Student abstracts

P1

NEUTRON CRYSTALLOGRAPHY OF MEMBRANE PROTEINS

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Hydrogens play a crucial role for protein function and involved in almost every mechanism. They are critical in understanding the function of various proton pumps such as bacteriorhodopsin (BR) and cytochrome oxidase C. Their light or redox driven action and unidirectional proton pumping mechanism motivates the structural study of these membrane proteins. With the advancement in technology, neutron crystallography is used to locate hydrogen as it is not visible by X-ray crystallography [1]. Since it requires larger crystals, no neutron structures of these membrane proteins have been determined yet. In order to maximize crystal size, we need large amount of protein to feed the crystals. Thus, we focused on optimizing the largescale production of membrane protein. In the initial stage, we used OmpF as a model system due to its high stability, yield and solubility in aqueous solutions to determine the neutron structure at a later stage [2]. A comparative study for the production of OmpF was done considering various parameters such as temperature, media, optical density at wavelength of 600 nm and inducing conditions by Isopropyl -D-1-thiogalactopyranoside (IPTG). The most challenging part was to avoid the improper folding of protein and extraction of outer membranes to the maximum. Thus, after systematic trials with different conditions, we were able to optimize the protocol for large scale purification of OmpF. We also reproduced the published crystallization conditions for OmpF. The next step will be to grow crystals large enough for neutron studies.

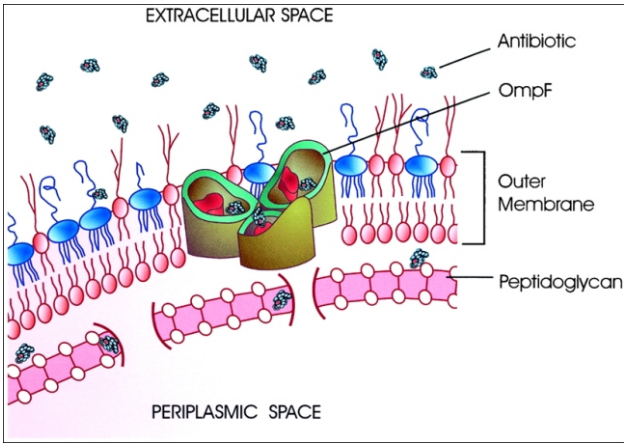

Figure 1. A trimeric OmpF channel in the lipid bilayer of the outer bacterial membrane is a pathway for penicillin antibiotics to the periplasmic space [3].

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P2

ANALYSING STRUCTURE, FUNCTION AND DYNAMICS OF INDUSTRIALLY IMPORTANT ENZYMES FROM *NECTRIA HAEMATOCOCCA* FOR BIOFUEL PRODUCTION

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The alarming levels of environmental pollution, economic growth, as the increasing fuel requirements are parameters that trigger scientific research activities in the field of biofuel production. Biofuels are expected to replace approx. 20% of the fossil fuel used by 2020 [1,2]. In this context and due to competitiveness of grains and biomass required for food-production research activities in the field of biofuel production shifted towards analyzing the potential of lignocellulosic biomass for bioethanol production. As we know today, fungal cellulases such as endoglucanase, cellobiohydrolase, -glucosidase and lytic polysaccharide monooxygenase play a vital role in hydrolysis of complex lignocellulosic biomass to simpler monomers, which can be further used to produce biofuels. Therefore, such enzymes are today in focus of investigations. [3, 4]

The fungus *Nectria haematococca* was recently isolated by the research group of Prof. W. Schäfer, UHH Botany and the initial secretome analysis showed its potential application in the degradation of vegetal cell walls, when grown on two different agro industrial residues.

Consequently, in terms of the proposed PhD research activities selected cellulolytic, hemicellulolytic and redox enzymes from *Nectria haematococca* will be analysed in detail, to score their potential for future industrial applications. The so far selected enzymes belong to GH families GH5, GH6, GH7, GH10, GH11 and GH12. Further a lytic polysaccharide monooxygenase (AA9), polysaccharide lyase (PL3) and carbohydrate esterase (CE5) are selected for the proposed investigations.

For detailed structure-function-analysis selected genes (C7ZH33 and C7Z9N2) from *Nectria haematococca* are cloned into pET 52-b and pGEX-6P-1. Enzymes were expressed by different strains of *E. Coli.* following standard procedures. Target enzymes were always expressed as inclusion bodies. Purification of extracted enzymes is performed by using Tris buffer pH 8 with 1% Dimethyl[3-(propyl]. azaniumyl}propane-1-sulfonate. Purification was done applying standard procedures and fractions obtained from chromatography have been concentrated by using different centrifugal concentrator devices and enzymatic assay has been done by using Xylan as substrate. Unfortunate activity assays showed that the target enzymes were inactive. Due to current solubility problems and observed inactivity we need to change the expression system, and plan to use such as yeast or insect cells to express the active form of target enzymes. Presently cloning is being done to transfer the constructs into pFASt-Bac-Hta for insect cell expression.

In near future the individual enzyme specificities will be investigated applying selected and different substrates. To obtain X-ray suitable crystals dynamic light scattering (DLS) and circular dichroism spectroscopy will be used to obtain homogeneous sample suspensions. The three-dimensional structures will provide essential information about the enzyme's functionality and specificity. Structural data will be used for further functional optimization via site directed mutagenesis aiming to design enzymes suitable for industrial applications to treat lignocellulosic waste material for the effective production of biofuels.

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P3

LOCALIZATION OF AN EPITOPE RECOGNIZED BY A PROMISING PROTECTIVE ANTIBODY ON THE SURFACE OF GLYCOPROTEIN E OF TICK-BORNE ENCEPHALITIS VIRUS

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Tick-borne encephalitis virus is a flavivirus that causes more than 10 000 cases annually. High affinity protective antibody ch14D5a against glycoprotein E of tick-borne encephalitis virus was constructed earlier in our laboratory [1]. It is planned to create a therapeutic drug on the basis of this antibody, so it is necessary to localize the epitope recognized by this antibody on the surface of glycoprotein E. By testing different recombinant fragments of glycoprotein E, it was shown that the antibody binds to the domain D3 of the glycoprotein E, presumably within the *a* and *b* strands. A more precise determination of the epitope will be performed by x-ray diffraction. In order to do this, a Fab fragment of ch14D5a antibody, as well as a single-chain variable fragment were generated and examined by size exclusion chromatography, as well as by dynamic light scattering. The affinity of the antibody fragments to the recombinant domain D3 measured by the SPR-based biosensor was about 40 nM. Complexes of the domain D3 of glycoprotein E with Fab fragment of ch14D5a antibody, as well as with single-chain variable fragment have been obtained. As a result of the initial screening of the crystallization conditions, prismatic crystals ans microcrystals were obtained for the Fab+D3 complex. Further studies are necessary for the preparation of diffraction-quality crystals as well as the determination of the structure of the complex. We believe that localization of the epitope recognized by the antibody ch14D5a will allow us to estimate variability of glycoprotein E in this region, so it will be possible to determine the suitability of this antibody for treating various subtypes of tick-borne encephalitis virus. In addition, information about recognized epitope may shed light on the mechanism of protection activity of this antibody.

The research was supported by the Russian Science Foundation (project 17-74-10146) and by the Ministry of Education of the Russian Federation (project MK-6575.2018.4).

