Friday, September 23, Session III

L12

INTEGRATIVE STRUCTURAL STUDY OF ANTIBIOTIC-INACTIVATING ENZYME FROM *STENOTROPHOMONAS MALTOPHILIA*

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An opportunistic bacterial pathogen *Stenotrophomonas maltophilia* causes a serious number of infections worldwide. This species possesses significant antibiotic resistance that has been further broadened owing to the ability to acquire antibiotic-resistance genes and mutations [1]. Based on a bioinformatic analysis of its sequenced genomes, we selected for our research a not yet experimentally characterised protein, sequentially related to antibiotic-inactivating enzymes.

The target protein was expressed in *Escherichia coli* strain Lemo21 (DE3) and subsequently purified using affinity and size-exclusion chromatography. Results from the spectrophotometric assay in the UV-VIS range indicated that the protein catalysed the enzymatic modification of antibiotics.

Moreover, the enzyme was crystallized and a dataset of diffraction images was collected at a synchrotron radiation source. The diffraction was strongly anisotropic: the proposed high-resolution cutoff, reported in *Aimless* [2] using the criterion of $CC_{1/2} > 0.30$, was in a range from 2.43 Å to 1.92 Å, depending on the direction in the reciprocal space. Hence, the anisotropic correction was carried out with *STARANISO* [3]. The phase problem was solved using molecular replacement in *MoRDa* [4], followed by refinement of the structure model in *REFMAC*5 [5]. Paired refinement on the anisotropic data was performed with *PAIREF* [6] to determine the diffraction limit of 1.95 Å.

The asymmetric unit contains two homodimers. Each dimer is associated through two disulfide bridges. Nonetheless, the further analysis of oligomerization, conducted with mass structural spectrometry and small-angle X-ray scattering, revealed that the enzyme is in a monomeric state in solution. The overall molecular structure is analogous to the family of tetracycline destructases [7] or the reductase from the biosynthesis pathway of abyssomicines [8]. However, the arrangement of the putative substrate-binding pocket differs significantly. The crystal structure provides the basis for a further *in silico*, *in vitro* or *in crystallo* investigation of the complexes with antibiotic substrates or potential inhibitors.

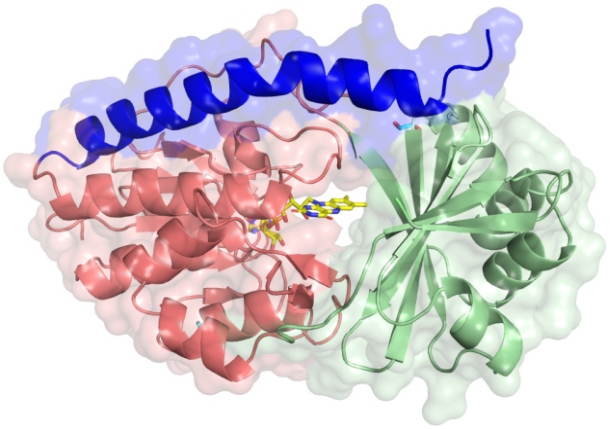

Figure 1. Crystal structure of a monomer of the antibiotic-inactivating enzyme from *Stenotrophomonas maltophilia*. The FAD-binding domain is coloured in red, the substrate-binding domain in green, and the C-terminal helix in blue. Flavin adenine dinucleotide (FAD) is displayed in stick representation (carbon atoms in yellow).

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L13

DEVELOPMENT OF MONOCLONAL ANTIBODIES AGAINST 3-NITROTYROSINE-ABETA SUSPECTED OF BEING ASSOCIATED WITH ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is one of the major medical challenges of our century, affecting 55 million patients worldwide [1]. This progressive, as yet incurable, neurodegenerative disorder, primarily affects people over the age of 60 years. In the brain of an AD patient, the formation and breakdown of amyloid beta protein (A) are out of balance, leading to increased aggregation of A to the point where normal cognitive functions are obstructed. This usually manifests with changes in memory, behaviour and ultimately interferes the control over the body functions [2].

Passive immunotherapy, that is directed against Amyloid beta, has so far proven to be the most promising therapy option. Accelerated approval of Aduhelm (Aducanumab), a monoclonal antibody targeting A [3], was recently granted by the U.S. FDA [4]. Showing undesirable side effects and minor effectiveness, the use of this drug is controversial [5]. One reason is presumably the physiological function of A . Therefore, the search continues for alternative approaches that address only the pathological aggregated A . Here, post-translationally modified Aâ forms are of great interest, since they are often specific found in deposited A .

