Friday, March 21, Session III

L9

CAN SEQUENCE-, STRUCTURE- AND SUGAR-NONSPECIFIC NUCLEASES BE HARNESSED?

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Fungi, trypanosomatids, plants and some pathogenic bacteria code for 3'-nucleases/nucleotidases which belong to the S1-P1 nuclease family [1]. While for example in *Leishmania amazonensis* its membrane-attached 3'-nucleotidase is suspected to be a virulence factor, in plants they have a role in growth and cell death. The role of bacterial enzymes from this family is not clear.

S1-P1 nucleases are metalloenzymes of MW ~25-40 kDa, typically zinc-dependent, relying on a cluster of three divalent metals in a surface cavity of an almost fully -helical fold. The cluster, binding the phosphate group after the cleaved bond, is accompanied by the nucleobase-binding site 1 which is responsible for stabilization of the –1 nucleotide with respect to the cleaved O3'-P3' bond. These enzymes can act as endonucleases and nucleotidases. They cleave DNA, RNA, single strands, double strands, viroids, some modified nucleotides, oligonucleotides and genomic DNA, substrates which are structure or unstructured, without any significant sequence preference. These properties make them ideal candidates for biotechnological applications [1].

Interestingly, even if their fold basically does not change across the biological species, their activity profiles can differ dramatically, in some cases being basically a ss DNase with negligible activity towards double strands, in some cases more of an RNase, and for example in plants showing comparable activity towards all types of nucleic acids. Some eukaryotic representatives (tomato) require glycosylation to maintain the enzyme stable, while the bacterial versions with very close structure are stable at high concentrations without such modifications.

We have studied S1-P1 nucleases from plants, fungus, and two bacterium species [2-5]. Crystallographic studies, mutagenesis, numerous product/ligand complexes helped us better understand the structure-function questions, such as active site remodelling, sensitivity to metal replacement, key mobility elements in the active site and more. Recently, for the first time we have identified a "supernuclease" capable of previously unseen rates for this enzyme class, uncovered the key region for RNA/DNA preference, which opens door to "tailor-made" optimization, and discovered its high activity towards cyclic-di-GMP, the bacterial second messenger [6]. The crystallization properties of some representatives enable studies at high resolutions, making it possible for us to start asking questions about protonation states of enzyme and its substrate upon encounter.

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This work was supported by the Czech Science Foundation (25-17546S), and by the Czech Academy of Sciences (86652036). CIISB, Instruct-CZ Centre of Instruct-ERIC EU consortium, funded by MEYS CR infrastructure project LM2023042 and European Regional Development Fund-Project No. CZ.02.01.01/00/23_015/0008175 is acknowledged for providing access to all facilities at CMS in BIOCEV for this project.

L10

OLIGOMERIZATION AS A REGULATORY MECHANISM IN PLANT ADENOSINE KINASE ACTIVITY

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Adenosine kinase (ADK) catalyses ATP-dependent phosphorylation of adenosine and cytokinin ribosides in plants. Ado is primarily synthesized through the activity of S-adenosyl homocysteine (SAH) hydrolase [1] and purine nucleoside phosphorylase (PNP) catalysing Ado ribosylation [2]. Since SAH hydrolase participates in the S-adenosyl methionine (SAM) cycle, the accumulation of Ado can lead to its feedback inhibition, thereby disrupting the cycle and impairing SAM-dependent transmethylation. To prevent this, ADK role in removing Ado is pivotal [3].

We examined the substrate preferences, oligomeric states, and structures of ADKs from moss (*Physcomitrium patens*) and maize (*Zea mays*), complemented by metabolomic and phenotypic analyses. Unlike monomeric human and protozoal ADKs, maize and moss enzymes formed dimers at higher concentrations. Structural and kinetic studies revealed an inactive dimer with active sites blocked by the other subunit. The moss ADKs, with a higher dimerization propensity, showed tenfold lower activity than maize ADKs. Monomeric structures in a ternary complex captured the open-to-closed state transition upon substrate binding. Our findings suggest that oligomerization modulates plant ADK activity, with dimerization serving as a negative feedback mechanism to regulate adenosine and AMP levels [4]. The regulation is even more complex as the plant ADKs form heterocomplexes with SNF1/SnRK1/AMPK [5]. ADK helps to maintain SnRK1 activity which turns off energy-consuming biosynthetic pathways as well as turns on ATP-generating reactions upon various stresses [5, 6].