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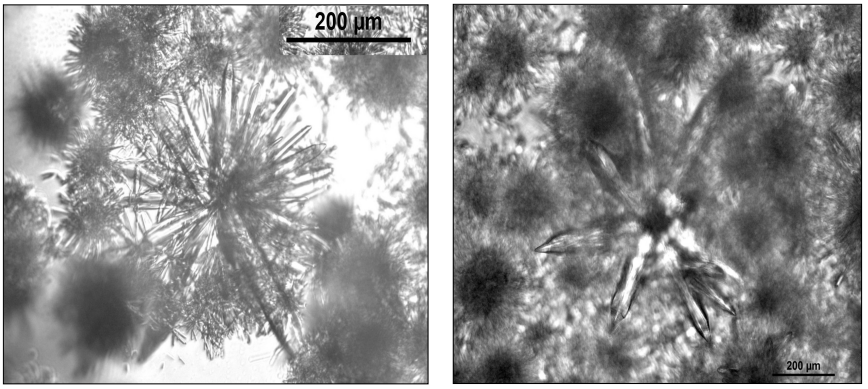

Figure 1. Crystals obtained for Fab+D3 complex as a result of initial screening.

P4

PRELIMINARY CHARACTERIZATION OF CLINICALLY SIGNIFICANT CYTOMEGALOVIRUS UL144 GLYCOPROTEIN

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The prevalence of human cytomegalovirus (HCMV) ranges from 40 to 100 % worldwide. A primary HCMV infection results in a lifelong latent or persistent infection. In healthy people, HCMV infection is often asymptomatic, but in newborns and immunosuppressed individuals, it can cause life-threatening disease. HCMV encodes functions which limit host defenses during productive and latent phases of infection. Studies of viral gene functions have identified a wide range of HCMV gene products with immunomodulatory roles that may enhance the capacity of this virus. One group of these viral genes shares the common property of having acquired the capacity to mimic cellular cytokines or their receptors. These include chemokines and their receptors, which represent a cytokine subset that mediates chemoattraction. One of such protein with immunomodulatory potential in attenuation of congenital antiviral response is UL144. Here, we present the cloning, isolation, expression and purification of such recombinant HCMV UL144 glycoprotein in baculovirus *Sf9* insect system. By preliminary liquid chromatography-tandem mass spectrometry (LC-MS/MS) we have identified up to 6 peptides of HCMV UL144 genes that has proved the sample origin. By using size-exclusion chromatography, we have shown this is in sufficient quality and it has the corresponding molecular weight of monomer. Prepared protein is being further used for biophysical (SPR binding, DLS/SLS) as well as crystallographic (structure-functional) studies.

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P5

TWO STRUCTURAL MECHANISMS FOR INHIBITORY REGULATION OF *FASCIOLA HEPATICA* CATHEPSIN L1

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Fasciolosis caused by the liver fluke *Fasciola hepatica* is a worldwide spread parasitic disease of ruminants responsible for considerable economic losses in the cattle industry and is now recognized as an emerging human disease. Proteases of parasitic helmints represent promising therapeutic targets. This work is focused on cysteine protease cathepsin L1 from *F. hepatica* (FhCL1). It plays a crucial role in nutrition, invasion and migration of adults of *F. hepatica.*

We identified two novel structural mechanisms for natural regulation of proteolytic activity of FhCL1 that are mediated by endogenous inhibitors of *F. hepatica*. First, we determined the structure (at 1.7 Å resolution) of mature FhCL1 in complex with the propeptide, which is generated from the FhCL1 zymogene and functions as an autoregulatory inhibitor. Second, we determined the structure (diffracting at 1.6 Å resolution) of the inhibitor FhCY2, a member of the stefin family, which contains a reactive site for binding to the FhCL1 active site.

P6

VALIDATION OF COMPUTATIONAL PROTEIN ENGINEERING METHODS BY X-RAY CRYSTALLOGRAPHY

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Computationally designed libraries hold a much larger abundance of improved enzymes variants than (semi)-random libraries. The Groningen Biocatalysis group is developing strategies for improving stability and enantioselectivity of enzymes, which combine computational enzyme design with "in-silico" screening by molecular dynamics simulations[1]. The strategy for improving thermo- [2] and solvent-stability [3](termed FRESCO) has produced enzymes variants of an epoxide hydrolase and two dehalogenases with much higher unfolding temperature and high cosolvent resistance. The strategy for engineering enantioselective enzymes (termed CASCO) has resulted in enantiocomplementary variants of an epoxide hydrolase for the production of highly enantioenriched (S, S)- and (R, R)-diols**[4]**.

The FRESCO and CASCO methods need further improvement to enhance the effectiveness and reliability of the computational methods. Evaluation of the designed protocols will be carried out with the use of protein crystallography by solving high resolution structures of the re-engineered variants of target enzymes. The 3D structures will be used to provide a structural basis explaining the observed effects of mutations, in particular for designs where the observed effects deviate from the predicted effects. Deviations between the predicted and observed structures will be examined to allow adjustments of the computational tools, such that the reliability of the predictions is improved. Solved structures will serve as input for computational tools to predict activities that can be compared to experimental data.

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University of Groningen, ES-Cat MSC-ITN 2017-2021. This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 722610.

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P7

NANOCRYSTAL DETECTION AND SCORING BASED ON ADVANCED SECOND HARMONIC GENERATION SIGNAL DETECTION

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The size of protein crystals required for diffraction data collection at high brilliant and micro-focus X-ray sources is continuously decreasing. This creates a strong demand to develop and establish new methods to analyze, score and optimize protein nano- and micro-crystal suspensions for serial crystallography. To support and facilitate this demand, a new microscope based setup was designed and constructed, based on detecting second harmonic generation (SHG) signals of the particles in sample suspensions. This method has the advantage of allowing reliable differentiation of amorphous and crystalline particles. The setup and instrument enhances the already available signal sensi-

tivity to such an extent that detection of relatively small crystals size and crystals with higher symmetry, known to produce rather weak signals, is now possible. Further, the instrument is equipped with additional channels, which are capable of detecting the third harmonic generation signal, and furthermore, also use three-photon excited UV-fluorescence all in parallel, to provide complementary information about the crystalline sample suspension.

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P8

STRUCTURE-FUNCTION STUDIES OF THE NON-STRUCTURAL PROTEINS FROM SINGLE-STRANDED +RNA VIRUSES

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The Picornaviridae family is small (22 to 30 nm) non-enveloped icosahedral viruses that use single-stranded positive-sense RNA (ss+RNA) as genome and are responsible for causing a wide range of diseases in both humans and animals (e.g., Poliovirus-PV, Aichivirus-AiV, Hepatitis C virus-HCV, etc.). Their genome (~7.5 kb) translates into a single polyprotein that is proteolytically prosed into capsid/structural VP1-4 proteins and non-structural (NS) proteins (2A; 2BC-2B, 2C; 3AB-3A, 3B; and 3CD-3C, 3D) which include both mature and stable intermediates that are crucial for the viral life cycle.

Picornaviruses replicate inside a hybrid membrane platform/membranous web/replication organelle (RO) within the host cell to shield from immune responses. Cryo-EM images present ROs as a highly curved and multilamellar structures [2;3]. Several 3D structures of non-structural proteins from picornaviruses are available, but only for the soluble domains, including the recent crystal structure of the soluble domain of EV71-2C protein [4]. Currently, recombinant 3A, 3AB full length wild-type/mutated proteins with membrane spanning regions are being prepared and reconstitution into liposomes and soluble domain of 2C proteins from AiV, FMDV and Sicinivirus, which are believed to adopt a slightly different structure and a novel oligomerization mechanism is prepared for crystallographic studies. Recent work from our group has structurally elucidated that 3A proteins act as molecular harnesses to hijack the host ACBD3 protein, that interacts with the host lipid kinase PI4KB to generate negatively charged membranes enriched in PI4P [5]. Additionally, it was also discovered that negative charge and 3B protein co-operate to recruit 3Dpol forming the replication complex [6]. We expect to reconstruct the whole viral replication cycle "in vitro", which will allow us to establish how ss+RNA viruses hijack human host factors to sculpt membrane into their ROs and to better understand its formation and the molecular mechanisms bridging virus and host.

