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Mini-Review

Peptidyl-tRNA hydrolase and its critical role in protein biosynthesis

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Peptidyl-tRNA hydrolase (Pth) releases tRNA from peptidyl-tRNA by cleaving the ester bond between the peptide and the tRNA. Genetic analyses using *Escherichia coli* harbouring temperature-sensitive Pth have identified a number of translation factors involved in peptidyl-tRNA release. Accumulation of peptidyl-tRNA in the cells leads to depletion of aminoacyl-tRNA pools and halts protein biosynthesis. Thus, it is vital for cells to maintain Pth activity to deal with the pollution of peptidyl-tRNAs generated during the initiation, elongation and termination steps of protein biosynthesis. Interestingly, while eubacteria possess a single class of peptidyl-tRNA hydrolase, eukaryotes possess several such activities, making Pth a potential drug target to control eubacterial infections. This review discusses the aspects of Pth that relate to its history and biochemistry and its physiological connections with various cellular factors.

Introduction

For various physiological reasons, not all the ribosomes that begin to translate an mRNA reach the termination codon. A significant subpopulation of the translating ribosomes stalls in between the decoding cycles and poses a serious impediment to mRNA translation (Manley, 1978; Jorgensen & Kurland, 1990). Often such stalled ribosomes, and the ones translating short open reading frames (Heurgue-Hamard *et al.*, 2000), release peptidyl-tRNAs as a part of a mechanism that allows the reuse of the ribosomes. Peptidyl-tRNA hydrolase (Pth), an esterase, originally identified in *Escherichia coli* and yeast, releases tRNA from peptidyl-tRNAs by cleaving the ester bond between the C-terminal end of the peptide and the 2′- or 3′-hydroxyl of the ribose at the end of the tRNA (Cuzin *et al.*, 1967; Kössel & RajBhandary, 1968). Pth is also capable of hydrolysing an amide bond between the peptide and the 3′-amino group of the modified ribose at the end of the tRNA in synthetic substrates (Jost & Bock, 1969). However, peptidyl-tRNAs bound to 70S ribosomes are resistant to hydrolysis by Pth (Vogel *et al.*, 1971), and termination codon dependent peptide release proceeds in the absence of Pth. These observations rule out a function of Pth as a release factor, surmised at the time of its discovery. However, the functional significance of association of Pth with the 30S ribosomal subunit (Kössel, 1970) remains unclear.

The *pth* gene encoding Pth was first identified in *E. coli* (García-Villegas *et al.*, 1991) at 27·1 min in the genome. Genes encoding Pth have been recognized in organisms belonging to all three kingdoms of life. *E. coli* and other eubacteria possess Pth (Pth1). Archaea possess a different class of Pth, known as Pth2. Interestingly, eukaryotes possess multiple Pth activities, including orthologues of the eubacterial and archaeal enzymes.

Pth is a key protein at the crossroads to the function of several translational factors (Fig. 1). Here, we discuss the genetic and biochemical aspects of Pth which have added substantially to our understanding of the mechanism of protein biosynthesis.

Substrate specificity and protection of fMet-tRNA^{fMet} from hydrolysis by Pth

Studies on *E. coli* Pth have shown that the N-blocked aminoacyl moiety attached to elongator tRNAs but not the initiator tRNA^{fMet} is a substrate for Pth; and the enzyme is specific for the bond formed by L-amino acids (Cuzin *et al.*, 1967; Kössel & RajBhandary, 1968). The resistance of fMet-tRNA^{fMet} to Pth is physiologically relevant in prokaryotes, where it is utilized for initiation and needs protection from hydrolysis by Pth. Notably, a mismatch at the top of the acceptor stem (positions 1 and 72), unique to the prokaryotic initiators, is a hallmark of their resistance to Pth (Kössel & RajBhandary, 1968; Schulman & Pelka, 1975; Dutka *et al.*, 1993). The amino acid attached to the initiator tRNA^{fMet} also contributes to its resistance to Pth (Thanedar *et al.*, 2000).