The 3-nitrotyrosine modification at position 10 ($3NY^{10}$) of A is suspected to be involved in the pathogenic course of AD [6]. Two highly specific $3NY^{10}$-A antibodies are now generated, which can be used as tool for further investigation and beyond shall be developed into a drug to fight A deposits in AD patient's brain, while leaving the physiological A unaffected. In context of these work, the antibodies will be characterised by a variety of biochemical and kinetic analyses (ITC, ELISA, TEM, Aggregation studies). For a better understanding and evaluation of the interaction between the antibody and its target, the macromolecular structure shall furthermore be determined by X-ray crystallography.

So far, one of two antibodies was co-crystalised using the responding F_{ab}-fragment and the $3NY^{10}$-A target-peptide. After initially no crystals could be obtained by the sitting drop vapor diffusion method using commercial available random matrix screens at 96-well format, precipitate from one of the former screenings was used as a microseed. Suddenly, about 20 % of the tested commercial available crystallisation conditions were suitable for crystal growth. Hence obtained stretched hexagonal crystals have been tested for their diffraction pattern and the condition with the best crystal was selected for further optimisation. Due to change to hanging drop vapor diffusion in a 15-well format and variation of the seeding concentration, crystal size and thickness could be controlled. This slightly improved a problem of the crystal: different planes of the crystal are slightly staggered in their orientation, making it difficult to collect only one set of diffraction data at a time. Currently the diffraction data obtained are used in building and refinement of the structure.

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L14

STRUCTURAL STUDIES OF PURINE NUCLEOSIDE PHOSPHORYLASE INHIBITORS

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Purine nucleic acid metabolism is an essential process which occurs in almost all the cells. Synthesis can occur in two ways: from precursors in time and energy-consuming *de novo* pathway or through much preferred salvage pathway which recycles intermediates of purine degradation pathway [1].

Purine nucleoside phosphorylase (PNP) is an enzyme that hydrolyzes ribose from inosine and guanosine in presence of inorganic phosphate producing hypoxanthine and guanine which are further degraded to xanthine which can be recycled via salvage pathway. Activity of human PNP (hPNP) is increased in various pathologies, such as different types of cancer and autoimmune diseases and this makes PNP a target in drug discovery. Additionally, salvage pathway is essential for parasites, such as *Mycobacterium tuberculosis* (tuberculosis causing pathogen), where PNP (MtPNP) activity is important for transition from latent to active infection [2].

Both hPNP and MtPNP form homotrimers from monomers with molecular weight of 32 kDa and 28 kDa respectively. These enzymes share low sequence homology (35%), but overall fold and active site are conserved. Each subunit forms an active site in proximity of subunit-subunit interaction region, where all but one residue belong to the parent subunit. Active site can be divided into three regions based on the substrate moieties and inorganic phosphate binding positions.

Currently, there are several inhibitors that entered clinical trials in the last decade. Overall, they are composed from purine-moiety linked connected to a sugar-region-binding moiety. All of these inhibitors are characterized by low selectivity and specificity which leads to serious side-effects [2].

In this project we utilize X-ray crystallography to gain structural information for design of PNP inhibitors with high specificity and selectivity. Our compounds contain three moieties that occupy all three regions of the active site and their affinity and selectivity to hPNP and MtPNP is optimized through structure-assisted inhibitor design approach.

Both enzymes were prepared by heterologous expression in *E. coli* in high yields and purity required for crystallographic studies. Crystallization conditions were identified through wide screening and optimization and diffraction data were collected at synchrotron BESSY II, Helmholtz-Zentrum, Berlin. We solved and refined eight crystal structures to resolutions 1.6-2.6 Å, five structures of hPNP and three structures of MtbPNP.