[1] Zell et al., 2017; [2] Belov et al., 2014; [3] Harak et al., 2015; [4] Guan et al, 2017; [5] Klima et al., 2017 and [6] Dubankova et al., 2017.

Figure 1. Poliovirus 1 (serotype 1) genome organization and processed capsid/structural and non-structural proteins (arrow and arrow heads shows major and minor cleavage sites) [1].

P9

CRYSTALLIZATION AND REFINEMENT OF THE LSM FAMILY ARCHAEAN PROTEINS WICH REPRESENTS A MINIMAL LSM CORE

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Proteins of the Sm-like family (Lsm) are found in three domains of life. They provide biogenesis and functioning of various RNA molecules in the cells. They are defined by the ability to adopt the Sm fold, which is comprised of a 5-stranded -sheet and an N-terminal -helix. Bacterial Lsm proteins called Hfq exhibit chaperone activity promoting interaction between regulatory sRNA and mRNA during regulation of translation [1]. Eukaryotic Sm proteins are core proteins of the spliceosome while eukaryotic Lsm proteins are involved in the mRNA degradation [2]. Functions of the archaeal Lsm proteins (SmAP) in a cell have been studied poorly, although there are some data on their participation in the processing of some RNA (such as tRNA).

In order to study functions of an archaeal Lsm protein (SmAP) *Halobacterium salinarum* and *Haloarcula marismortui* were chosen as its sources. These proteins are homologues and they have remarkable differences of the sequences compared with the other SmAP proteins. SmAP proteins from *H. salinarum* and *H. marismortui* have no unstructured regions; they are represented by a minimal Lsm core. These proteins were isolated and purified in preparative scale, then they were crystallized and a high-resolution diffraction dataset for the protein from *H. salinarum* was collected at ERSF in Grenoble. Despite the fact that proteins are 78% identical, it seems not possible to obtain good protein crystals from *H. marismortui*. Crystal structure of SmAp protein of *H. salinarum* allows creating a model for determination the structure of its homologue from *H. marismortui*. In addition, this structure allows to design site-specific mutagenesis of the surface of HmaSmAP in order to increase its crystallization capability.

At the moment HsaSmAP structure is under determination and refinement.

The work is supported by RFBR grant #18-04-00222 and YTF grant for the advanced course PC18-001.

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P10

BINDING MODE BETWEEN TRANSIENT RECEPTOR POTENTIAL (TRP) MEMBRANE CHANNELS AND PDZ3 DOMAIN

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PDZ domain interaction modules are found in multi domain scaffold proteins which regulate different biological processes, such as clustering ion channels, targeting signaling complexes and maintaining several cellular structures [1]. PDZ domain contains six strands (A to F) and two helices (A and B) forming partially opened barrel. We recognize two interaction modes between PDZ domains and target proteins: canonical, when B/ B groove of PDZ domain binds to short sequence at the C termini of ligand, or non-canonical, when PDZ domains recognize internal peptide sequence or ligand is binding away from the B/ B groove [2]. Transient receptor potential (TRP) protein superfamily is highly conserved in yeast, invertebrates and vertebrates. They are involved in various physiological processes, such as stimuli sensation and ion homeostasis. Mutations in these nonselective cation channels are related to human diseases, such as cancer and neurological disorders [3]. Project is focused on finding novel binding sites in interaction of various transient receptor potential (TRP) membrane channels N and C termini with highly abundant PDZ protein domain by using steady-state fluorescence anisotropy measurements, and X ray crystallization to better understand the interaction mode. We expect non-canonical interaction mode in PDZ3 binding with TRP membrane channel. Human PDZ3 domain from Tight junction protein ZO1 will be prepared with molecular biology tools and predicted sequences from TRP membrane channels to bind PDZ domain will be chemically synthesized as peptides. Macromolecular crystallization of the complex reveal canonical or non-canonical binding mode and uncover amino acids which play the key role in interaction.

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P11

TEST OF GENETIC CODE EVOLUTION HYPOTHESES: REVERSE EVOLUTION OF RNA BINDING DOMAIN OF RIBOSOMIAL PROTEIN L11

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Although extant proteins consist of 20 different amino acids, it has been proposed that primordial proteins consisted of a smaller set of "early" amino acids and that additional "modern" amino acids have gradually been recruited into the genetic code [1-3]. This naturally leads to the questions: can structured and functional proteins be constructed using the "early" amino-acid alphabet? Can extant proteins be reverse-evolved while preserving their structure/function?

To test this, protein databases have been inspected to select model extant protein candidates with different structural folds. Our preliminary search contains proteins with both catalytic and binding/interaction functions. The first target analyzed was RNA-binding domain of ribosomal protein L11.The selected protein target was "reverse-evolved" in vitro into variants where the "modern" amino acids were randomized by "early" ones. The libraries of randomized genes were incorporated into a genotype-phenotype linkage to be compatible with an appropriate library display (mRNA display [4]) and selection method. The selection of successful candidates was based on conservation of structure and/or function and the most "successful" variants will be characterized analyzing the new structure and the new complex protein/RNA by X-Ray crystallography.

This research will inform us of the essentiality of "modern" amino acids for building protein structure/function and thus will provide a direct test of the hypotheses about early proteins. In addition, proteins constructed from a limited amino acid alphabet are of importance in protein engineering and synthetic biology. Finally, this area touches upon the very basic link of protein sequence-structure-function that lies at the core of many biotechnological and biomedicine problems and has express implications for construction of artificial biochemistries.

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P12

THE STRUCTURE AND FUNCTION OF HUMAN MITOCHONDRIAL HEAT SHOCK PROTEINS

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Molecular chaperones belong to a special class of heat shock proteins (Hsp) that assist the folding, unfolding and formation of proteins' quaternary structure as well as play an important role in their transport into the correct subcellular compartment. The Hsp70 protein family has been found in both, prokaryotes and eukaryotes. Like other chaperones, Hsp70 perform their physiological function exclusively in complex with other co-chaperones. In general, Hsp70's chaperone function is regulated through cycles of ATP binding, ATP hydrolysis and ADP release mediated precisely by the activity of its co-chaperones [1]. In human mitochondrial Hsp70 system, these include co-chaperones with J-domains, such as Hsp40 (2 isoforms: Tid-1L and Tid-1S), which stimulate the ATP hydrolysis [2]; nucleotide exchange factors (2 isoforms: GrpE1 and GrpE2), which recycle Hsp70 into an ATP-bound state that permits the efficient release of substrate [3]; and the Hsp70 escort protein Hep1 involved in increasing of Hsp70 ATPase activity [4]. To date, no full Hsp70 protein has been successfully crystalized, only the structures of sepa-

rate Hsp70 domains have been solved so far [5]. As well as none of the quaternary structure of any Hsp70 co-chaperone is known. Therefore, our work is focused on isolation and purification of human mitochondrial Hsp70 and its co-chaperones GrpE, Hep1 and Tid1 and their crystallization, either individually or in complex with Hsp70, in order to solve the crystal structures and ultimately understand the interactions between Hsp70 and its substrates and how these influence the Hsp70 chaperone activity.

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P13

CORE FACILITY FOR CRYSTALLOGRAPHIC AND BIOPHYSICAL RESEARCH TO SUPPORT THE DEVELOPMENT OF MEDICINAL PRODUCTS

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The project "Core facility for crystallographic and biophysical research to support the development of medicinal products" (cfcb.uw.edu.pl) is funded by the TEAMTECH Core Facility programme from the Foundation for Polish Science (www.fnp.org.pl). The project will establish a **Core Facility for Crystallography and Biophysics** (CFCB UW) at the Biological and Chemical Research Centre, University of Warsaw under the supervision of **Prof. dr hab. Krzysztof Woźniak** (crystal.chem.uw.edu.pl).