Hydrolysis of peptidyl-tRNAs by Pth increases substantially for substrates with two peptide bonds compared to those with a single peptide bond (a diaminoacyl-tRNA or an N-blocked aminoacyl-tRNA). The rates increase further with increase in chain length to three or four peptide bonds. However, a further increase in the chain length does not lead to a significant increase in the rates. Thus, a peptidyl-tRNA

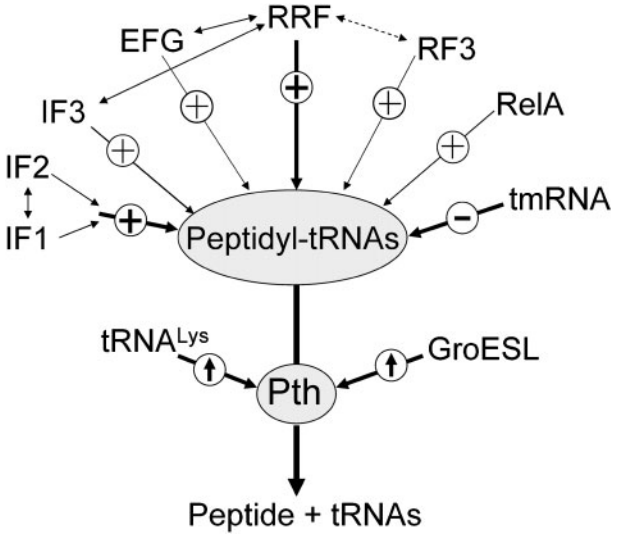

Fig. 1. Genetic interactions between various factors leading to an increase (+) or a decrease (−) in peptidyl-tRNA drop-off from the ribosomes. Peptidyl-tRNAs are processed by Pth. The factors connected to the pool of peptidyl-tRNAs with a thick arrow have a direct effect on the peptidyl-tRNA drop-off. The factors connected through thin arrows most likely function via indirect pathways, which may be mediated by the genetic interactions between various factors (shown by two-way arrows). For example, initiation factor 3 (IF3) and elongation factor G (EFG) function via the ribosome recycling factor (RRF) pathway. Although there is evidence to suggest that release factor 3 (RF3)-mediated peptidyl-tRNA drop-off may occur through a functional interaction with the RRF pathway, its mechanism is unclear (shown by a dotted two-way arrow). Initiation factors 1 and 2 (IF1 and IF2) both lead to increased drop-off of peptidyl-tRNAs by 'abortive initiation', and their simultaneous overproduction is synergistic. Although RelA enhances peptidyl-tRNA release, the mechanism of its action is unknown. $tRNA^{Lys}$ and GroESL rescue the Pth^{ts} phenotype by mediating their positive effects (↑) on Pth. It is quite likely that the chaperone activity of GroESL stabilizes the temperature-sensitive Pth. The $tRNA^{Lys}$ allows an increase in the level of Pth during the transition from the permissive to non-permissive temperature for sustained growth of the strain (see text for details).

containing three or four peptide bonds represents an optimal substrate for Pth (De Groot *et al.*, 1969; Shiloach *et al.*, 1975). In addition, the phosphate group at the 5′ end (position 1) of the tRNA is important in the substrate recognition by Pth (Schulman & Pelka, 1975). However, in $tRNA^{His}$, which possesses a (−1):73 base pair, the phosphodiester group between the −1 and 1 positions contacts the enzyme (Fromant *et al.*, 2000). Interestingly, of the two reaction products, only the tRNA is inhibitory to Pth (Jost & Bock, 1969). It is likely that the tRNA part of the substrate establishes the primary set of interactions with the enzyme. The contacts established by the peptidic moiety are plausibly secondary, but important for proper alignment of the substrate onto the enzyme. In stark contrast to Pth, Pth2 utilizes $fMet\text{-}tRNA^{fMet}$ as a substrate, and the 5′ phosphate group of the tRNA is not an important recognition element for it (Fromant *et al.*, 2003), which suggests that the mismatch at the top of the acceptor stem of the eubacterial initiators is not capable of protecting $fMet\text{-}tRNA^{fMet}$ from hydrolysis by Pth2. This study also showed that Pth2 could replace Pth in *E. coli*. Possibly, other mechanisms contribute to protect $fMet\text{-}tRNA^{fMet}$ from hydrolysis by Pth2. Indeed, initiation factor 2 (IF2) is known to protect $fMet\text{-}tRNA^{fMet}$ from Pth (Thanedar *et al.*, 2000).

