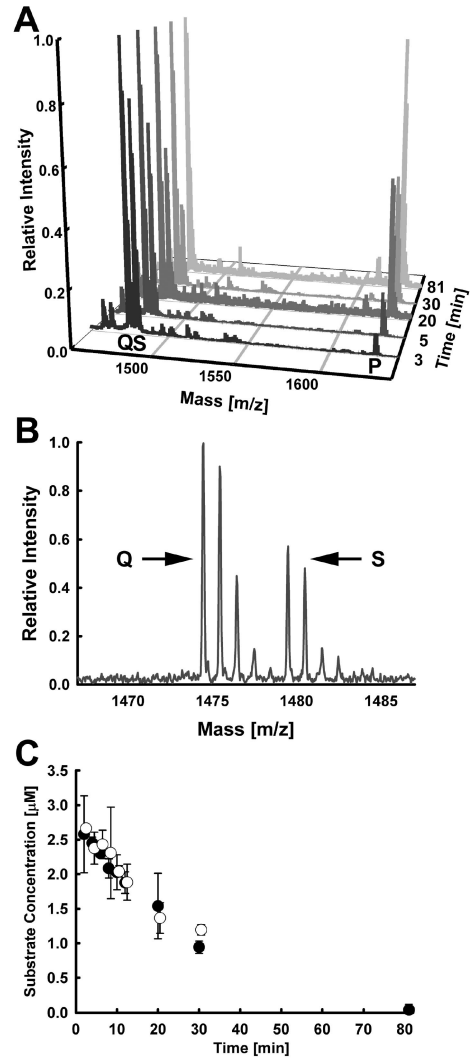

Figure 3. Peptide standards to quantify peptide bond formation: (A) MALDI-TOF mass spectrum of sequential time points of L/F transferase reaction containing a heavy substrate peptide (S, m/z 1478). At the indicated time, the enzymatic reaction was quenched with a solution containing an equimolar amount of the light peptide standard (Q, m/z 1473). During the reaction, formation of the product peptide (P, m/z 1625) is also observed. (B) Close up view of the substrate (S) and standard (Q) peptide from the 20 min time point. (C) Reaction time course of substrate disappearance. Two reactions are shown: using a heavy peptide substrate (as described in part A) compared to a light peptide standard (○) or vice versa (●).

function of time (Figure 3C). Experiments were typically carried out in complementary duplicates, using the heavy peptide as a substrate and the light peptide as a standard in the reaction quench as described above or vice versa using a light peptide substrate and heavy peptide quench. Figure 3C reveals that essentially no difference in measurements are observed for reactions using heavy or light peptide substrates, indicating that isotopic labeling does not affect the reaction rates.

The product formation can also be quantified using an isotopically labeled product peptide. However, in our studies on the addition of various unnatural amino acids (as described below) it would be significantly more challenging to obtain all the standard peptides required. More importantly, when comparing the incorporation of different amino acids into product peptides, errors in determining the absolute concentrations of different

Table 1. Initial Rates of and Kinetic Parameters of L/F Transferase Catalyzed Peptide Bond Formation

substrate peptide (μM)	initial rate (μM min^{-1})	appparent rate constants
	Phenylalanine[a]	
0.59	0.039 ± 0.002	$K_{m\ (apparent)} = 3.2\ \mu M$
1.2	0.068 ± 0.003	$k_{cat\ (apparent)} = 0.22\ min^{-1}$
2.4	0.089 ± 0.032	
3.6	0.139 ± 0.003	
5.9	0.189 ± 0.022	
	Br-Phe[a]	
0.59	0.028 ± 0.004	$K_{m\ (apparent)} = 4.3\ \mu M$
1.2	0.044 ± 0.007	$k_{cat\ (apparent)} = 0.20\ min^{-1}$
2.4	0.079 ± 0.027	
5.9	0.151 ± 0.003	
	azidoPhe[a]	
0.59	0.023 ± 0.005	$K_{m\ (apparent)} = 2.2\ \mu M$
1.2	0.048 ± 0.022	$k_{cat\ (apparent)} = 0.11\ min^{-1}$
2.4	0.047 ± 0.008	
3.6	0.079 ± 0.017	
	acePhe[a]	
0.59	0.0057 ± 0.0013	$K_{m\ (apparent)} = 18\ \mu M$
1.2	0.012 ± 0.003	$k_{cat\ (apparent)} = 0.17\ min^{-1}$
2.4	0.018 ± 0.001	
3.6	0.032 ± 0.004	

[a] Amino acid residues on the aminoacyl-tRNA^{Phe} substrate that is transferred to the peptide substrate.

product peptides would directly reflect on analysis. With our current procedure the same substrate is used for all reactions, which improves the accuracy of comparisons.

