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Introduction

Peptidyl-tRNAs are thought to be inconspicuous molecules found in all cells. is is mainly due to cells having developed e cient means to recover tRNA from the unusable peptide bound form. However, the signi cance of peptidyl-tRNA and its enzymatic hydrolysis is nally being discovered, particularly in regard to impact on protein biosynthesis and cell viability.

In the course of translation, ribosomes stall on average 10% of the time [1]. If not rescued, disassembly of the translation machinery results in the release of peptidyl-tRNA. Peptidyl-tRNA is also generated from translation of minigenes or short ORFs [2-4]. Build-up of peptidyl-tRNA is lethal due to tRNA starvation [5]. e importance of preventing peptidyl-tRNA accumulation is apparent from the multiple mechanisms cells employ to recycle them [6,7]. ese mechanisms depend on whether peptidyl-tRNA is part of the stalled ribosome or has been released. In stalled ribosomes, peptidyl-tRNA is recycled by ribosome associated proteins with peptidyl-tRNA hydrolase function [8,9]. If the ribosome is disassembled and peptidyl-tRNA is released, the complexity of tRNA recycling mechanisms di ers greatly between prokaryotes and eukaryotes. In most bacteria there is a single, essential peptidyl-tRNA hydrolase enzyme, Pth1, responsible for removing the peptide from peptidyl-tRNA [10]. In certain bacterial species, a Pth2 homolog has been identi ed [11] but has not been structurally or functionally characterized. In eukaryotes, there is an emerging network of enzymes that recycle peptidyl-tRNAs, including mitochondrial associated Pths and Pth domain containing proteins [12-14].

From a biochemical point of view, peptidyl-tRNA is somewhat of an enigma having dual character – part peptide, part nucleic acid, Figure 1. is dual character leads to tremendous heterogeneity. Peptidyl-tRNA isolated from any given cell is a mixture of more than 40 tRNA molecules and considerably larger number of peptides [15]. Even with all of the possible di erences, the character of the tRNA component is fairly uniform – a typical “clover leaf” structure with large negative charge. Each has an attached peptide of variable length and composition. e nature of the peptide component is much more heterogeneous and largely determined by size and composition. e presence of a shorter peptide does not change the properties of the molecule much from the properties of tRNA itself. A larger peptide chain can, however, signi cantly alter the molecular properties and have implications for the choice of methods to analyze peptidyl-tRNA enzymatic hydrolysis.

Herein we review the methods that have been developed to monitor the cleavage of the peptide-tRNA ester bd,8rmin9xdtionpep0.6(er

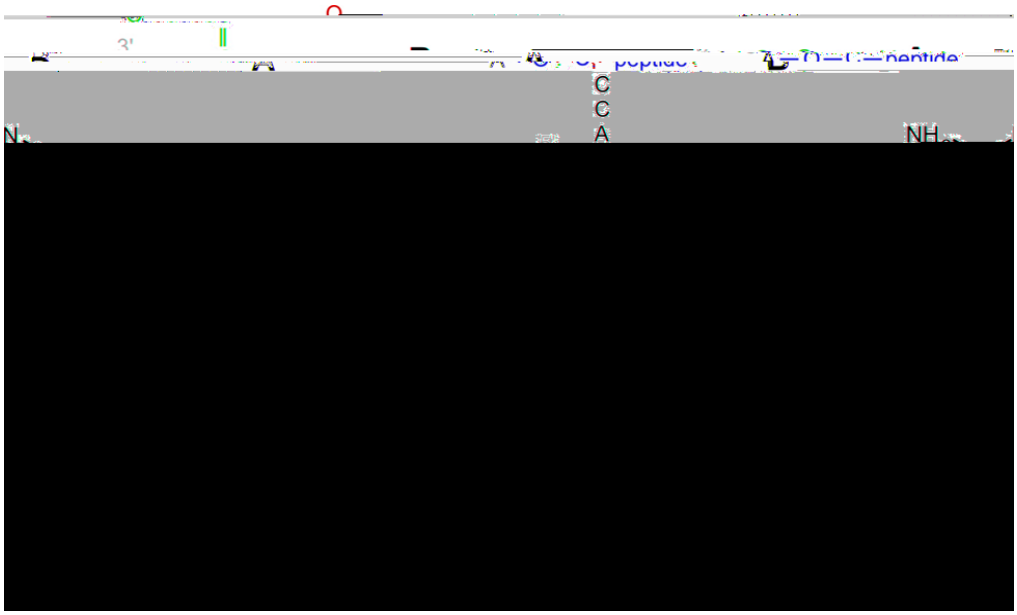

Figure 1: A. An example of peptidyl-tRNA. The peptide (red) is attached through an ester bond to the 3' end of tRNA (black), in this case $tRNA^{Pro}$. **B**. Close up of the ester bond between ribose 3'-hydroxyl group and C-terminus of the peptide chain. Note that the ester bond can also be formed between ribose 2'-hydroxyl group.