The mission of the new Facility is focused on analysis of proteins and small chemical compounds (molecules) leading to crystallization trials for academic and commercial users. The project will enable the study of challenging biochemical and pharmaceutical problems, with emphasis on drug development and collaborations with the local research groups. Work at CFCB UW will be carried out in an interdisciplinary way, including both wet biology **("BIO")** and chemical crystallography **("CHEM")** techniques as well as theoretical approaches including structure modelling, bioinformatics and computational methods. Biology and chemistry team members will work in synergy complementing their knowledge, skills and experience. Apart from services and collaborations, postdoctoral and PhD researchers are expected to carry out their own research projects dedicated either to small molecule or protein crystallography.

Young scientists working in the project will benefit from mentoring and exchange visits with the project partners, **Prof. Wladek Minor** (University of Virginia, USA) and **Prof. Ben Luisi** (University of Cambridge, UK). Work at CFCB will include, among others, collaboration with biotech/pharmaceutical companies, such as the WPD Pharmaceuticals or the Pharmaceutical Institute in Warsaw (Poland).

This work was supported by the TEAMTECH Core Facility programme from Foundation for Polish Science (TEAM TECH CORE FACILITY/2017-3/4, Nr. 0032).

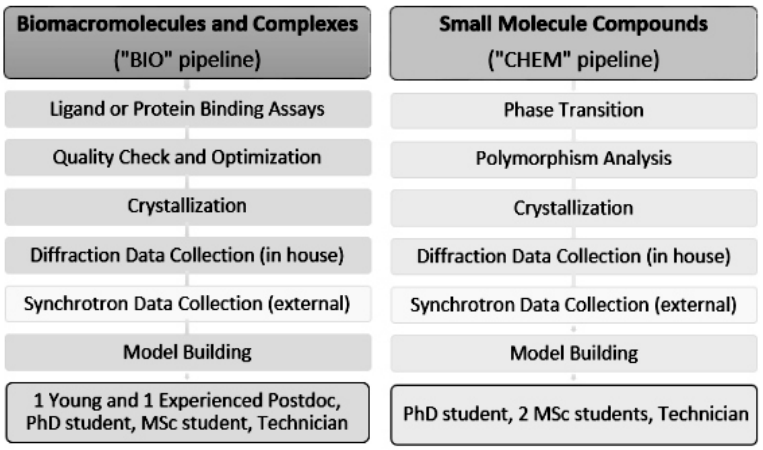

Figure 1. Schematic view of the main pipelines of the Core Facility for Crystallography and Biophysics

P14

INVESTIGATIONS OF ACCESSIBILITY OF T2/T3 COPPER CENTRE OF TWO-DOMAIN LACCASE FROM *STREPTOMYCES GRISEOFLAVUS* AC993

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Laccase (EC 1.10.3.2, pdiphenol:dioxygen oxidoreductase) is a copper containing enzyme catalyzing oxidation of phenolic substrates and some inorganic ions with reduction of dioxygen to water. Laccases of fungi consist of three domains, among bacterial laccases there are both three domain and two domain structures. It is known that two-domain laccases (2D) are more thermostable than three domain (3D) ones, stable against inhibitors and are functionally active in the alkaline pH area. In the structures of the 3D laccases, the copper ion of the T2 center and the pair of copper ions of the T3 center are connected by channels with the surface of the protein. We have performed a comparative structural analysis and showed that the channels for 2D and 3D laccases differ in length and width.

Recently we showed that conservative His165 belonging to second coordination sphere of T2/T3 copper centre of 2D laccase Streptomyces griseoflavus (SgSL) is the "gate" of the channel leading through the interdomain cavity to the Cuá ion of T3 center. Further, we suggested the presence of a second channel leading to the Cuá ion of T3 center and that conservative Ile170 can prevent passage of oxygen to this center.

For the verification of our hypothesis, we have obtained two mutant forms with substitutions Ile170 to large amino acid residue Phe (I170F SgL) and small residue Ala (I170A SgL) and crystallized them. Crystal structures of I170F SgL and I170A SgL were obtained with high resolution -1.95 Å and 1.98 Å respectively. Analysis of structures of mutant forms showed that channel was completely blocked by the side chain of Phe in position 170. The substitution Ile170 to Ala make the channel a few large, however, we did not observe the penetration of water into this channel.

The oxidizing activity of mutant forms was measured using as substrate ABTS at pH=4.5 and 2.6-DMP at pH=9. The results of biochemical studies showed that both substitutions of Ile170 decrease the activity of the enzyme. Taking into account the structural and functional data we guess that Ile170 is not a channel gateway and its true role has not yet been clarified.

This research was supported by the Russian Foundation for Basic Research (No. 18-04-00270).

P15

STRUCTURAL, BIOCHEMICAL AND BIOPHYSICAL ANALYSIS OF CRYPTOCHROMES IN *PLATYNEREIS DUMERILII*

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The changes in light conditions allow most organisms to anticipate changes in their surrounding and to adapt / change their behavior and physiology. The 24h day/night activity cycle also called the circadian rhythm governs several physiological and behavioral processes in a circadian manner. The transcriptional and translational feedback loop of several regulatory genes governs the circadian clocks. The regulatory genes are partially shared between insects and the mammalian/vertebrate/invertebrate systems indicating a possible evolutionary function.

The bristle worm *Platynereis dumerilli* has a circalunar (monthly) in addition to a circadian (daily) clock which regulates its maturation and helps it to adjust its reproduction to tidal rhythms. *Platynereis dumerilli* possess 3 different kinds of cryptochromes, one each similar to the drosophila (l-CRY), mammalian repressor (tr-CRY) and plant (p-CRY) cryptochromes. *Platynereis* is suitable model to study the interplay between circadian and non-circadian clock components and the influence of this on the behavior of the organism. It has already been established that the circalunar clock of the worm affects the circadian clock at several levels and the circalunar clock persists even when the circadian clock is disrupted. The proposed model does not include the cryptchromes l-CRY and p-CRY as their functions are still unclear. This study focuses to characterize l-CRY and p-CRY spectroscopically by UV/VIS and fluorescence spectroscopy and structurally by X-ray crystallography and SAXS.

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P16

STRUCTURAL BASIS OF INTERACTIONS BETWEEN 14-3-3 PROTEINS AND THE 14-3-3 BINDING MOTIFS OF CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE KINASE

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Calcium/calmodulin-dependent protein kinase kinases (CaMKK1 and CaMKK2) are members of the Ca^{2+}/calmodulin-dependent kinase (CaMK) family involved in adiposity regulation, glucose homeostasis and apoptosis. These upstream activators of CaMKI, CaMKIV and AMP-activated protein kinase are negatively regulated by phosphorylation, which also triggers an association with the scaffolding protein 14-3-3. Studies have shown that CaMKKs bind to various 14-3-3 isoforms and that the conserved N-terminal motif containing phosphorylated S74 in CaMKK1 (S100 in CaMKK2) functions as the primary 14-3-3 binding site. Furthermore, a second 14-3-3 binding motif located at the C-terminus containing phosphorylated S475 in CaMKK1 (S511 in CaMKK2) has also been suggested. Although the C-terminal 14-3-3 binding motif (sequence RSLpSAP) is a canonical "mode I" 14-3-3 binding site, the N-terminal motif (sequence RKLpSLQE) contains a Gln residue at the position +2 relative to the phosphorylated residue pSer. Bioinformatics survey of 14-3-3 binding sites revealed that Gln is seldom found at +2 because the Pro residue and, to a lesser extent, Ser, Gly and Asp also, are frequently found at this position. To elucidate the structural basis of interactions between 14-3-3 proteins and the 14-3-3 binding motifs of CaMKK, we solved the crystal structures of phosphopeptides containing both 14-3-3 binding motifs of CaMKK bound to 14-3-3. The structures showed that both phosphopeptides interact with the amphipathic groove of 14-3-3 similarly to other 14-3-3 complexes. Nevertheless, in the case of the N-terminal motif, the interaction between the side-chain of Gln at the position +2 relative to pS and the phosphate group appears to abruptly change the direction of the polypeptide chain.