Substrate Concentration Dependence of L/F Transferase Activity. Having established the feasibility of measuring L/F transferase activity by mass spectrometry, we investigated the kinetic parameters of the multicomponent enzyme system. Absolute kinetic parameters can be obtained in a two substrate enzymatic system by maintaining one substrate at saturating concentrations and measuring the concentration dependence of the other. With biomolecules like aminoacyl-tRNAs it is extremely challenging to obtain sufficient amounts of material to ensure saturating concentrations due to its solubility and instability. As a result of measurements being made at subsaturating concentrations of aminoacyl-tRNA, our reported measurements for K_m and k_{cat} are apparent $K_{m(app)}$ and apparent $k_{cat(app)}$ values.

Enzymatic reactions were measured over a range of substrate peptide concentrations to determine apparent kinetic parameters. Reactions were measured at initial substrate concentrations ranging from 0.59 to 5.9 μM. Initial reaction rates and substrate concentrations for the transfer of phenylalanine are listed in Table 1. Analysis of the initial rates of the reactions results in the determination of an apparent K_m (3.2 μM) and an apparent k_{cat} (0.22 min^{-1}).

Unnatural Amino Acid Substrates. It has been determined previously that L/F transferase will utilize tRNAs aminoacylated with a range of unnatural amino acids as substrates.[3−6] These experiments have qualitatively demonstrated that unnatural amino acids are transferred to a substrate polypeptide. Here we use the MALDI-TOF MS method to quantitatively compare tRNA substrates aminoacylated with unnatural amino acids.

A significant technical challenge for investigating unnatural amino acid with respect to L/F transferase activity is the generation of the aminoacyl-tRNA substrates. Aminoacylation of

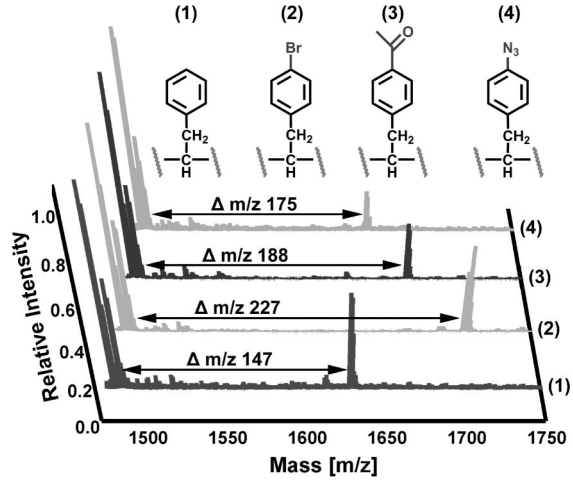

Figure 4. Enzymatic transfer of unnatural amino acids. MALDI-TOF mass spectra of the L/F transferase reactions where (1) phenylalanine, (2) BrPhe, (3) acePhe, and (4) azidoPhe are appended to the N-terminus of a substrate peptide. The change in *m*/*z* upon conversion of the substrate to the indicated product peptides is shown for each reaction.

tRNA^{Phe} with unnatural amino acids for L/F transferase investigations has been done both chemically[3−5] and enzymatically.[6] For this work, enzymatic aminoacylation is used.

Mutant phenylalanyl-tRNA synthetases (PheRS) can enzymatically aminoacylate tRNA^{Phe} with unnatural amino acids. It has been previously demonstrated[36] that an A294G mutation to the α-subunit of PheRS enables the synthetase to aminoacylated tRNA^{Phe} with 4-bromo phenylalanine (BrPhe) or 4-azidophenylalanine (azidoPhe). Inclusion of a second mutation to the α-subunit of PheRS (A294G:T251G double mutant) enables aminoacylation with a phenylalanine analogue containing a ketone group (acePhe).[25] The side chains of these unnatural amino acids are illustrated in Figure 4.

