
Session 3. Proteins — Structure and Function

Lectures

L3.1

The mechanism of RNase H activity in DNA repair and reverse transcription

Marcin Nowotny

International Institute of Molecular and Cell Biology, Laboratory of Protein Structure, Warsaw, Poland

e-mail: Marcin Nowotny <mnowotny@iimcb.gov.pl>

RNases H are nucleases that cleave ribonucleotides in RNA/DNA hybrid duplexes. Two classes of these enzymes have been described — RNases H1 and RNases H2. The first requires a stretch of at least four ribonucleotides in the hybrid for the cleavage to occur. In addition to its cellular form it also exists as a domain of reverse transcriptases — enzymes converting single-stranded RNA to double-stranded DNA. Reverse transcriptases participate proliferation of retrotransposons and retroviruses such as HIV and RNase H activity is essential for this process. The second type is RNase H2 which specifically cleaves single ribonucleotides embedded in the DNA. Such single ribonucleotides are mistakenly incorporated by DNA polymerases and lead to genome instability. Therefore, ribonucleotide excision by RNase H2 protects the genomic DNA. In the talk the mechanisms of various RNases H will be discussed as elucidated based on crystal structures of RNases H2 and reverse transcriptases complexed with their nucleic acid substrates. RNase H2 utilizes a unique mechanism in which two divalent metal ions are coordinated at the active site. The RNA-DNA junction present in the substrate is specifically deformed so that its phosphate group participates in metal ion coordination. Therefore, only in the presence of the proper substrate the metal ions can be bound and promote catalysis which assures enzyme's specificity.

For reverse transcriptases an important part of their mechanism is the regulation of the RNase H activity. Our crystallographic and SAXS studies showed that for example in the monomeric reverse transcriptase from XMRV virus the RNase H domain is very mobile and gets organized only when the proper RNA/DNA substrate is present. Very different mechanisms exist for dimeric reverse transcriptases such as the HIV enzyme and the enzymes from retrotransposons. These mechanisms will also be discussed.

L3.2

Crystal structures of two neurotoxic phospholipases A₂ and identification of their interaction sites with various biological targets

Grazyna Faure

Institut Pasteur, Unité Récepteurs – Canaux, Department of Neuroscience, France

e-mail: Grazyna Faure <grazyna.faure-kuzminska@pasteur.fr>

Snake venoms are a rich source of phospholipases A₂ (PLA₂) with distinct biological functions and with a high potential for medical and pharmaceutical applications.

We focus here on structural and functional studies of two neurotoxic PLA₂, the monomeric ammodytoxin from *Viper ammodytes ammodytes*, and heterodimeric crotoxin from *Crotalus durissus terrificus*. Recently we have determined at high resolution their three-dimensional structures. These two enzymes are i) potent β -neurotoxins which target neuronal or peripheral presynaptic receptors and ii) potent anticoagulant molecules, which directly interact with human coagulation factor Xa. Comparative structural analysis of PLA₂ isoforms allowed us to detect local conformational changes and delineate the role of critical residues in the anticoagulant and neurotoxic functions of these neurotoxic PLA₂ (Saul *et al.*, 2010). The crystal structure of crotoxin revealed details of the binding interface between its acidic (CA) and basic (CB) subunits and helps us to understand the role played by critical residues of the CA subunit in the increased toxicity and stability and reduced enzymatic activity of the crotoxin complexes (Faure *et al.*, 2011).

More recently, we identified a new target of these PLA₂ and described their novel function as a potential pharmacological effector of human Cystic Fibrosis Transmembrane Regulator (CFTR) Cl⁻ channel activity (Faure *et al.*, submitted). We demonstrated the physical and functional interaction of wild-type and mutated CFTR with PLA₂. A 3D structural model of the complex will be presented. The potentiating and correcting effects of CB on mutated CFTR (the $\Delta F508$, most frequent mutation responsible of cystic fibrosis) renders this PLA₂ attractive for potential clinical applications and offers an original perspective to investigate the pharmacotherapy of CF. An overview of these new data will be presented.

References:

- Saul FA *et al* (2010) *J Struct Biol* **169**: 360–369.
Faure G *et al* (2011) *J Mol Biol* **412**: 176–191.