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We acknowledge SOLEIL for providing synchrotron radiation facilities in using PROXIMA 1 and 2 beamlines (proposals ID 20170872, 20191181, and 20210831). We would like to thank Mgr. Anna Krnáčová for HPLC-MS analysis and Pharm.Dr. Jitka Široká, Ph.D. for measurement of HRMS data. We acknowledge CF Biomolecular Interactions and Crystallography of CIISB, Instruct-CZ Centre, for the assistance of AUC measurements, supported by MEYS CR (LM2023042) and European Regional Development Fund-Project "UP CIISB" (No. CZ.02.1.01/ 0.0/ 0.0/ 18_046/0015974). The work was supported by GA21-07661S from the Czech Science Foundation, IGA_PrF_ 2023_012 from Palacký University, and ANR-11-IDEX -0003. This work benefited from the I2BC crystallization platform supported by FRISBI ANR-10-INSB-05-01.

L11

SHEDDING LIGHT ON THE SECRETS OF NanoLuc, ITS MECHANISM, AND ALLOSTERIC BEHAVIOUR

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NanoLuc luciferase, a small enzyme renowned for its exceptionally bright bioluminescence, has found widespread applications in biotechnology and biomedicine since being designed in 2012 [1]. However, the mystery behind NanoLuc's light-emitting reaction, crucial for developing next-generation bioluminescent systems, remained unsolved. In this study, we made significant progress in understanding NanoLuc's mechanism by combining various laboratory and computational techniques, including crystallography, kinetic measurements, molecular docking, and molecular dynamics simulations with enhanced sampling.

One of the most intriguing features of NanoLuc is its small size, consisting of just 171 amino acid residues. This is a stark contrast to luciferases from sea pansy *Renilla reniformis* (311 residues) and firefly *Photinus pyralis* (550 residues). We confirmed that NanoLuc is monomeric in solution but can also crystallize as a homotetramer under certain conditions. We have also identified two substrate binding sites (Fig. 1): the catalytic site inside NanoLuc monomer, and an allosteric binding site on the oligomerization interface of NanoLuc crystals [2]. Moreover, we used adaptive sampling and adaptive steered molecular dynamics (ASMD) simulations to study the ligand behavior with the NanoLuc monomer and dimer. Importantly, we have demonstrated that introducing mutations in the allosteric site can enhance the bioluminescent reaction occurring in the active site.

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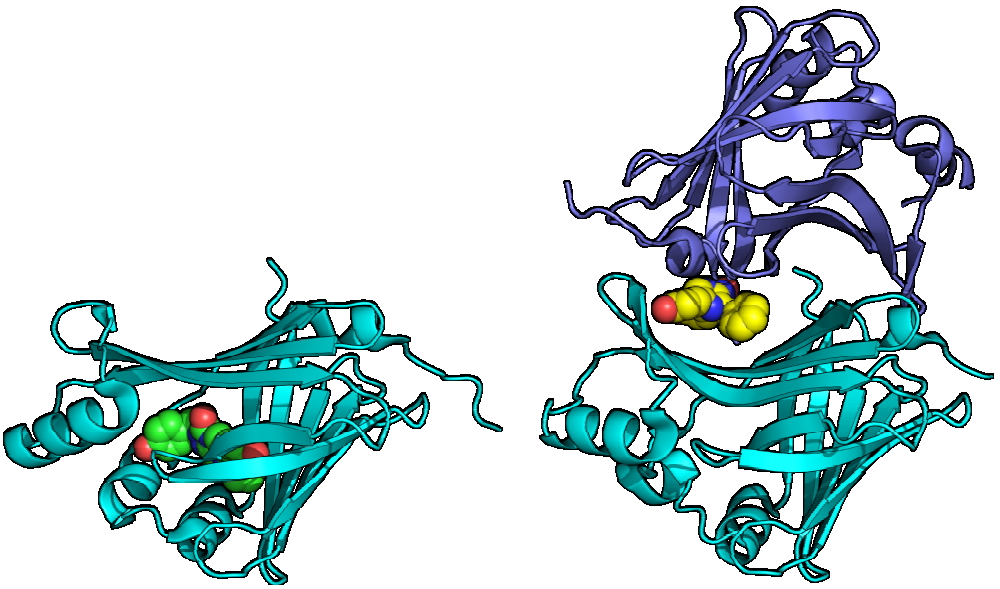

