**MS03-1-9 Structural and functional studies of cyclodipeptide synthases with RNA microhelices mimicking their tRNA susbtrates**
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**Abstract**
Cyclodipeptide synthases (CDPS) divert aminoacyl-tRNAs to produce cyclodipeptides and complex derivatives, diketopiperazines, which constitute a broad class of natural products synthesized by microorganisms and possessing pharmacological properties. Most CDPS have a relaxed specificity and often produce several cyclopeptides. This hinders the identification of specificity determinants. To overcome this problem, we selected Nbra-CDPS from Nocardia Brasiliensis. Nbra-CDPS has the advantage of using different substrates, Ala and Glu tRNA that target the first and second CDPS pocket respectively, thus, synthesizing cyclo-Ala-Glu (cAE) as the main product. A recent study of Gondry's team (Canu et al, 2020) shows that CDPS interact mainly with the acceptor arms of tRNAs (also termed miHx). A major objective of our study is to identify the CDPS amino acid residues responsible for substrate specificity. The substitution of these residues will allow to generate enzymes that can use non canonical amino acids. Ultimately, the intent of the project is to produce by an ecological biosynthesis process, various diketopiperazines with high therapeutic potential.
We aim in this study to solve by X-ray crystallography the high resolution structure of the complex between Nbra-CDPS and miHx and to prove the interest of using miHx as mimick of tRNA substrates for functional and structural studies. We determined twelve crystal structures of Nbra-CDPS crystallized in presence of acylated and nonacylated-miHx. Data analysis showed the presence of the enzyme alone, without MiHx. We have identified some obstacles that may hinder the study of the complex. In particular, the substrate Ala-miHxAla is not stable and deacylates significantly. Thus, we recently developed the use of more stable analogues of the substrate through amide bond formation (miHx-NHAla) for structural and biophysical studies. Functional studies are in progress to study new small molecules binders of Nbra-CDPS.
To explore the interaction of Nbra-CDPS with the tRNA substrate we use a catalytic-dead mutant by introducing a S34A mutation that blocks the first substrate at the pocket. Recently we produced another variant of the protein without the C-terminal his-tag to avoid any interference with interaction area access nearby. We measured the affinity of these CDPS variants for miHx by BioLayer Interferometry and EMSA. We deduced a KD in the nanomolar range for miHxAla by both approaches.
NMR studies were performed on isotopically labelled Nbra-CDPS and highlighted its interaction with a non-acylated miHxAla by 15N HSQC spectra. The preliminary NMR assignment provided first molecular information of residues involved in the recognition with MiHx.
In addition, new strategies are being deployed to further characterize the interaction. Firstly, synthesis of miHx with functional groups allowing to specifically cross-link the miHx with the CDPS pocket are in progress. Secondly, we initiated a study to analyse by cryoEM the 3D structure of a dimeric form of Nbra-CDPS in complex with two Ala-tRNA (MW about 100kDa). Preliminary negative staining images of this complex have been collected.
In conclusion, the combination of all these approaches will allow us to decipher the molecular mechanism of recognition between CDPS and their substrates and thus guide the engineering of these enzymes.

**References**
Nicolas Canu, Carine Tellier, Morgan Babin, Robert Thai, Inès Ajel, et al.. Flexizyme-aminoacylated shortened tRNAs demonstrate that only the aminoacylated acceptor arms of the two tRNA substrates are required for cyclodipeptide synthase activity. Nucleic Acids Research, 2020, 48 (20)