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P17

STRUCTURAL STUDIES OF INTERMEDIATE FILAMENTS

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Intermediate filaments (IFs) form one of the three filament systems of the eukaryotic cytoskeleton. Six different types of IFs provide reinforcement to the cell to withstand external stress. Our study is focused on the structure of vimentin that was shown to have distinct roles in actin organization, cell signaling, cancer cells dissemination and wound healing.

Like all IF proteins, vimentin consists of the central conserved -helical domain flanked by more variable "head" and "tail" domains. Two parallel central domains form -helical coiled coil. Such dimers associate into tetramers that in turn combine into octamers and higher oligomers, ultimately yielding mature filament. To date, the detailed molecular architecture of IFs is not known. To address this challenge, we are using both X-ray crystallography and electron microscopy (EM).

A common stumbling block of crystallographic studies is to grow a crystal. Full-length vimentin is not suitable for crystallization as it has a tendency towards self-assembly into filaments. Dividing the filament into multiple overlapping fragments solved the problem for dimer structure, but the molecular architecture of the tetramer remains a challenge [1].

In order to stabilize the tetrameric assemblies of truncated vimentin we use N- and C-terminal fusions with several helical bundle domains, following the strategy recently applied to myosin fragments [2]. In addition, to stimulate crystal formation, fusions with larger globular domains are being made.

In parallel, we are exploring the use of EM towards resolving the molecular structure of assembled filaments as well as higher assembly intermediates.

Ultimately, we hope to combine all sources of experimental information as well as recent advances with *in silico* modelling [3] towards obtaining novel insights into the molecular organization of IFs (Fig.1).

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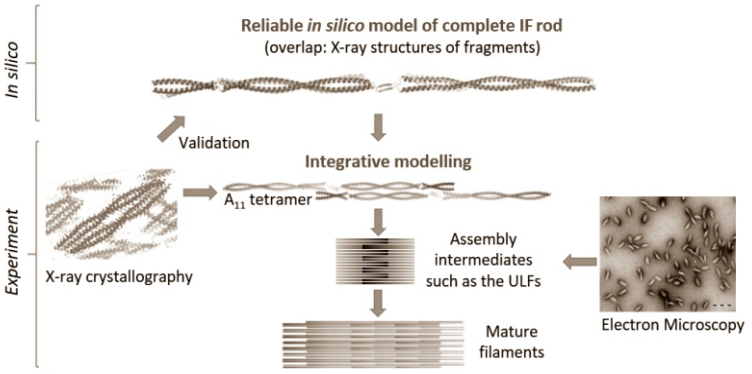

Figure 1. Workflow of the project: combination of different approaches to obtain information about vimentin structure.

P18

CRYSTAL STRUCTURE OF -CARBONIC ANHYDRASE FROM *CANDIDA ALBICANS*

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Pathogenic yeasts of the genus Candida represent the most prevalent cause of mycotic diseases worldwide. They behave as opportunistic pathogens, which means that they can live in human hosts as harmless commensals, being kept under the control by the host immune system. One of the key survival strategies of fungal pathogens is the ability to proliferate in different carbon dioxide (CO_2) concentrations. CO_2 is among the most important gases for living organisms. In nature, the concentration of CO_2 is balanced by interconversion to hydrogen carbonate (HCO_3, bicarbonate) however, its average amount required by organism is much greater than the amount produced spontaneously from CO_2. Therefore, bicarbonate production requires a fine tuned regulation. For this reason, a highly diverse family of enzymes has evolved that are able to accelerate the interconversion reaction up to 10 000-fold. The members of this family, carbonic anhydrases (CAs) are able to catalyze reversible hydration of CO_2 to bicarbonate. CAs evolved in all three domains of life, and are divided into six, evolutionarily unrelated classes (and) that independently arose from different precursors during convergent evolution. ß-CAs are present in many pathogenic microorganisms but not in the mammalian hosts and therefore represent possible target for drug development.

We determined the crystal structure of CA form *Candida albicans* (CaNce103) at 2.2 Å resolution. CaNce103 assembles as a tetramer, with the active site localized at the interface between two monomers. At the bottom of substrate pocket, the zinc ion is coordinated by three highly conserved residues Cys78, His133 and Cys136 in addition to water molecule. Activity assays of full length and truncated versions of CaNce103 indicated that the N-terminal region is indispensable for enzymatic activity.

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PRELIMINARY RESULTS OF A STRUCTURAL STUDY ON THE POLYMERASE WZY: A DIFFERENT APPROACH TO BACTERIAL DRUG RESISTANCE

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The integrity of the Lipoplysaccharide (LPS) layer is essential for bacterial survival and for the pathogenic activity of bacteria. In septicemia caused by Gram-negative organisms [1] such as Pseudomonas aeruginosa, an opportunistic pathogen involved in most of nosocomial infections, the endotoxic shock depends on LPS activation of immunogenic receptors [1]. The Wzy-dependent pathway is one of the biochemical routes that lead to the formation of LPS [2]. In this pathway, oligosaccharides are bound to a lipid carrier and transferred from the cytoplasm to the periplasm. The Wzx protein, a flippase, is involved in this first step [3]. The polysaccharide formation is catalyzed by the polymerase Wzy that adds a single O-Antigen unit to the reducing end of a sugar polymer [2], generating a new glycosidic bond. Finally, polysaccharides are channeled to the outer membrane through the protein Wza.

Considering that proteins belonging to the Wzy-pathway play an essential role in the correct LPS assembly and in cell wall formation [1], the aim of the present work is to structurally characterize Wzy through X-Ray crystallography in order to understand its molecular mechanism. Due to the low sequence homology between different bacterial strains, Wzy is a promising target for a new generation of antibacterial drugs.

Here we present the preliminary results of this study. The gene encoding for P. aeruginosa Wzy was cloned in a bacterial overexpression vector with a GFP and a Histidine tag at the C-term. Using fluorescence measurements and Western Blotting analysis, the expression protocol was optimized. The over-expressed protein was solubilized in different detergents and degree of solubilization and stability of the protein were tested. Affinity chromatography and size exclusion chromatography were employed for protein purification. Wzy have been characterized by Circular Dichroism, and a preliminary screening of crystallization conditions has been carried out.

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P20

EXPRESSION, PURIFICATION AND ENZYMATIC ACTIVITY OF HUMAN ALDO-KETO REDUCTASE 1C2 (AKR1C2)

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Aldo-keto reductases are enzymes involved in reduction of aldehyde and ketone groups of various substrates using NADPH and NADH as cofactors. AKR1C2 is a human isoform that acts on a wide spectrum of compounds as substrates, including steroid hormones, which can affect the progression and development of certain diseases, such as hormone-dependent types of cancer. In addition, AKR1C2 binds bile acids and is strongly inhibited by some of them. To date, several crystal structures of AKR1C2 complexed with cofactor and different inhibitors have been resolved.