Figure 1. Two distinct substrate binding sites of NanoLuc – the catalytic site (left) and the allosteric site (right).

L12

CDK2-BASED CDK7 MIMIC AS A TOOL FOR STRUCTURAL ANALYSIS: BIOCHEMICAL VALIDATION AND CRYSTAL STRUCTURE WITH SY5609

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Structure-based drug design on CDK7 has not been extensively used due to the lack of a well-established platform for this kinase. CDK7 requires expression in insect cells [1,2], and until recently, there have been only few X-ray structures of CDK7 in the PDB – relatively low-resolution monomeric CDK7 in complex with ATP [1] and with inhibitor LDC4297 (PDB 8p4z). Only very recently, high-resolution cryo-EM structures have been determined for CDK7/cyclin H/MAT 1 complexes with ATP analogue and number of inhibitors including THZ1, samuraciclib and dinaciclib.

In this work, we present CDK2m7 – a CDK2-based CDK7 mimic that can be expressed in *E. coli* in a fully active form. Similar approach has been successfully used for several other kinases, including CDK4 [3]. We identified and mutated 12 CDK residues involved in contacts with ligands to mimic the sequence of CDK7 (Figure 1). CDK2m7 was expressed in *E. coli* in the Thr160-phosphorylated form and co-purified with a fragment of cyclin A2 (cycA, residues 175–432), separately expressed in *E. coli.*

To confirm the suitability of the introduced mutations in CDK2m7, inhibitor binding was analysed using a panel of CDK inhibitors with varying selectivity. Potent CDK7 inhibitors showed no inhibition of CDK2, whereas CDK2m7 was strongly inhibited in nanomolar concentrations. We further determined the crystal structure of active