[^{14}C] peptides, were separated with paper chromatography, visualized using ninhydrin staining, and quanti ed by scintillation counting of the excised peptide band. Early initial insight into the enzymatic activity of bacterial Pth1 was gained using this methodology [16,17]. Several subsequent modi cations have been reported. e amount of hydrolyzed peptide was determined using scintillation of the supernatant obtained by centrifugation of the TCA quenched reaction mixture omitting the paper chromatography separation step [18] and N-acetyl-[^{14}C]-aminoacyl-tRNA was used instead of peptidyl-tRNA [19]. While Pth1 is not able to hydrolyze aminoacyl-tRNA, it does catalyze the cleavage of N-acetyl-aminoacyl-tRNA. Introducing N-acetylated-aminoacyl-tRNA thus simpli ed the preparation of the substrate and, like the omission of the paper chromatography step, expedited the assay.

Radioactively labeled peptidyl-tRNA has also been generated using an *i i* translation system with [^{35}S]-Methionine incorporated into TnaC peptide [20]. is peptidyl-tRNA has not been used as a substrate for Pth activity assays but, made in su cient quantity, would be an excellent speci c substrate. In addition, this system could be readily modi ed for production of other speci c peptidyl-tRNA as in the case of uorescently labeled peptidyl-tRNA below. Upon enzymatic hydrolysis, the remaining nonhydrolyzed substrate as well as the hydrolyzed peptide could be precipitated, separated using PAGE, and visualized by autoradiography. Quanti cation of hydrolysis would then be determined based on band intensity.

e methods employing radioactively labeled amino acids are well suited for determination of Pth enzyme kinetic parameters. ey are extremely sensitive, can be used for a wide range of signal intensities (i.e. substrate concentrations), and quanti cation can be readily performed. On the other hand, they require the use of a speci c peptidyl-tRNA, production of which is a multistep process that may be unnecessarily demanding for certain applications. e use of radioactivity may also be undesirable or problematic. It has also been shown that the rate of Pth1 hydrolysis depends on peptide composition [18] and length [21] which is not accounted for by using N-acetyl-aminoacyl-tRNA or a single peptidyl-tRNA species.

Electrophoretic Separation of tRNA

Separation of tRNA using low pH urea polyacrylamide gels was introduced to study the levels of aminoacylation in *E. c li* [22]. Total RNA isolated from the cell was separated on an acid-urea polyacrylamide gel and tRNAs (free, aminoacyl, and peptidyl) detected using a speci c nucleotide probe labeled with [^{32}P]-ATP (i.e. a Northern blot), Figure 2. is technique was further simpli ed by using minigels instead of large gels traditionally used for nucleic acid separation [23]. e analysis could be further expedited by employing nonspeci c nucleotide staining [24,25]. Compared to those employing radioactively labeled substrate, this method makes observation of both the hydrolyzed product (tRNA) and the nonhydrolyzed peptidyl-tRNA possible on one gel. However, it is limited to the use of peptidyl-tRNA with very short peptides because, in general, larger peptides tend to have more positive charge at lower pH. is prevents peptidyl-tRNA from entering the gel.

Methods using electrophoretic separation of tRNA are well suited for determination of Pth enzymatic activity. ey allow the use of heterogeneous peptidyl-tRNA directly isolated from cells and hydrolysis for the entire population. Speci c peptidyl-tRNAs can also be used. e fact that these methods are relatively nondemanding in terms of substrates, yet work with natural substrates, makes them very attractive for many applications. e ability to perform enzymatic hydrolysis with heterogeneous, bulk peptidyl-tRNA isolated from bacterial cells, separate the reaction products on a minigel, and stain for detection makes the analysis of peptidyl-tRNA enzymatic hydrolysis achievable in any biochemistry laboratory.

Fluorophore Conjugated Amino Acids

e most recent development in the analysis of enzymatic peptidyl-tRNA hydrolysis has been the introduction of uorophores conjugated

to amino acids. e concept is the same as for [^{14}C] studies but is less hazardous and has extended utility from the ability to detect changes in uorescence anisotropy. Initial reports of uorescent peptidyl-tRNA hydrolysis used BODIPY® FL succinimidyl ester for the labeling of lysyl-tRNA^{Lys} used in downstream applications [26]. Similarly, Oregon