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Georges Lahoud
Thomas Jefferson University

Ya-Ming Hou
Thomas Jefferson University, ya-ming.hou@jefferson.edu

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Georges Lahoud and Ya-Ming Hou

Thomas Jefferson University, Department of Biochemistry and Molecular Biology, Philadelphia, Pennsylvania 19107, USA. E-mail: ya-ming.hou@jefferson.edu

Aminoacylation of tRNA is the cellular process to provide aminoacyl donors for the ribosome synthesis of polypeptides. New research highlights an unexpected structural overlap between enzymes involved in this process and those involved in the biosynthesis of cyclodipeptides, an important class of bioactive molecules.

The canonical role of tRNA in ribosomal protein synthesis begins with aminoacylation catalyzed by aminoacyl-tRNA synthetases (aaRSs). Each aaRS activates its cognate amino acid with ATP and esterifies the intermediate aminoacyl-AMP (aa-AMP) to the 3' terminal ribose of tRNA to

form the product aminoacyl-tRNA (aa-tRNA), which enters the ribosome according to the tRNA anticodon. Although the ribosome machinery is highly efficient, thanks to protein factors that ensure rapid capture of aa-tRNA to support protein synthesis, recent work has shown that some aa-tRNAs can escape capture and instead participate in peptide synthesis outside the ribosome. For example, cyclodipeptide synthases (CDPSs) hijack aa-tRNAs to make cyclodipeptides[1] (**Fig.1a**), but how they accomplish this task was unknown. One page xx of this issue[2], Blanchard and colleagues report the crystal structure of one of these enzymes, revealing a surprising homology to aaRSs with interesting new insights into the mechanism of making cyclodipeptides from aa-tRNAs.

Cyclodipeptides and their derivatives are important diketopiperazine compounds with diverse biodefense activities; hence an understanding of their biosynthesis is expected to impact human health. While most cyclodipeptides are synthesized by the large non-ribosome peptide synthetases in microorganisms, albonoursin biosynthesis relies on a small enzyme, the AlbC protein of *Streptomyces noursei*, to synthesize the cyclodipeptide precursor cyclo(L-Phe-L-Leu) (cFL)[3]. The AlbC activity is unorthodox[1]; it does not use free L-phenylalanine and L-leucine as substrates, but instead requires the pre-charged Phe-tRNA^{Phe} and Leu-tRNA^{Leu}. This unusual activity explains the lack of an ATP-binding motif in AlbC and relates it to proteins of two other families that use aa-tRNAs to make peptides: aa-tRNA protein transferases[4] and aminoacyl transferases[5]. An *in silico* search has identified several AlbC homologues[1], with subsequent biochemical experiments confirming their CDPS activity[1]. One of these is Rv2275 from *Mycobacterium tuberculosis*, which uses two molecules of Tyr-tRNA^{Tyr} to synthesize cyclo(L-Tyr-L-Tyr) (cYY) as an intermediate in mycocyclosin biosynthesis.

Although a recent protein sequence profile analysis has suggested that CDPSs may be related to the aa-tRNA-synthesizing aaRSs[6], the low homology between the two families of enzymes

precluded any speculation as to the structural features of CDPSs. Thus, the crystal structure of Rv2275 provided by Blanchard and colleagues is a surprise: the protein is structurally most closely related to TyrRS and TrpRS, the class Ic members of aaRSs. The aaRSs have been divided into two major classes according to their ATP-binding motifs: class I enzymes use the common protein fold known as the Rossmann fold to bind ATP for generating the intermediate aa-AMP and converting it to aa-tRNA, whereas class II enzymes bind ATP in an antiparallel core of β-strands. Unlike the monomeric structure of all other class I enzymes, class Ic enzymes are distinguished by a dimeric structure with the two active sites interdigitated at the dimer interface. Rv2275 is also a dimer, possessing similarly arranged active sites while containing notable structural rearrangements that would block ATP binding (**Fig.1b**). Importantly, of the many conserved CDPS-signature residues that cluster near a putative amino acid-binding pocket of Rv2275 is Ser88, whose spatial position is a determinant of the ATP-independent active site and is relevant to catalysis. Indeed, the alanine substitution of Ser88 completely abolishes the enzyme activity and prevents the formation of a crucial reaction intermediate. This intermediate, when formed In the wild-type Rv2275 after incubation with [^{14}C]-Tyr-tRNA^{Tyr} and separation of the tRNA, contains the radiolabel and is stable enough to survive even in denaturing condition with 5 M urea, suggesting that it is a tyrosinylated covalent enzyme complex. These observations form the basis for a proposed mechanism of Rv2275 that may be generalizable to other CDPSs.