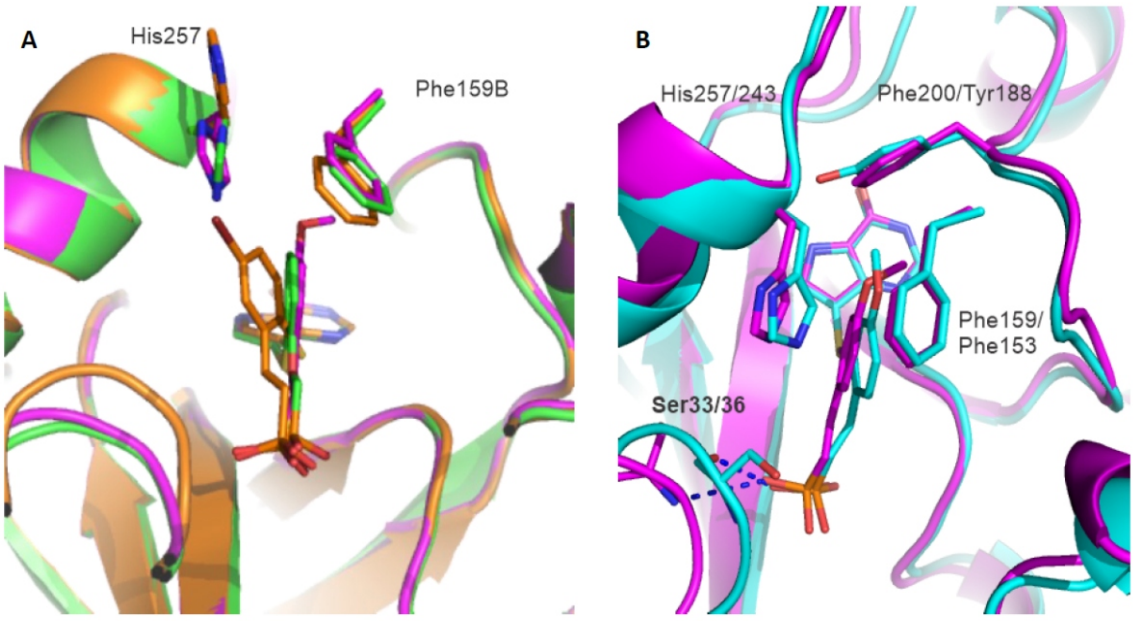

Figure 1: A. Overlay of three hPNP active sites with different inhibitors bound; B. Overlay of hPNP (magenta) and MtbPNP (cyan) with JS-375 bound to the active site.

Structure analysis showed that all the inhibitors bind to the active sites in the expected way: mimicking binding of substrate. Overall, modifications at different positions of central sugar-mimicking moiety increase inhibitor affinity towards MtbPNP while decreasing the affinity towards hPNP. These changes can be explained by differences in flexibility of the active sites that allows MtbPNP to accommodate these modifications.

This structural information is crucial for in understanding structure-activity relationship and can further be used to modify this class of compounds.

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L15

STRUCTURE- AND ACTIVITY-GUIDED DRUG DESIGN OF 7-DEAZAPURINE CYCLIC DINUCLEOTIDE ANALOGUES ACTIVATING STIMULATOR OF INTERFERON GENES (STING) RECEPTOR

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The cyclic GMP-AMP synthase - Stimulator of Interferon Genes (cGAS-STING) immune pathway is pivotal in detecting pathogen-associated (PAMPs) and damage-associated molecular patterns (DAMPs) [1]. Cyclic dinucleotides (CDNs) serve as second messengers transducing signals between cGAS and STING, if dsDNA is detected in the cytosol by cGAS [2]. Activation of STING by CDNS leads to downstream signal transduction resulting in induction of expression of proinflammatory cytokines (TNF- , IL-1) and the type I interferons (IFN- , IFN-) [3]. The cGAS-STING pathway thus has a crucial role in defence against pathogen infection, immune surveillance of tumour cells, and maintaining the normal immune functions of the body [4].

In this work, we describe the enzymatic and/or chemical synthesis of CDNs with 7-deazapurine modifications, with which we were able to introduce bulky substitutions while preserving the activity of modified CDNs when compared to unmodified. For the preparation of modified CDNs, we utilized mouse cyclic GMP-AMP synthase (cGAS) and bacterial dinucleotide synthases from *Vibrio cholerae* (DncV) and *Bacillus thuringiensis* (DisA). Moreover, the Suzuki-Myiaura cross-coupling reaction of 7-iodinated 7-deazapurine CDNs with aryl and heteroaryl boronic acids was used to introduce substituents too bulky for enzymatic synthesis. In total, we have prepared 24 new CDNs, which were characterised by biochemical, cell-based assays and for effect on human peripheral blood mononuclear cells (PBMCs). To explain the effect of these aromatic substituents we have solved four X-ray structures of complexes of 7-aryl substituted 7-deazapurine containing CDNs with human STING. These structures explain the reshaping of STING's binding site enabling the formation of a complex of STING with CDNs carrying such bulky substitutions. Moreover, potentially enables further modifications with even larger substitutions escaping the binding site, while still preserving further signal transduction of signal, which hasn't been possible until now.