Mechanism of action of Pth

The three-dimensional structures of Pth from *E. coli* (Schmitt *et al.*, 1997) and the Pth2 class of proteins from human (de Pereda *et al.*, 2004), *Archaeoglobus fulgidus* (Powers *et al.*, 2005) and *Sulfolobus solfataricus* (Fromant *et al.*, 2005) are known. However, structures of enzyme–substrate (or its analogues) complexes are not yet available. *E. coli* Pth consists of a single α/β globular domain assembled around a twisted mixed β-sheet. Three of the active-site residues, N10, H20 and D93, were identified as crucial for catalysis. Interestingly, in the crystal structure of *E. coli* Pth, the three C-terminal residues of one Pth molecule occupy the active-site groove of the other Pth molecule. This binding could mimic interaction of the peptidic moiety of the substrate with the enzyme. In this model, the cleavage site of the substrate was placed in close proximity to the highly conserved residues N68, N114 and H20. A positively charged protein area typified by K105 and R133, and a cluster of asparagines (N10, N21, N68 and N114), were shown to be important for interaction with the 5′ phosphate and the acceptor-TΨC helix of tRNA, respectively (Fromant *et al.*, 1999). Recently, H20 has been shown to play an essential role in catalysis (Goodall *et al.*, 2004). In contrast to Pth, the human Pth2 possesses a novel three-layered α/β fold consisting of a four-stranded anti-parallel sheet in its core surrounded by two α-helices on each side. Enzymes belonging to this class do not show a significant similarity to Pth, and are dimeric in solution (as opposed to Pth, which is monomeric). Mutational analysis of *S. solfataricus* Pth2 has shown that the conserved residues K18, D86 and T90 are critical for catalysis and form a part of the N-terminus of the α1 helix and the loop between the β3 and β4 strands (Fromant *et al.*, 2003).

Genetic interactions and the mechanism of peptidyl-tRNA 'drop-off'

Atherly & Menninger (1972) reported isolation of *E. coli* K-12 strains temperature sensitive for Pth (Pth^{ts}). Such strains, when shifted to non-permissive temperature, rapidly accumulate peptidyl-tRNA and arrest protein biosynthesis (Menninger, 1979). These strains have been instrumental in advancing our understanding of the role of Pth in protein biosynthesis, and have been extensively used to study the mechanism of peptidyl-tRNA drop-off. Genetic studies showed that RelA, RRF (ribosome recycling factor), RF3, IF1, IF2 and IF3 enhance peptidyl-tRNA release (see Fig. 1).

Conversely, downregulation or compromise in the function of many of these factors rescues the Pth^{ts} phenotype in *E. coli* (Menninger *et al.*, 1983; Heurgue-Hamard *et al.*, 1998; Singh *et al.*, 2005). Another class of suppressors includes tRNA^{Lys} and tmRNA, whose overexpression rescues the Pth^{ts} phenotype (Heurgue-Hamard *et al.*, 1996; Singh & Varshney, 2004). The observation that overexpression of tRNA^{Lys} (the tRNA that is depleted most rapidly in *E. coli*) rescues a Pth^{ts} strain of *E. coli* suggests that it is the unavailability of free tRNA that results in cell death. It is now known that increased availability of Lys-tRNA^{Lys} allows *E. coli* to maintain threshold levels of Pth (and possibly other crucial proteins) during the transition from permissive to non-permissive temperatures, which in turn sustains a supply of tRNA^{Lys} by recycling the peptidyl-tRNA^{Lys} (Vivanco-Dominguez *et al.*, 2006). This finding diminishes the possibility that accumulated peptidyl-tRNAs directly interfere with cellular processes. Yet another category of suppression, for instance by overexpression of GroESL, most likely improves the stability of the Pth when the cells are shifted to non-permissive temperatures. We discuss the mechanism of increase or decrease in peptidyl-tRNA drop-off by various factors as follows.