Here, we have expressed human AKR1C2 from E. Coli using a pET28 vector construct which was a generous gift from Professor Chris Bunce, University of Birmingham, UK. We have purified active AKR1C2 enzyme using affinity chromatography and gel filtration, and tested its activity via spectrophotometric assay which measures absorbance at 340 nm, representing NADPH consumption in the presence of phenanthrenequinone (PQ). AKR1C2 was shown to be active, and further steps include identifying potential inhibitors among steroid compounds synthesized by organic chemists at our Faculty of Sciences. In addition, we have run several molecular docking simulations of natural bile acids to already resolved AKR1C2 structures to develop a system that will aid in pre-selection of compounds for enzymatic activity inhibition tests. Once inhibitors have been identified, we will work towards co-crystallization of enzyme-cofactor complexes with inhibitors and investigate the structural basis of inhibition. Our results might be helpful in developing new drugs for treatment of hormone dependent types of cancer.

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P21

DRUGGING CHALLENGING E3 LIGASES: A NOVEL MULTIDISCIPLINARY APPROACH TO IDENTIFY SMALL-MOLECULES THAT BIND FBW7

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During normal cellular homeostasis, proteins are constantly synthesized and destroyed. The most common degradation pathway for proteins is the Ubiquitin Proteasome System (UPS), a highly regulated signalling cascade that is ultimately responsible for the controlled degradation of a large number of proteins. E3 ligases provide substrate specificity to this system, making them extremely attractive candidates as drug targets. However, the development of small-molecules against E3 ligases has led to limited success, in part because modulating their activity and regulation requires targeting protein-protein interactions. [1]

Fbw7 is an important E3 ligase and one of the most commonly deregulated proteins in human cancers. Indeed, 6% of cancers have mutations in the fbw7 gene. On one hand, the loss of activity of the mutated Fbw7 results in a loss of its tumour suppressor function and an upregulation of the natural and oncogenic substrate proteins: c-Myc, cyclin-E, Notch, etc. [2] On the other hand, the inhibition of Fbw7 has been proposed as an approach to sensitize cancer stem cells to chemotherapies. [3] However, so far, no potent small-molecules directly targeting Fbw7 have been reported. In this project, using a novel multidisciplinary approach, we aim to identify small-molecules that targets Fbw7 to disentangle the more convenient pharmacological strategy to manipulate it.

To identify *ligandable* allosteric sites in the Fbw7-Skp1 surface we have applied MDmix simulations. [4] Docking-based virtual screening applying Duck [5] filter has been performed to find potential *hits*. These potential *hits* have been tested by Surface Plasmon Resonance and confirmed by STD-NMR. Following this workflow, we have been able to identify molecules that target Fbw7 in the one digit micromolar range. In parallel, a fragment-based screening has been performed and several fragments have also been identified. Work is on-going to obtain structural information that will confirm us the binding site and binding mode of these *hits*.

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P22

CONTRIBUTE KNOWLEDGE ABOUT UNRESOLVED SITES OF THE PHF CORE BY X-RAY CRYSTALLOGRAPHY OF RECOMBINANT TAU PROTEIN IN COMPLEX WITH MULTIPLE ANTIBODIES

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Tau protein is a natively unfolded protein and plays an important role in stabilization of microtubules [1]. However, in Alzheimer disease and other tauopathies, tau protein is the constituent of neurofibrillary tangles [2]. Ultrastructurally, tau inclusions are made of paired helical filaments (PHFs) and straight filaments [3]. Structure of tau fibrils has been recently elucidated using cryo-electron microscopy at 3.4-3.5 Ĺ resolution, however, there were still unresolved sites at both N and C terminal regions of the filament core [4]. Structural study of recombinant PHF core tau protein fragments in complex with PHF core conformation specific monoclonal antibodies may help to elucidate missing parts. Conformational antibody MN423, which recognizes a compact fold of the PHF core, was selected for this task [5].

In my work I have prepared the recombinant PHF core fragment dGAE containing tau residues 297-391 (numbering according to the tau2N4R) together with recombinant MN423 Fab, DC8E8 Fab and DC25 Fab for crystallization experiments with tau. MN423 can induce a PHF core conformation and remaining two antibodies can help with creating crystal contacts. After screening cocrystallization conditions and crystal growth optimization, the structure will be solved using X-ray crystallography.

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P23

ELUCIDATING THE STRUCTURAL BASIS OF LINKAGE SPECIFICITY IN THE UBIQUITIN LIGASE HUWE1

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Protein ubiquitination is a posttranslational modification that regulates protein functions on all levels. Consequently, deregulation of ubiquitination is implicated in various human diseases, including neurodegeneration, inflammation, and cancer.

Ubiquitination reactions are driven by a catalytic cascade of E1, E2, and E3 enzymes (ubiquitin ligases). The large number of E3 ligases - over 1000 estimated in the human proteome - mediate the recognition of target proteins for modification, thus providing key specificity factors in ubiquitin signalling. Moreover, the particular functional outcome of ubiquitination for the target protein is dependent on the type of ubiquitin modification that is formed. Substrates may be modified with single ubiquitin molecules or chains of diverse linkage specificities that trigger distinct downstream responses.

Our study aims to reveal how a particular E3 enzyme, HUWE1, encodes linkage specificity. HUWE1 is a key player in tumor biology and, therefore, an attractive therapeutic target.

To understand how HUWE1 synthesizes ubiquitin chains we will dissect the interactions of this ligase with ubiquitin by a combination of structural and functional tools with a distinct focus on X-ray crystallography.

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P24

CRYSTALLIZATION OF TRANSCRIPTIONAL REPRESSORS DEOR AND EFFECTOR-BINDING DOMAIN OF GNTR FROM *BACILLUS SUBTILIS*

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DeoR is involved in carbon catabolite repression in *B. subtilis* as the local repressor of the deoxyribose and deoxyribonucleoside catabolism. The structure of the C-terminal effector-binding domain of DeoR has already been reported [1]. However, to understand fully the allosteric effects of the repressor during the metabolic regulatory process, it is necessary to resolve the 3D structure of the full-length protein in complex with its operator DNA. To achieve this, we initiated structural studies by X-ray diffraction. Recombinant DeoR was prepared by heterologous expression in *E. coli* BL21 (DE3) and purified with yield of 3.4 mg per L of bacterial culture. Biochemical and biophysical analysis revealed that protein forms dimer and binds DNA duplex derived from the operator sequence. Crystallization of DeoR in complex with DNA was carried out using the vapour diffusion sitting- and hanging-drop techniques. Needle-shaped crystals were obtained and presence of DNA was confirmed by fluorescence microscopy in the presence of SYBR-Gold®. Diffraction data were obtained to a resolution of 3.9 Å and structure was solved by molecular replacement. While model is being refined, crystallization conditions are being optimized to obtain better-diffracting crystals.

GntR is also involved in carbon catabolite repression in *B. subtilis* but as a local repressor of D-gluconate catabolism. Although it was the first characterized protein from GntR family [2], any part of its structure has not been solved yet. As the first step to approach this we initiated structural studies of the C-terminal effector-binding domain (C-GntR) in complex with its effector D-gluconate. Recombinant C-GntR was prepared by heterologous expression in *E. coli* BL21 (DE3) and purified with yield of 21.4 mg per 1 L of bacterial culture. Biochemical and biophysical analysis revealed that protein forms dimer. Crystallization of complexed C-GntR was carried out using vapour diffusion techniques. Obtained crystals diffracted X-ray to a resolution of 2.2 Å. Attempts to solve structure by molecular replacement failed and thus crystals for experimental phasing will be prepared.

This project was supported by the Ministry of Education of the Czech Republic (programme "NPU I") project LO1304.

