From functional study of tRNA CCA-adding enzymes toward their characterization by serial X-ray crystallography

My PhD work was carried out under the supervision of Dr. Claude Sauter (ARN – IBMC – CNRS, University of Strasbourg) and Prof. Dr. Mario Mörl (Biochemistry and Molecular Biology, University of Leipzig) in the context of a cotutelle program supported by the French-German University. Its main focus was the application of novel X-ray crystallography approaches to the study of specific class of polymerases, called *CCA-adding enzymes*, catalyzing the addition of the 3' CCA tail of transfer RNAs (tRNAs).

The last decade has seen important technology developments in the field of structural biology, especially in X-ray crystallography. The introduction of linear electron accelerators called X-ray free electron lasers (XFEL) represents a real revolution to explore enzymatic catalysis at the atomic scale. Based on serial time-resolved X-ray crystallography, this system is used to follow and monitor chemical and biological reactions happening from the femto to the micro-second scale (Chapman et al., 2011) . Under the impulse of this technology, new generation synchrotrons started to develop biocrystallography beamlines dedicated to time-resolved experiments such as T-REXX beamline at DESY synchrotron (Hamburg) (Pearson & Mehrabi, 2020).

This PhD project aims to develop a reproducible pipeline for sample preparation and characterization suitable for time-resolved serial crystallography experiments. Our focus is put on tRNA nucleotidyl transferases or CCA-adding enzymes which catalyze the sequential addition of the CCA trinucleotide at the 3' position of tRNAs. This reaction is crucial, as the CCA end is the universal site of amino acid binding to tRNAs which then transport them to the ribosome. These nucleotidyltransferases distinguish from all the other polymerases as they do not require any nucleotidic template to fulfill their action (Augustin et al., 2003) .

In order to explore and obtain snapshots of the different steps of the CCA addition, we chose a set of three different class II enzymes. Our first candidate consisted of the CCA-adding enzyme from a psychrophilic bacteria *Planococcus halocryophilus* (PhaCCA) living at high salt concentration and negative temperatures in the arctic permafrost (Mykytczuk et al., 2013) . The second was identified in the mosquito roundworm parasite *Romanomermis culicivorax* (RcuCCA), the organism presenting the smallest functional tRNAs ever observed (from 42 to 46 nucleotides) (Jühling et al., 2018) . Finally our last candidate corresponded to a potential last common ancestor (AncCCA) of gammaproteobacteria group enzyme designed by my team in Leipzig.

To begin with, we needed to determine the biochemical properties of selected enzymes. As the study was already done for PhaCCA as well as AncCCA, I could take part in the investigation of RcuCCA which is able to process minimal tRNAs lacking both T- and D-arm. While conventional

tRNA:nucleotidyl transferase complexes show an interaction and anchoring on tRNA T-arm (Weiner, 2004) , the main question concerned elements of RcuCCA giving the specificity for such non-standard substrates. The study included the human (HsaCCA) and *Escherichia coli* (EcoCCA) as control enzymes. First, my host team in Leipzig established that RcuCCA is capable to bind and add full CCA end to canonical tRNA as well as HsaCCA and EcoCCA. However, only RcuCCA could process miniaturized tRNAs from *R. culicivorax*. Then a set of chimeras were designed by injecting into HsaCCA fragments from RcuCCA supposed to be involved into the tRNA binding. Thus we analyzed the affinity of this set of chimeric enzymes for small tRNAs by electrophoretic mobility shift assay (EMSA). Combining biochemical results and a computational model RcuCCA, we highlighted the important role of two lysines from the catalytic core in the small tRNA binding stability and correct orientation of 3’ primer into the catalytic site. All results described above were published in International Journal of Molecular Sciences (Hennig et al., 2020).

Once biochemical characteristics of an enzyme are established, crystallization experiments can start. On the one hand, we were trying to grow large crystals suitable for “traditional” synchrotron analysis. On the other hand, the objective was to prepare microcrystalline samples dedicated to new generation of serial X-ray crystallography experiments. In this exploration, we combined conventional methods, meaning vapor diffusion in microplates, microseeding and batch, with more advanced methods described in this thesis. One of these techniques used a microfluidic device, called ChipX, developed by my laboratory in Strasbourg. Designed for counter-diffusion experiments, ChipX allows to screen eight different crystallants in a wide range of concentration and of supersaturation states. Furthermore, ChipX can be directly used at room temperature on standard synchrotron setups. Based on the concept of serial crystallography, we collected several datasets on a selection of crystals and combined data to reconstruct the macromolecule structure. The final version of ChipX was presented in IUCr Journal where the structure of psychrophilic PhaCCA was solved in its apo form as well as with non-hydrolizable CMPcPP (de Wijn et al., 2019). In addition we elaborated a user-friendly protocol for any crystal grower who is not familiar with such devices. Requiring few microliters of macromolecule solution and basic laboratory equipment, we showed in a video published in Journal of Visualized Experiments, how to prepare a ChipX in few minutes and use it for data collection at standard synchrotron beamline (de Wijn et al., 2021) .