It was suggested that an incorrect peptidyl-tRNA in a ribosome wherein the tRNA anticodon does not match the mRNA codon (e.g. as a consequence of incorrect accommodation of the aminoacyl-tRNA or frame-shifting following peptide bond formation in the ribosome) preferentially dissociates from the ribosome during protein synthesis (Menninger, 1979). Such a phenomenon occurs frequently in RelA^{+} strains to maintain high accuracy of protein synthesis. However, RelA-deficient strains, where the accuracy of protein synthesis is not strictly monitored, allow incorrect peptidyl-tRNAs to continue chain elongation, decreasing their drop-off (Menninger *et al.*, 1983).

RRF activity leads to accumulation of excess peptidyl-tRNA in the cell, and for this function, EFG is required (Rao & Varshney, 2001). More recently, involvement of IF3 in this pathway has been elucidated (Singh *et al.*, 2005). Thus, RRF, EFG and IF3 most likely function through a common pathway of enhancing peptidyl-tRNA release by RRF-mediated recycling of the stalled ribosomes. Further, it was observed that RF3 enhances peptidyl-tRNA release in the presence of RRF and EFG, and the Pth^{ts} phenotype could be better rescued by simultaneous deficiency of RF3 and RRF in *E. coli* (Heurgue-Hamard *et al.*, 1998). However, the biochemical mechanism of co-ordination between RRF and RF3 is unclear.

A synergistic effect of simultaneous overexpression of IF1 and IF2 on drop-off of peptidyl-tRNAs (with short peptidic moieties) has been discussed (Karimi *et al.*, 1998). Briefly, upon binding of the 50S subunit to the 30S initiation complex, both IF2 (bound to a non-hydrolysable GTP analogue) and IF1 are present in the 70S complex. The P-site-bound fMet-tRNA^{fMet} becomes non-reactive to puromycin, suggesting that IF2 influences the location of initiator tRNA on the ribosome (Allen *et al.*, 2005). Under physiological conditions, joining of the 50S subunit to the 30S initiation complex triggers hydrolysis of the IF2-bound GTP and departure of these factors to produce a 70S initiation complex competent to move into the elongation phase. Thereafter, aminoacyl-tRNAs are continuously recruited to the A site, and IF1 and IF2 do not get an opportunity to rebind. However, in the stalled ribosomes, the empty A site may prompt rebinding of IF1 and IF2, which could destabilize the ribosome-bound peptidyl-tRNA and lead to its drop-off. Such a mechanism of peptidyl-tRNA drop-off ('abortive initiation') could be important in regulating translation of an mRNA containing rare codons at the beginning of the reading frame, especially under starvation.

How does tmRNA decrease the peptidyl-tRNA drop-off? When alanylated tmRNA (SsrA) is recruited to the A-site of the stalled ribosome, the peptidyltransferase activity transfers the peptide from the P-site-bound peptidyl-tRNA to the alanine on the -CCA end of the tmRNA. Consequently, the tRNA sequestered as peptidyl-tRNA in the stalled complex is released as free tRNA. As the tmRNA-mediated mechanism utilizes the peptidyltransferase activity of the stalled ribosomes to liberate the tRNA (as opposed to peptidyl-tRNA drop-off), the need for Pth to recycle such tRNAs is bypassed. The function of tmRNA thus decreases the peptidyl-tRNA load in the cell (Singh & Varshney, 2004).