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L16

STRUCTURAL STUDIES ON SECOND GENERATION SOLUBLE ADENYLYL CYCLASE INHIBITORS AND CHARACTERIZATION OF A PUTATIVE ATPASE DOMAIN

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Cyclic AMP is an important second messenger that plays a key role in numerous signal transduction pathways. In mammals, cAMP is produced from ATP by either G-protein regulated transmembrane adenylyl cyclases or bicarbonate-regulated soluble adenylyl cyclase. The soluble isoform is for example associated with skin and prostate cancer, sperm capacitation and motility, offering a target for pharmaceutical drug and male contraceptive development [1]. In contrast to the catalytic domain, little is known about the C-terminal region of sAC. Based on a prediction of the domain composition, mammalian soluble adenylyl cyclase belongs to the "signal transduction ATPases with numerous domains (STAND) family. A sAC ortholog was discovered in Mycobacterium tuberculosis, comprising the consecutive genes Rv0891c and Rv0890c, serving as model for further analysis. For future characterization of the predicted NTPase domain and its partner domains, first experiments were performed using the C-terminal region of the orthologous enzyme. Initial enzyme activity assays using an UPLC system confirmed its ATPase activity and will allow its further characterization.

Recent pharmacologic evaluation of sAC inhibitors for usage as on-demand, non-hormonal male contraceptives suggested that both, high intrinsic potency and long residence times are essential design elements for successful contraceptive applications. We focused on purification, crystallization and structure determination of novel sAC inhibitor complexes by protein X-ray crystallography and obtained multiple datasets yielding excellent density for the inhibitors and indicating molecular features that might cause their differences in potency and other drug properties [2].

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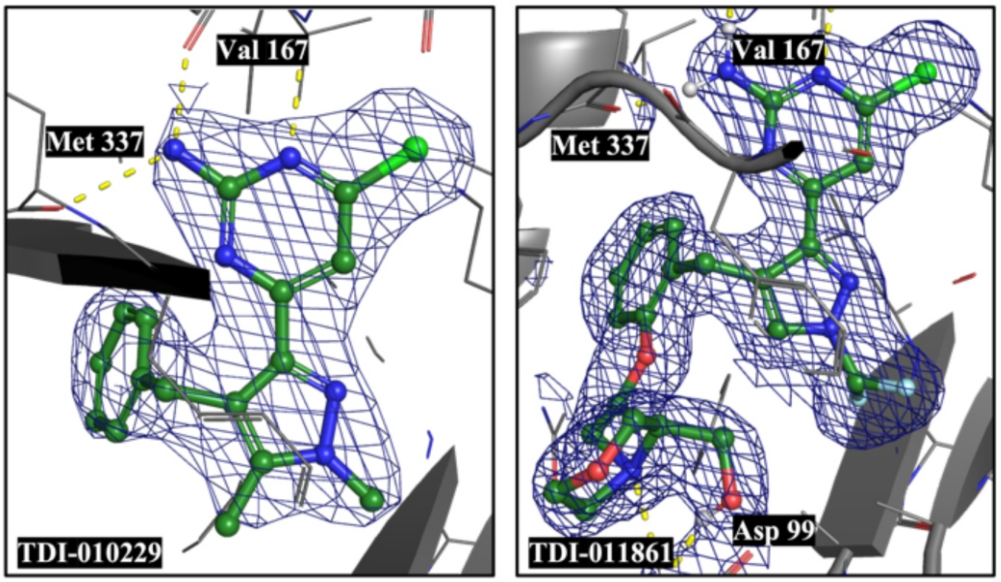

Figure 1: Crystal structure of hsAC in complex with TDI-010229 (left) and TDI-011861 (right). $2F_o$-F_c map contoured at 1s.