In the proposed mechanism (**Fig.1a**), the Ser88 hydroxyl performs nucleophilic attack on the carbonyl ester of the first aa_1-$tRNA_1$, leading to the formation of the covalent enzyme-aa_1 intermediate and the release of the deacylated $tRNA_1$. Structural data suggest that the active site should rearrange to accommodate the entry of a second aa_2-$tRNA_2$, upon which the α-amino group of the tRNA-bound aa_2 would attack the enzyme-bound aa_1 to generate the tRNA-

bound dipeptide aa_1-aa_2. Following a second nucleophilic attack by the α-amino group of aa_1 on the carbonyl ester of aa_2 is the formation of the product cyclodipeptide and the release of $tRNA_2$. Mutational analysis implicates the involvement of a general base in the active site (the CDPS-signature residue E233 of Rv2275) that would accelerate proton abstraction of the α-amino group of aa_2-$tRNA_2$ to promote nucleophilic attack and would also assist in the protonation of the deacylated $tRNA_2$ to facilitate product release.

The proposed mechanism highlights two remarkable features of making cyclodipeptides on tRNA. One is the sequential use of aa-tRNAs as the donors of already activated amino acids, thus affording CDPSs with a small structure (without the amino-acid activation domain) to catalyze a complex reaction. The second is the covalent trapping of the first amino acid on CDPSs while arranging the second aa-tRNA for the chemistry step. This covalent strategy may be necessary to compete for the cellular pools of aa-tRNAs, which after synthesis on aaRSs are typically captured by elongation factor EF-Tu for rapid delivery to the ribosome. However, even with the first amino acid stabilized, the competition for the second aa-tRNA is formidably more challenging because the covalent mechanism is no longer invoked. Intriguingly, their structural homology to class Ic aaRSs suggests that CDPSs should bind aa-tRNA favorably, because class I aaRSs generally exhibit preferentially high affinities to the aa-tRNA product[7], and because the dimeric Ic structure has been suggested to serve as a barrier to prevent direct release of aa-tRNA to EF-Tu[7]. These considerations provide a useful framework to appreciate the unexpected structural homology.

The current structure should be a springboard to jump start additional structural work on the CDPS enzymes, preferably in complexes with bound substrates. Such structures will help to address unresolved questions, pertaining to their selectivity of aa-tRNA, the determinants for the

sequential entry of aa-tRNA, and the operational relationship of the two active sites. Together with more detailed biochemical analysis, new insights are likely to emerge enabling the design of novel cyclodipeptides for medical purposes.

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Legend to Figure 1:

(a) Peptide synthesis from aminoacyl-tRNAs. Aminoacylation of a tRNA by an aaRS (blue), using an amino acid (aa) and ATP as substrates, generates an aa-tRNA that is typically released to GTP-bound EF-Tu (purple) and delivered to the ribosome (grey) for synthesis of a new peptide bond on a nascent polypeptide. However, some aa-tRNAs are hijacked by CDPSs (green) to make cyclodipeptides, using a covalent trapping strategy that fixes the aa from the first aa-tRNA (red) on the enzyme while making a dipeptide with the second aa-tRNA (metallic).

(b) Structural homology of Rv2275 and TyrRS. The active site of Rv2275 contains a putative amino acid-binding site (aa site) structurally homologous with the aa site of the class Ic TyrRS, but features a “closed” loop conformation (blue) that would block ATP binding as opposed to the

“open” loop conformation of the ATP site in TyrRS. Drawn with PyMol based on coordinates of Rv2275 (PDB 2X9Q) and TyrRS (PDB 1VBM).