Essentiality of Pth

In *Saccharomyces cerevisiae*, deletion of both of the identifiable *pth* genes, individually or in combination, still leaves it viable (Menez *et al.*, 2002a; Rosas-Sandoval *et al.*, 2002). However, disruption of the gene encoding Pth1 (PTH1) in *S. cerevisiae* decreased its growth on non-fermentable carbon sources, suggesting a mitochondrial location of this protein (Fromant *et al.*, 2003; Sickmann *et al.*, 2003). In fact, the Pth2 (PTH2) is also known to be present in mitochondria (Sickmann *et al.*, 2003; Jan *et al.*, 2004). On the other hand, Pth is essential for the survival of *E. coli* even when many of its suppressors (such as downregulation of RRF, deletion of RF3, overproduction of tRNA^{Lys} and tmRNA) are integrated into a RelA^{-} strain (Singh & Varshney, 2004). Although additional Pth activities have not been identified in yeast, the fact that the Pth activity is essential for protein biosynthesis is a good indicator of the presence of additional activities in yeast (and other eukaryotes). Many of these activities may be the ones that are crucial in salvaging tRNAs from the peptidyl-tRNAs that accumulate in the cytosol. At least in rabbit, one of the phosphodiesterases cleaves between the C and A, the last two nucleotides in the tRNA part of the peptidyl-tRNA (Gross *et al.*, 1992). The released tRNA can be repaired before its reuse.

Concluding remarks

As summarized in Fig. 1, the suppressor analysis of Pth^{ts} strains has contributed significantly to our understanding of the mechanism of protein biosynthesis. The genetic

interactions of Pth with IF1, IF2, IF3, EFG, RelA, tmRNA, RF3 and RRF have revealed its crucial requirement to salvage tRNA from the peptidyl-tRNAs released during all steps of protein biosynthesis. Notably, among all the factors known to influence peptidyl-tRNA drop-off, only tmRNA decreases the peptidyl-tRNA load in the cell; all others lead to an increase. The peptidyl-tRNAs so dropped are channelled through a single pathway of Pth-mediated recycling, highlighting the critical role of Pth in protein biosynthesis. Positive effects of GroESL and $tRNA^{Lys}$ are mediated directly by increasing the levels of Pth. However, an issue that has remained unclear relates to the size of the peptidic moieties in the dropped-off peptidyl-tRNAs. The sizes of these are small (the majority of them with peptides of up to seven amino acids) in the population arising from 'abortive initiation' (Heurgue-Hamard *et al.*, 2000). Such peptidyl-tRNAs may well be the most predominant form of the peptidyl-tRNAs that accumulate in the cell. However, the peptidyl-tRNAs that are dropped off the translating ribosomes, most likely to maintain accuracy during translation (Menninger, 1976), could be expected to contain peptidic moieties that have already emerged out of the peptide tunnel of the 50S subunits. Additionally, genetic evidence also suggests dropping-off of longer peptidyl-tRNAs from the ribosomes (Menez *et al.*, 2002b). How are these peptidyl-tRNAs dropped off and recycled? One untested possibility is that after disassembly of the stalled ribosomes, these peptidyl-tRNAs remain anchored to the 50S subunit. The tRNAs may then be released by the action of free or the 30S-subunit-bound (Kössel, 1970) Pth.

Finally, a wide variety of potentially lethal infectious diseases, including tuberculosis, bacterial pneumonia, childhood meningitis, infections of wounds and burns, syphilis and gonorrhoea, are caused by eubacteria. Considering the alarming rise in the incidence of bacterial resistance to known antibiotics, and bearing in mind that eubacteria possess a single Pth whereas eukaryotes possess multiple forms (Pth1, Pth2 and more), Pth offers an important potential target for developing new drugs to control eubacterial infections.

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