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P25

NADPH OXIDASES AND THEIR ROLE IN REDOX SIGNALING: STRUCTURAL AND BIOCHEMICAL STUDIES FOR THE DEVELOPMENT OF NEW INHIBITORS/MODULATORS AS A PATHWAY TO CANCER THERAPY

Joana Reis,[1] Francesca Magnani,[1] Elisa Millana Fananas,[1] Marta Ceccon,[1] Marta Massari,[1] Simone Nenci,[1] Elvira Romero,[2] Marco W. Fraaijeb[2] and Andrea Mattevi[1]

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NADPH-oxidases (NOXs) are the only known enzymes whose sole function is ROS generation. Moreover, mammals contain several enzyme isoforms: Nox1-Nox5, Duox1 and Duox2, which differ with respect to their subunit composition, regulation, specific activities, and ROS product(s). These integral membrane proteins are known to perform delicate signaling roles that are specific for each of the seven isoforms present in humans and which modulate pathways that induce and sustain cell proliferation [1].

Recently, our research group has been successful in accomplishing the first crystal structure of the cytosolic dehydrogenase (DH) and trans-membrane (TM) domains of NOX using a bacterial ortholog which is highly similar (40% sequence identity) to human NOX5 [2]. The structural analysis had revealed the architecture and the arrangement of the redox cofactors in the catalytic core of these integral membrane enzymes. Furthermore, the structural elucidation hinted towards features that could be exploited for the design of selective drugs against distinct NOX isoforms [2].

In this framework, our group has been currently focused in the study of the three-dimensional structure of the

full-length NOX core subunit. This will clarify the mechanism related with the electron transfer from intracellular NADPH to the external heme, where the ROS generation takes place. Accordingly, we are currently working on the screening of detergents which might limit protein degradation and enhance stability. The development of mutants targeting the domain linker region whose flexibility might lead to proteolysis is another successful strategy to obtain protein suitable for structural and biochemical studies. This work will also be supported on the development of ligands as useful tools to help expression, purification, and crystallization of the TM-DH core subunit by stabilizing its structure. The development of isoform-specific NOX inhibitors and modulators can act as powerful tools to investigate the biology of ROS signaling and consolidate the role of NOXs in cancer.

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P26

CRYSTALLIZATION AND STRUCTURE DETERMINATION OF NEW ENE-REDUCTASES

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Ene-reductases (ERs) (EC 1.6.99.1) from the Old Yellow Enzyme (OYE) family are versatile flavin-dependent catalysts that have been applied to the asymmetric *trans*-reduction of a broad range of activated C=C-bonds at the expense of NAD(P)H cofactor [1]. These flavoenzymes exhibit a remarkably broad range of substrate acceptance, catalyzing the reduction of -unsaturated ketones, aldehydes, carboxylic acids and derivatives (such as esters, lactones, cyclic imides), nitriles and nitro compounds, yielding products with a variety of biotechnological and pharmaceutical applications [2].

Here we report the crystal growing conditions and the three-dimensional structures of seven new ene-reductases from different organisms (*i.e.* bacteria, fungi and algae). Till now five ene-reductases belonging to the "classical" and two belonging to the "thermophilic-like" ERs subclasses have been crystallized and their structure solved with good to moderate resolution.

The different strategies developed for their crystallization and the structural peculiarities of the different ERs subclasses are also discussed.

In order to understand biocatalysts stereoselectivity and the key interactions with substrates and cofactors, attempts to soak crystals with NAD(P)H analogues and different compounds are also reported.

P27

INITIAL CRYSTALLIZATION STUDIES OF BTLA PROTEIN IN COMPLEX WITH PEPTIDE BASED INHIBITORS OF BTLA/HVEM COMPLEX FORMATION

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Melanoma is considered to be one of the most dangerous skin cancers that occurs in humans. It usually develops from naturally occurring pigment cells – melanocytes which undergone inappropriate transformation [1]. The constantly increasing incidence of melanoma causes that this disease remains a big challenge for contemporary oncology. The promising way of inhibiting the development of cancer seems to be a therapy based on blocking immune checkpoints, e.g. HVEM/BTLA. B and T lymphocyte attenuator (BTLA) is an immunoglobulin-like receptor which highest expression was observed on the surface of B lymphocytes and $CD4^+$ T cells [2]. BTLA has the ability to form complex with the HVEM (Herpesvirus entry mediator) protein present, for example, on the cells surface of melanoma. Complex between HVEM/BTLA proteins inhibits the activation of $CD8^+$ T lymphocytes. Blocking the interaction between these two receptors with small molecules (peptides or peptidomimetics) can be the way to stimulate the body's immune response.

Up to now the crystal structure of the full-length HVEM protein and its ligands has not been determined. However in 2005 the crystal structure of complex of

extracellular domains of BTLA and HVEM was solved. This structures shows that BTLA binds to the N-terminal domain of HVEM, specifically to region 2633 which is in Cysteine Rich Domain 1 (CRD1) [3]. This information allowed to design and characterize peptide-based inhibitors of BTLA/HVEM complex formation.

The development of the efficient method of expression and purification of human BTLA was necessary to determine crystal structure of BTLA in complex with peptide based inhibitors of BTLA/HVEM complex formation. Protein used for crystallization was expressed in *E.coli* and purified using affinity column followed by size exclusion chromatography. Peptides were synthesized by solid-phase peptide synthesis method.

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P28

CRYSTAL STRUCTURES OF THE CELL WALL PROTEINS CWP8 AND CWP6 FROM *CLOSTRIDIUM DIFFICILE*

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The cell wall of Gram-positive bacteria is a surface, exoskeletal organelle composed of peptidoglycan, secondary polymers and a wide variety of proteins. Several types of cell wall proteins are non-covalently attached via cell wall binding domains in some cases forming surface protein layers (S-layers). Of the two conserved S-layer-anchoring modules composed of three tandem SLH or CWB2 domains, the latter have so far eluded structural insight. In *C. difficile* 630, one of the most important nosocomial pathogens causing antibiotic-associated diarrhea that can lead to potentially lethal pseudomembranous colitis, there are 29 cell wall proteins (CWPs), including the major S-layer precursor, SlpA, sharing the CWB2 module. We have recently reported the crystal structures of multi-domain cell wall proteins Cwp8 and Cwp6 that revealed a triangular trimer of 2x2 Rossmann-fold CWB2 domains and the structural basis of the intact module fold necessary for its binding to the cell wall substantiating previous reports that unperturbed three tandem CWB2 repeats mediate non-covalent binding to the anionic secondary polymer PSII. The trimer binding arrangement contains highly conserved residues and is shared between CWB2 and the SLH S-layer/cell wall-anchoring modules suggesting a common or convergent evolutionary origin. Additional structural insight into the molecular organization of *C. difficile* cell wall is provided by showing partial structural similarity of the N-terminal parts of Cwp8 and SlpA. Based on biochemical characterization of Cwp6 and its C-terminal domain structural alignment with PDB deposites, we also conclude that Cwp6 is a zinc-dependent N-acetylmuramoyl-L-alanine amidase belonging to an Amidase_3 (PF01520) family. A comparison with previously reported EM and AFM data of S-layers suggests that *C. difficile* S-layers are complex oligomeric structures, likely composed of several different proteins.

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OPTIMIZATION OF THE CRYSTALLIZATION BENCH FOR CRYSTALLIZING THE MEMBRANE PROTEINS

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While large volume crystals (~ mm^3) are required for neutron protein crystallography, small uniform-size microcrystals (~ 10 - 20 m) are used for time-resolved experiments in X-ray protein crystallography. In case of membrane proteins finding suitable crystallization conditions represent one of the most challenging parts. To overcome this obstacle the crystallization bench was designed in our lab a few years ago. It uses dialysis method while allowing the variation of the temperature and the chemical composition during the experiment to find the best condition of crystallization of globular proteins [1]. The goal of my work is to develop the current crystallization bench as well as the rational optimization strategies of crystal growth based on knowledge of equilibrium phase diagrams for crystallization of membrane proteins.