FT5

NEW PROTEIN REACTIVE CENTRES AGAINST ASPARTIC PROTEASES

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Aspartic proteases and proteolytic systems controlled by these enzymes play a critical role in several human pathologies such as cancer, neurodegenerative and immune diseases. Cathepsin-like proteases are also essential for viability or virulence of important human pathogens, parasites, and pests. Therefore, strong efforts have gone into the design and testing of compounds that could become new drugs [1]. Unlike the serine protease family, naturally occurring inhibitors of aspartic proteases are relatively rare, and only three of them have been structurally characterized so far in complex with a target protease [2-4].

Here we present new crystal structures of cathepsin D in complex with three proteinaceous inhibitors: potato inhibitors pAPI-1 and pAPI-2 from the plant Kunitz family, and equistatin domain 2 (Eqd2) from sea anemone belonging to the thyropin (thyroglobulin type-1-like) family. The inhibitors show distinct designs of structural binding motifs that are based on disulfide-stabilized loops forming a network of interactions in the extended non-primed part of the enzyme active site. Our results serve as a basis for the development of biomimetic inhibitors of medicinally relevant aspartic proteases.

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FT6

STRUCTURAL BASIS OF PROMOTER MELTING IN *SPIROCHAETA AFRICANA*

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The transcription systems in the phylum spirochaetes are poorly characterized at the molecular level yet are of significant evolutionary and medical importance. Pathogenic spirochaetes easily move through the mammalian tissues, penetrate blood vessels, cross the blood-brain barrier and cause serious diseases, such as lyme disease, relapsing fever and syphilis. At the same time, many spirochaetes are non-pathogenic free-living species. Notably, spirochetal RNA polymerases (RNAPs) are naturally resistant to rifampicin, the best-known transcription inhibitor in clinical use. Spirochaetes evolved independently from other bacterial phyla and are not related to the well-established model organisms *Escherichia coli* and *Bacillus subtilis*, suggesting that the regulation of transcription include distinct and novel strategies.

Transcription is the first event in the highly regulated process of gene expression and is divided into three phases, initiation, elongation and termination that determines the start and the end of the transcription unit. To initiate transcription, RNAP together with sigma factor (holoenzyme) recognizes promoter motifs on the DNA template and start RNA synthesis. Many regulatory factors associate with RNAP during the initiation step and modulate its activity, among them DksA, CarD and GreA/B. Sequence alignments identified additional or distinct domains of some of these transcription factors in spirochaetes compared to most other bacteria phyla suggesting they might act through a different molecular mechanism. Here we present the cryogenic electron microscopy (cryoEM) structures of Spirochaeta africana (*Sfc*) RNAP in complex with sigma

factor 70 (^{70}) and promoter DNA at 3.0–3.4 Å resolution. *Sfc*RNAP together with the housekeeping *Sfc* ^{70} factor bind to the promoter DNA in an open complex in which the duplex DNA is unwounded and the transcription bubble is formed. The structure reveals important insights into the overall architecture of *Sfc*RNAP and the initiation complex and forms the basis for further functional and structural analyses of spirochaete-specific transcription factors and their regulation.