When the appropriate mutant PheRS and unnatural amino acid are used in a L/F transferase reaction, the mass spectra of the reaction clearly demonstrates the transfer of the unnatural amino acid to the substrate peptide (Figure 4). Enzymatic addition of either acePhe or BrPhe to the substrate peptide results in the predicted shifts to the observed *m*/*z* of 227 and 188 units, respectively. Analysis of azidoPhe reveals a change in *m*/*z* of 175 versus a predicted shift of *m*/*z* 189. This inconsistency is predicted to be a result of photodecomposition of the azidoPhe amino acid upon MALDI-TOF analysis. AzidoPhe is an amino acid which has historically been used as a UV photocrosslinker,[37,38] so it is not unexpected that a photodependent reaction of the amino acid is observed upon irradiation with the UV laser of the MALDI-TOF instrument.

The rates of amino acid transfer for the three unnatural amino acids were measured at several different peptide substrate concentrations. The results are listed in Table 1, and the Lineweaver−Burk plot for analysis is shown in Figure 5. The data from both BrPhe and azidoPhe experiments demonstrate that the apparent K_m and apparent k_{cat} for the enzymatic transfer of these

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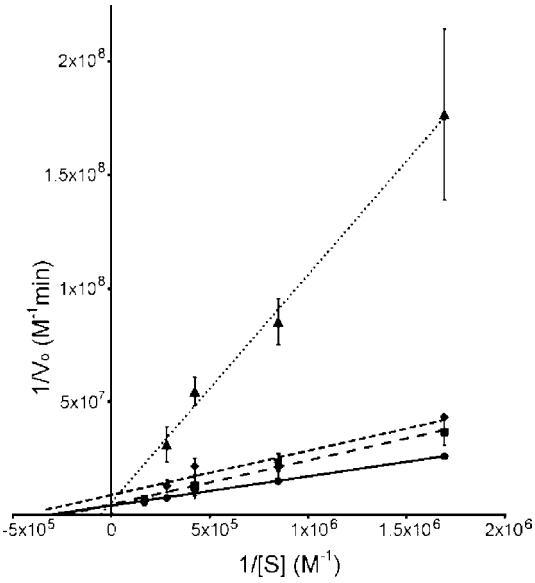

Figure 5. Kinetics of the enzymatic transfer of unnatural amino acids. A Lineweaver−Burk plot for the transfer of amino acids to a substrate polypeptide. Presented data are for phenylalanine (●, solid line), BrPhe (■, dashed line), azidoPhe (◆, long dashed line), and acePhe (▲, short dashed line).

two unnatural amino acids are indistinguishable (within 2-fold) from the kinetic parameters for the transfer of the cognate amino acid, phenylalanine. Conversely, analysis of the kinetics of acePhe transfer reveals a ~5-fold increase in the apparent K_m for the peptide substrate of the reaction and no significant difference in the apparent k_{cat}. The increased K_m suggests impaired binding of the peptide substrate for the reaction with the largest amino acid (acePhe).

DISCUSSION

A quantitative MALDI-TOF mass spectrometry assay to measuring tRNA dependent nonribosomal peptide bond formation was developed for several reasons. First, unlike conventional methods it does not require radioisotope [^{3}H]- or [^{14}C]-labeled amino acids, which enables investigations of unnatural amino acids, for which the radiolabeled derivatives are not commercially available. Second, procedures do not require additional sample handling steps such as trichloroacetic acid (TCA) precipitations or gel electrophoresis. Samples are ready for analysis almost immediately after the addition of a reaction quench. Third, the procedure is amendable to relatively high throughput procedures. Modern MALDI-TOF MS plates are compatible with 96, 384, and 1536 well formats and MALDI-TOF measurements are very rapid, with each sample being analyzed in just a few seconds.

The basis of the MALDI-TOF approach is the use of peptide substrates and standards that contain either light or heavy isotope labels. The application of isotopic labeling of standards for kinetic analysis by mass spectrometry has been described for a variety of other systems such as prenylation,[39] lipase activity,[40] and ribosome assembly.[41,42] This is the first report of MS application for quantitative kinetic analysis of tRNA dependent peptide bond formation. For this investigation, a robust alkylation procedure with bromoethane and deuterated bromoethane was adapted[31] to generate light and heavy peptides that differ in mass by 5 Da. Nonetheless, other strategies generating light and heavy peptide pairs would suffice. A variety of chemical labeling procedures are described in a review of quantitative proteomics.[43]

Investigations of L/F transferase are of interest for several reasons. Recent crystal structures of the L/F transferase in complex with substrate analogues and product polypeptides provided the framework for the recent proposed mechanism for ribosome-free peptide bond formation by this enzyme.[15] A robust activity assay enables further in-depth structure−function studies of this enzyme. Beyond mechanistic investigations, several researchers have been developing methods using L/F transferase as a tool for specific N-terminal labeling of proteins with radioisotopes[16] or unnatural amino acids for biotechnological applications.[3−6] A simple activity assay would facilitate further development and engineering of this enzyme to alter substrate specificity. Additionally this procedure may be useful in the studies of the related FemABX peptidyl transferases which are involved in peptidoglycan synthesis of some gram positive bacteria, such as species of *Streptococcus* and *Staphylococcus* genera.[44−46]