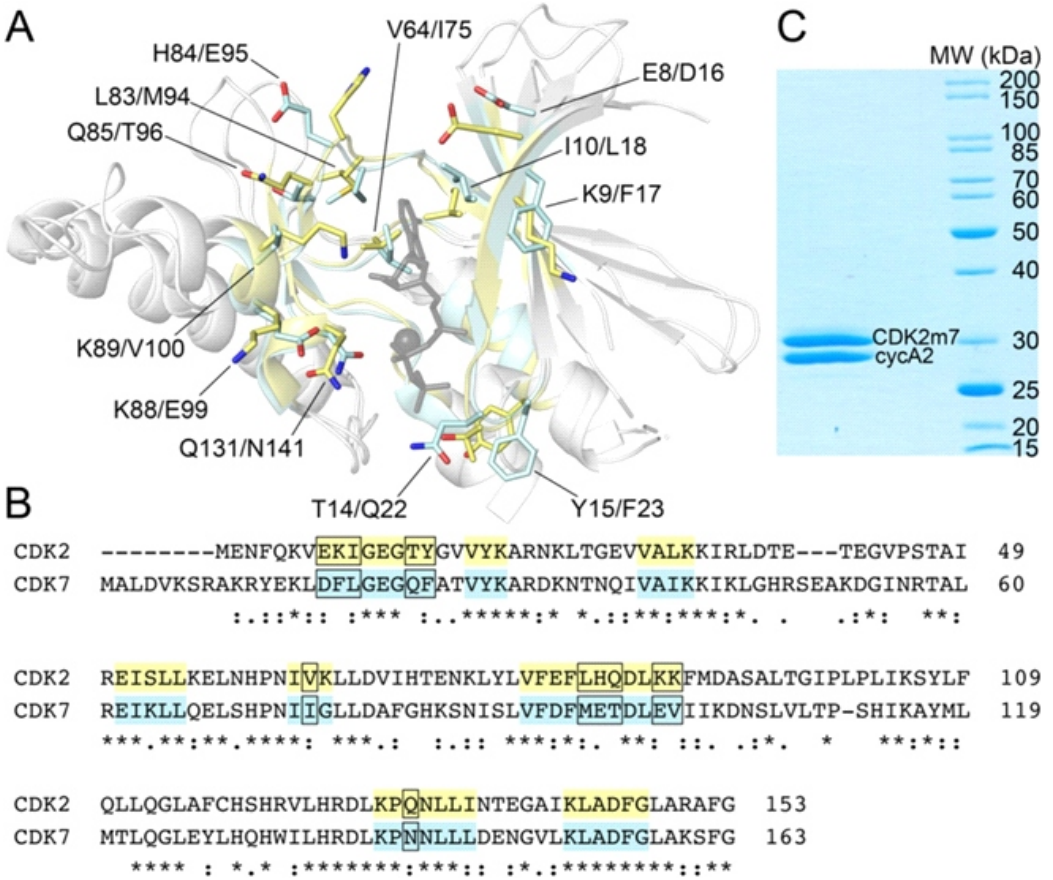

Figure 1. Design of CDK2m7. Structural (A) and sequence (B) alignment of CDK2 (PDB 8fp5) and CDK7 (PDB 1ua2). Regions are shown in yellow and cyan in CDK2 and CDK7, respectively. Mutated residues are shown as sticks (A) and highlighted by boxes (B). (C) Coomassie-stained SDS-PAGE gel documenting the purity of the CDK2m7/cyclin A2 (cycA2) complex.

CDK2m7/cycA in complex with inhibitor SY5609 [4] at the resolution of 2.2 Å. It was clear that CDK2m7 maintains the integrity of the active site, documented by similar conformation of the side-chains of the mutated residues as in the structures of the CDK7/cycH/MAT1. Moreover, binding of the inhibitor corresponded well with the previously performed molecular docking of SY5609 into the active CDK7 complex (PDB 7b5q) [4]. In conclusion, CDK2m7 mimics well the CDK7, could be produced in *E. coli* in a fully active, phosphorylated form in complex with cyclin A2. CDK2m7 could be used in biochemical measurements as well as in structure-assisted design of CDK7 inhibitors.

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We would like to thank Dr. Petr Pachl from IOCB Prague for diffraction data collection and Dr. Martin Lepšík of High Performance Computing Core Facility of IOCB Prague for advice on inhibitor geometry and binding. We acknowledge SOLEIL for provision of synchrotron radiation facilities and we would like to thank Dr. Martin Savko for assistance in using beamline PX2a. The work was supported by project National Institute for Cancer Research (Programme EXCELES, ID Project No. LX22NPO5102) - Funded by the European Union - Next Generation EU.

L13

CRYSTALLIZING THE FUTURE: UNVEILING NEW INNOVATIONS AT MOLECULAR DIMENSIONS

Paul Driver

Molecular Dimensions

Molecular Dimensions has long been a leader in providing comprehensive workflow solutions that empower structural biologists to overcome challenges in protein crystallization. In this presentation, the speaker will offer an in-depth update on the company's latest commercial innovations. First, a novel ultrafiltration concentrator will be introduced—a breakthrough product that eliminates the need for a centrifuge, delivers gentle concentration of recombinant proteins, and achieves exceptionally high recovery rates. In addition, the talk will spotlight a new lipid screen developed to evaluate the optimal lipids for membrane protein workflows, a vital step in enhancing the stability and function of these challenging targets. Furthermore, an updated suite of crystallisation screens will be presented, each designed to streamline the process of protein structure determination. By detailing these advancements, the presentation underscores Molecular Dimensions' commitment to innovation and its ongoing role in driving the evolution of structural biology research.