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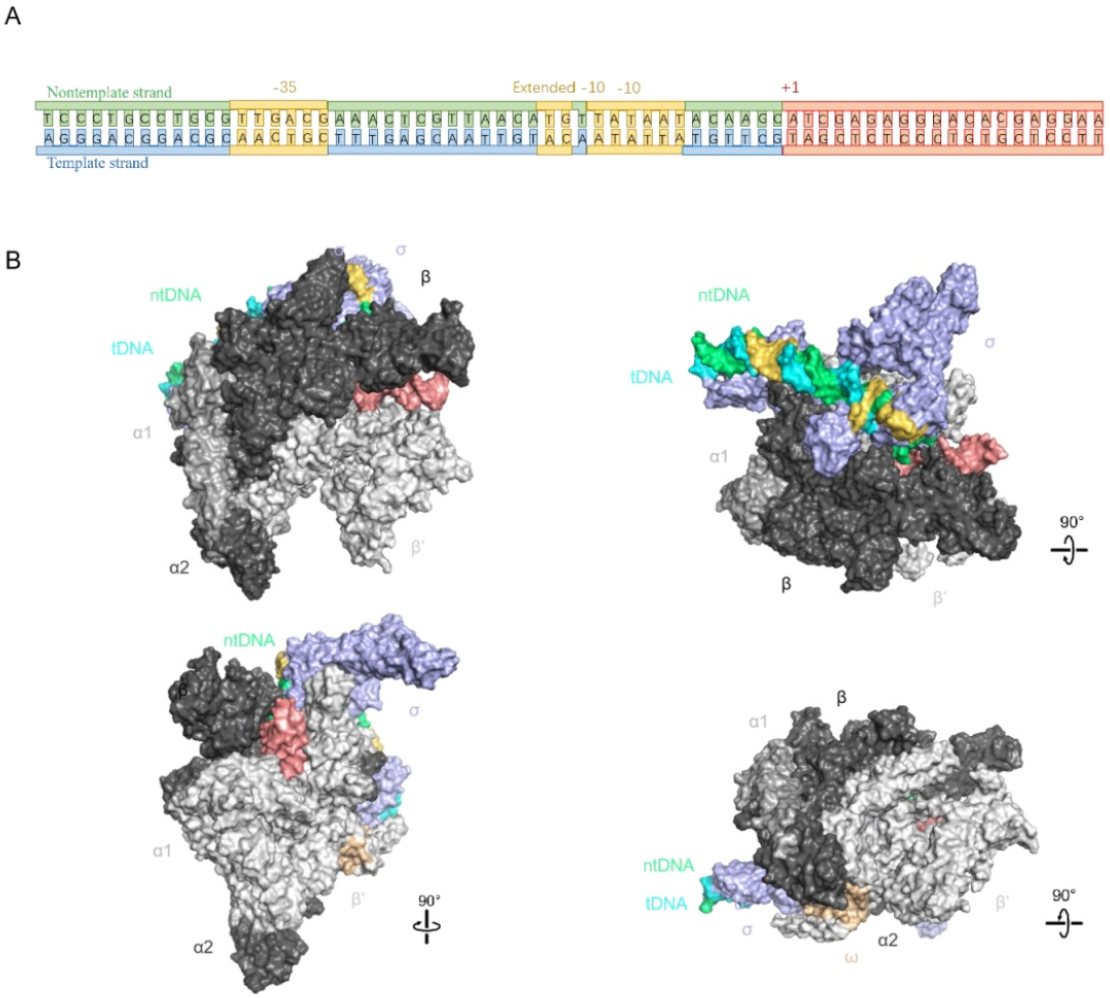

Figure 1. *Sfc* ^{70} initiation complex. A. Promoter DNA scaffold (-46 to +20) used for cryoEM. The -35 and -10 motifs are indicated. +1 refers to the transcriptional start site. B. Overall architecture of the *Sfc*RNAP- ^{70} open promoter complex. Subunits are indicated.

CL2

RIGAKU ADVANCES IN X-RAY AND ELECTRON CRYSTALLOGRAPHY

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The latest range of Rigaku Oxford Diffraction instrument configurations for structural biology will be summarised and illustrated with a number of specific example applications.

The XtaLAB Synergy platform with sealed tube or rotating anode microfocus sources on one side of the four-circle goniometer and a series of Hybrid Photon Counting (HPC) X-ray area detectors on the other, allows for versatile configurations perfectly adapted to the researcher's needs. These systems can be supplied with the sample changing robot (XtaLAB Synergy FLOW), or further equipped with an Intelligent Goniometer Head (IGH) for automated crystal centering or the plate scanning device XtalCheck-S.

Furthermore, our X-ray diffraction instruments are complemented by the new XtaLAB Synergy-ED – a fully integrated electron diffractometer, creating a seamless workflow from data collection to structure determination of three-dimensional molecular structures. The XtaLAB Synergy-ED is the result of an innovative collaboration to synergistically combine our core technologies: Rigaku's high-speed, high-sensitivity photon-counting detector (HyPix-ED) and state-of-the-art instrument control and single crystal analysis software platform (CrysAlis^{Pro} for ED), and JEOL's long-term expertise and market leadership in designing and producing transmission electron microscopes.

CL3

TUNDRA, AN AFFORDABLE MICROSCOPY FOR SAMPLE SCREENING TO HIGH RESOLUTION STRUCTURES

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Electron cryo-microscopy (cryo-EM) single particle analysis (SPA) method has become one of the dominating methods for high resolution structure determination of a wide variety of biological macromolecules. Such high resolution structures facilitate understanding of their functions, mechanism of action and protein ligand/drug interactions. With an increase in the popularity of cryo-EM, the need for accessibility, ease of use and improved efficiency has also increased. Tundra cryo-TEM operating at 100kV with semi-automated grid loading system and automated SPA data collection, makes EM accessible to scientists from diverse Life Science backgrounds. It enables novice users as well as users with varied level of EM experience to carry out sample optimization and obtain high resolution 3D structures to gain insights into biological macromolecules important to human health.