The applicability of our presented method was demonstrated by investigating two aspects of the L/F transferase catalyzed reaction. First, the apparent K_m and k_{cat} values for the standard reaction of phenylalanine transfer to a polypeptide were determined. Second, the rates of transfer of three different unnatural amino acids from tRNA to a polypeptide substrate were measured.

The enzymatic parameters determined by mass spectrometry (listed in Table 1) are in agreement with the values determined previously by conventional methods. The reported K_m values range from 1.6[32] to 5.4 μM[15] for protein substrates, while we have determined a K_m of 3.4 μM for a synthetic peptide substrate. It is noteworthy that none of the previously utilized protein substrates are of biological significance as there are still no known in vivo substrates for L/F transferase. The largest discrepancy between these data concerns the apparent k_{cat} value for the reaction where a previous study had reported rates 10 times faster than our measured rate of 0.24 min^{-1}. These differences could simply result from the fact that observed rate constants and not absolute rate constants were determined, due to measurements being made at subsaturating conditions (for our work and all previously published reports). Additionally, previous reports using conventional procedures do not contain internal standards such that errors in product quantification due to detection limitations and errors in determining the specific activities of the low energy isotopes of [^{3}H] and [^{14}C] labeled amino acids would directly reflect in

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the determination of the rate constants. Lastly, variations could arise from differences in the specific activity of different preparations of L/F transferase protein. Nonetheless, with all the possibilities for discrepancies the single order of magnitude difference is marginal and particular investigations will typically be internally controlled, such as comparison of enzyme mutants with wild-type.

For applications with unnatural amino acids, the efficiency of L/F transferase to utilize unnatural amino acid substrates may limit the applicability of this technology. A recent study by Taki and colleagues using large fluorescent analogues of alanine demonstrated the amino acid size limitation of L/F transferase.[5] Wild type L/F transferase did not transfer the largest unnatural amino acids to a substrate protein, but mutations introducing a larger amino acid side chain binding pocket into L/F transferase (M144A or F173A) enabled the transfer of even the largest amino acid. With more modestly sized amino acids such as acePhe (shown in Figure 4), there have been qualitative reports describing the amino acid to be less efficiently transferred by L/F transferase in comparison to phenylalanine.[4,6] The results of our investigations reveal almost identical rates of peptidyl transferase activity when using tRNAs aminoacylated with BrPhe or azidoPhe (Figure 5), suggesting efficient use of these unnatural amino acids. However, as was qualitatively demonstrated by previous investigations,[4,6] we observed reduced rates of acePhe transfer from $tRNA^{Phe}$ to the substrate peptide (Figure 5). Analysis of the Lineweaver–Burk plot reveals that the reduced amino acid transfer rates are a result of an increase in the K_m for the substrate peptide. It is possible that acePhe is near the size limit for the amino acid side chain binding pocket of L/F transferase and its binding may partially hinder or exclude binding of the peptide substrate. This is in agreement with X-ray crystallographic data where a structure of *E. coli* L/F transferase with a bound puromycin inhibitor,[13] the side chain of acePhe is slightly larger than that for puromycin. Analysis of the X-ray crystal structure reveals that the methyl-tyrosine amino acid side chain of the inhibitor is buttressed up against M144 (see Figure S-1 in the Supporting Information). Investigation of the structure reveals there is little or no extra space available to accommodate the slightly larger acePhe side chain, and binding may result in steric clashing. Potential use of engineered L/F transferase protein with the M144A or F173A mutations, which enable the transfer of the larger fluorescent amino acids,[5] may also increase the efficiency of acePhe transfer.

CONCLUSION

Quantitative MALDI-TOF mass spectrometry is a robust and rapid method for quantitative measurements of peptidyl transferase activity of the L/F transferase enzyme. The procedure described will enable more comprehensive structure–function investigations of L/F transferase and potentially aid in further engineering of this enzyme for altered substrate specificity. The MALDI-TOF procedure should also be applicable to investigations of other aminoacyl-tRNA protein transferases such as the eukaryotic Arg transferase that is of human medical interest due to its function in heart development[1] and apoptosis.[2]

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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