For decades, a main bottleneck and time-consuming step in crystallization was to determine the exact phase diagram of a macromolecule without having full control on the equilibration process occurring in the crystallization drop. With an increasing need for micro- or nanocrystals used at synchrotrons and XFELs our lab in collaboration with Xtal concept company (Hamburg) and team of Pr. Dr. Christian Betzel, acquired the Xtal Controller (XC900) technology allowing to (1) navigate into the phase diagram with controlled crystallant and water injection, (2) detect and monitor first

nucleation events in a crystallization drop thanks to the integrated dynamic light scattering (DLS) module, (3) visualize growing crystals into the experimental drop with a microscope coupled with a CCD camera. Using PhaCCA and lysozyme from hen egg white combined with Tb-Xo4 nucleant as models, we showed that we were able to (1) track efficiently early nucleii formation, (2) control the production of well diffracting crystals by playing on crystallization/dissolution cycles, (3) establish reproducible conditions to obtain stable nano- to micro-crystalline samples suitable for time-resolved experiments. All these results were gathered and published in the journal Crystals in 2020 (de Wijn et al., 2020).

It is important to mention the important amount of time spent on the optimization of preparation and purification of the three enzymes chosen for the project, as well as *in vitro* transcribed tRNAs, in Leipzig and in Strasbourg. Furthermore, all crystallization methods available in the Strasbourg team have been used in crystallization attempts. A special focus has been put on AncCCA which initially formed crystals badly diffracting around 6 to 7 Å at PX2A beamline (Soleil synchrotron, Saint-Aubin). For this project, we could have prior access as first users to a plate-screener system recently developed on PROXIMA2A beamline (Synchrotron SOLEIL, Saint-Aubin) for *in situ* serial experiments at room temperature (Jeangerard et al., 2018). This new system helps to quickly identify conditions leading to well-diffracting crystals without the need of direct crystal handling, mounting and flash-cooling. Despite all efforts, we have not obtained diffraction under 6 Å resolution so far, but few improvements are still running.

The last step of our pipeline is complementary to time-resolved experiments. It intends to determine "frozen?" structures of selected enzymes in order to better understand (1) the overall architecture of the protein; (2) identify atomic scale properties of the enzyme; (3) serve as a model for molecular replacement in time-resolved experiments. As we could solve five crystal structures of PhaCCA at high resolution (up to 1.8 Å) in its apo form as well as in complex with CTP, we combined this information with. Results from biochemical investigations by the Leipzig team To better understand the underlying cold-adaptation mechanisms of this enzyme. The main trend for cold adaptation stipulates that an increased flexibility should confer the enzyme the capacity to fulfill its role at low temperature. This assessment is compatible with the decreased fidelity activity proven for PhaCCA (Ernst et al., 2018). To pin down the exact structural features of this protein, we compared it to the closely-related analog coming from the thermophilic *Geobacillus stearothermophilus* (GstCCA). As expected our results showed an overall increased flexibility of PhaCCA, mostly affecting the non-conserved C-terminal side where a reduced amount (14%) of secondary structures was observed. In addition, the N-terminal motif C, acting as a spring element for head and neck domain reorientation during nucleotide switching, loses a helix capping element normally observed on helix α5. Taken together our high-resolution structures pinpointed adaptation strategies used by

RNA polymerases in extreme cold conditions. Our results were published in Computational and Structural Biotechnology Journal (de Wijn et al., 2021).

To conclude, this PhD work applied an ensemble tools and strategies available in crystallogenesis to characterize the structure and function of three class II CCA-adding enzymes by X-ray crystallography. Improvements still need to be done especially in the case of AncCCA and RcuCCA. However, our promising results should soon lead us to the step further, namely applying new serial crystallography protocols thanks to synchrotron and XFEL radiations to create a molecular movie of the 3'CCA addition in real-time and document with great atomic details the action of these fascinating polymerases.