In this abstract, we describe how Tundra was used for sample screening of a challenging small membrane protein that represents a crucial class of drug target for various diseases (Fig1). We solved structure of a challenging homo-pentameric human GABAA (gamma-aminobutyric acid type A) receptor, an important drug target for numerous neurological disorders to 3.4Å resolution (Fig2). In addition, we also solved high resolution structures of other important biological samples such as Adeno-Associated Virus6 that is used as viral vector in gene therapy and vaccines. These results clearly demonstrate potential of the Tundra microscope in drug discovery and how it will add great value at different stages of Cryo-EM workflow for our customers.

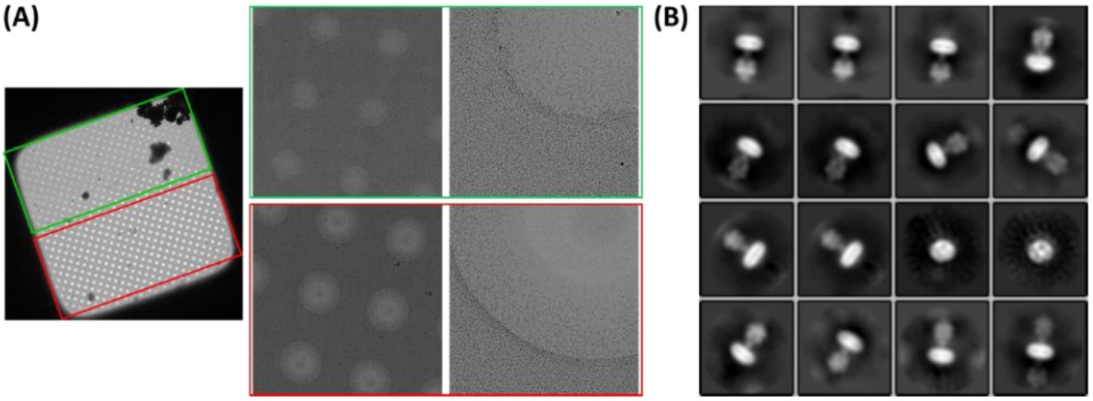

Figure 1. A. Sample screening of GPCR protein B. 2D class averages.

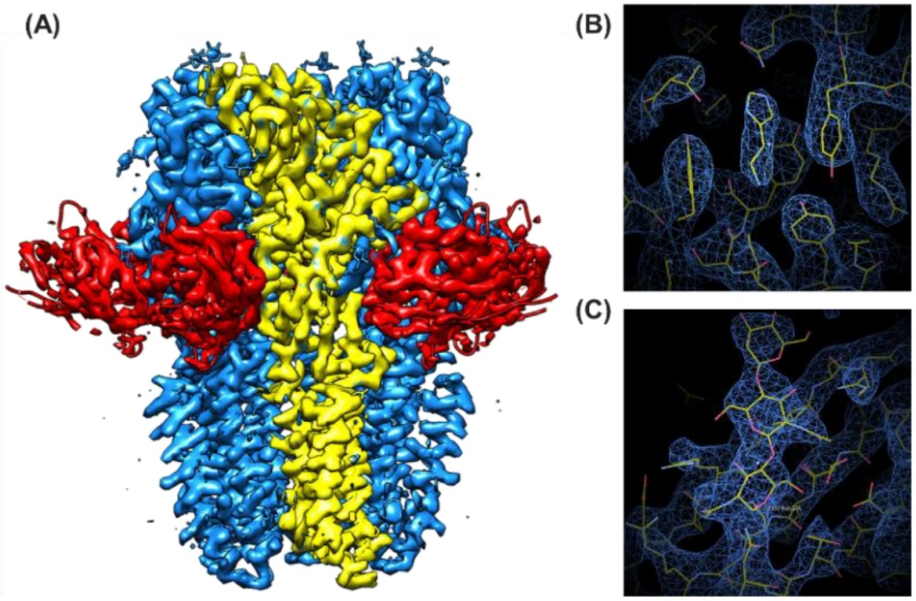

Figure 2. A). 3.4Å reconstruction of $GABA_A$ receptor from Tundra. B) ligand density and C) sugars.