We purified successfully two model membrane proteins, ShuA and AcrB, both expressed in Escherichia coli. As an example, for AcrB, we started with 4 litres (Luria Broth medium) of culture of E.coli cells. We induced the expression of AcrB by adding IPTG, we disrupted the cells with the Microfluidizer to isolate the membrane, and finally, we purified the protein by HisTrap column. At the end of the purification procedure, SDS-PAGE was used to evaluate the purity of the proteins. 7.2 mg of pure Acr B has then been used in initial crystallization screening to obtain first crystals. This has been done by conventional crystallization techniques (vapour diffusion). Currently, we are optimizing its crystallization by reducing the number of crystals per volume of crystallization solution. We will reproduce crystallization experiments in order to find the best crystallization conditions for our membrane models and the crystallization bench will be used to obtain suitable crystals for both the neutron macromolecular crystallography and fast (sub ms) time-resolved X-ray crystallography.

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P30

STRUCTURAL INSIGHTS INTO THE MECHANISM OF CLIC1 MEMBRANE INSERTION

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The Chloride Intracellular Channel (CLICs) proteins consist of a family of metamorphic proteins that exist in an equilibrium between a soluble and a membrane-bound state. The alteration of CLIC function has been involved in ischemia-reperfusion and different forms of cancer. CLIC1 has been directly linked with glioblastoma proliferative capacity. It can be found as a chloride channel or as a soluble reduced form with oxidoreductase activity. CLIC1 inhibitors block both its ion channel function and oxidoreductase activity. However, it is unknown what form or forms of the protein are relevant in the context of healthy and glioblastoma cells and how the equilibrium between them is affected in disease. In this study, we have assigned 90% of the backbone resonances and measured backbone dynamics in different conditions relevant to CLIC1 activity. The secondary structure in solution follows the X-ray structure. However, the region around helix 7 in the C-terminal domain displays elevated fast-time dynamics. We have also observed that pH produces alterations in the backbone dynamics both in the N and C terminal domains, modifying the overall shape of the protein as we have observed by SAXS, in particular the region around helix 7. Lipids titrations show that CLIC1 interacts with lipids with very different affinities. We have solved the X-ray structure of CLIC1 in the presence of lipids, and whereas the overall structure is similar to the soluble reduced monomer, the region around helix 7 is in a different orientation, suggesting that this region may be involved in the interaction with lipid membranes in a first step towards the insertion on the membrane.

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STRUCTURE OF CVHSP AND ITS CO-ASSEMBLY WITH OTHER MAMMALIAN MALL HEAT-SHOCK PROTEINS

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Cardiovascular heat shock protein (cvHsp) is expressed in cardiomyocytes shortly after myocardial infarction and in other muscle tissues. The cellular role of cvHsp is still unclear but its knockout can cause the collapse of skeletal muscle sarcomeric structure [1]. As a member of the small heat-shock protein (sHsp) family, it comprises a central "a-crystallin domain", flanked by flexible N- and C-terminal regions. SHsps have the tendency towards hetero-oligomerisation within the family, while the roles of those oligomers are to be revealed [2]. Previous biochemistry studies suggest that dimerised filamin C (FLNC) may be a potential interaction substrate of cvHsp [3].

Very little is known about the structure of cvHsp. Here I present native mass spectrometry (MS) data and preliminary structural studies to investigate the characteristics of cvHsp and its interactions with other sHsp family members. Unlike the other human sHsps, we find that cvHsp does not oligomerise via the assembly of dimers. Trace amount of dimers only appear above pH 7.5, and the majority of cvHsp remains to be monomeric (Fig. 1). We generated truncated constructs lacking the N- and C-terminal regions, or just the N-terminal region to study the co-assembly of cvHsp in detail as well as the crystal structure. We found that the core segment co-assembles with the cores of other sHsps, Hsp27 and B-crystallin. Although cvHsp can form an oxidised dimer, interactions between the heterodimers are confirmed to be non-covalent by collision induced dissociation (CID), which is a gas phase activation method in native MS. Trimers are also observed for both truncated constructs, which means further sequence optimisation is needed through proteolysis for the formation of crystal.

Future work will focus on determining the binding substrates of cvHsp and further structural study on cvHsp.

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EXPRESSION, PURIFICATION AND BIOPHYSICAL CHARACTERIZATION OF SELECTED PYRIDOXAL PHOSPHATE SYNTHASE COMPLEXES

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In terms of interdisciplinary research activities, we plan to analyse the structure-function and dynamics of selected pyridoxal phosphate synthase complexes of the vitamin B_6 metabolism, which are potential targets for structure based drug discovery investigations to treat infectious diseases. The investigations will focus in parallel and comparative, applying complementary bioanalytical and biophysical methods, on pyridoxal-5-phosphate (PLP)-synthase complexes of: *Plasmodium vivax*, *Mycobacterium tuberculosis* and methicillin resistant *Staphylococcus aureus* (MRSA), with a strong focus applying pulsed synchrotron- or XFEL radiation to perform time resolved structural investigations and utilizing data about the already published crystal structure of *Plasmodium berghei* (Pdx1) *and Plasmodium falciparum* (Pdx2) chimeric complex [1].

Malaria is still one of the leading causes of morbidity and mortality in developing countries, especially in Africa, but also in Asia and Latin America, caused by *Plasmodium* species [1]. Tuberculosis is a widespread and deadly infectious disease caused by *Mycobacterium tuberculosis* [2]. MRSA infections are known also as nosocomial and antibiotic resistant infections, causing worldwide a high number of fatal cases and known to be a substantial threat to global health [3].

The term vitamin B_6 collectively refers to the compounds: pyridoxal, pyridoxine, pyridoxamine and their related phosphate esters. The metabolically active forms are pyridoxal-5-phosphate (PLP) and pyridoxamine-5-phos-

phate (PMP). PLP is known as nature's most versatile cofactor and is involved in more than 100 enzymatic reactions [4]. Vitamin B_6 is synthesized through consecutive reactions mediated by the pyridoxal-5-phosphate (PLP)-synthase complex consisting of pyridoxal biosynthesis lyase PdxS (Pdx1) and glutamine amidotransferase PdxT (Pdx2) [2]. Pdx1 exist as hexamer-dodecamer equilibrium in solution. The dodecamer is formed by two interdigitating rings, each consisting of six Pdx1 molecules. Pdx2 subunits attach to the Pdx1 oligomer, and fully decorated complexes containing 12 Pdx2 subunits attached to a Pdx1 dodecamer [5].

We validated and established the bacterial expression and purification of both proteins of *Plasmodium vivax,* first as model system in preparation for crystallization experiments. Analysis of the recombinant proteins by size exclusion chromatography and Dynamic Light Scattering (DLS) revealed that Pdx1 is monodisperse and dodecameric in solution, whereas, Pdx2 appears as both in monomeric and multimeric forms, with a tendency to aggregate with time.

Interestingly, by applying monodisperse Pdx1 to Pdx2 aggregates a stable monodisperse complex validated by a DLS with radius distribution (R_H) of 9.7 1.3 nm and a corresponding Mw of approx. 600 KDa was observed. Showing a strong and unique interaction between Pdx1 and Pdx2, independently of the oligomerization state of Pdx2. Beside crystallization experiments we plan to analyse the interaction between the two proteins by time-resolved SAXS/SEC-SAXS, which will provide new insights into the structure and functional dynamicity of the complex enzyme machinery. Details will be presented.

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