L3.3

Structural and thermodynamic studies of ligand binding by bovine β -lactoglobulin

Krzysztof Lewiński¹, Joanna Loch¹, Piotr Bonarek², Agnieszka Polit²

¹Faculty of Chemistry, Jagiellonian University, Kraków, Poland; ²Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland

e-mail: Krzysztof Lewiński <lewinski@chemia.uj.edu.pl>

Bovine β -lactoglobulin (LGB), is a dimeric protein being one of the major components of bovine whey. Due to its ability to bind wide range of hydrophobic compounds, LGB is an object of intensive studies, since its discovery in 1934. The primary binding site is located in the centre of eight-stranded β -barrel and is surrounded by flexible loops. The conformational change of EF loop at pH around 7.0 regulates access to the binding site. Existence of other binding sites located on dimer interface was postulated by some authors but confirmed only in the structure of LGB-vitamin D3 complex. Physiological function of LGB has not been recognized. It is probably involved in transport of hydrophobic compounds and regulation of enzymatic activity of pregastric lipase. Potential utilization of modified LGB in clinical applications as molecular transporters requires better understanding of its binding properties and role of individual residues in the binding site. For that purpose, we have undertaken systematic structural and thermodynamic studies of LGB complexes with different ligands. Among studied ligands were 8- to 18-carbon saturated fatty acids, 18-carbon unsaturated linoleic and oleic acid as well as dodecyltrimethylammonium chloride (DTAC) and sodium dodecyl sulfate (SDS).

The crystal structures were typically determined with resolution 2.2–1.9 Å. In all structures ligand molecule was bound only in the β -barrel. We have find that position of ligand polar group strongly depended on length of the aliphatic chain, indicating that competition between polar and hydrophobic interactions is an important factor determining location of ligand in the binding site. It is interesting, that in case of 12-carbon ligands (lauric acid, SDS, DTAC) the chemical character of ligand polar group did not affect the position of its aliphatic chain, indicating that hydrophobic interactions prevailed even with the repulsion between positively charged DTAC and lysine residues located at the binding site entrance. The thermodynamic parameters have been determined by isothermal titration calorimetry. They revealed that binding of ligands is entropically driven and entropic component of ΔG increases with elongation of aliphatic chain.

The complexes of LGB with SDS, DTAC and lauric acid were studied for two most abundant LGB isoforms, A and B, to determine influence of substitutions D64G and V118A on crystal structure and thermodynamics of ligand binding.

L3.4

Monster crystal structure with 28 independent protein molecules

Mariusz Jaskolski^{1,2}, Joanna Sliwiak¹, Zbigniew Dauter³, Airlie McCoy⁴, Randy J. Read⁴

¹Center for Biocrystallographic Research, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznań, Poland; ²Department of Crystallography, Faculty of Chemistry, A. Mickiewicz University, Poznań, Poland; ³Synchrotron Radiation Research Section, National Cancer Institute, Argonne National Laboratory, Argonne, USA;

⁴Department of Haematology, University of Cambridge, Cambridge, UK
e-mail: Mariusz Jaskolski <mariuszj@amu.edu.pl>

Stress factors, such as pathogens, induce in plants the expression of so-called Pathogenesis-Related (PR) proteins, which have been divided into seventeen classes. PR proteins of class 10 (PR-10) are mysterious proteins with no unique biological function attributed to them despite their abundance and involvement in other processes, such as developmental regulation or symbiosis. Recent results, mostly from our group, strongly implicate the PR-10 proteins in phytohormone binding and regulation. The ligand binding occurs in a large cavity that is present in the hydrophobic core of the protein. PR-10/hormone complexes are studied *inter alia* using fluorescent probes, such as ANS. We have crystallized an ANS complex using a PR-10 protein from St John's wort (Hyp-1) with a controversial implication in the biosynthesis of hypericin. Solution of the apparent $P4_122$ crystal structure was impossible by standard molecular replacement methods because of evident tetrahedral twinning and an extremely bizarre modulation of the reflection intensities hkl with l periodicity of 7, which indicated seven-fold non-crystallographic translation along c . Ultimately, the structure was solved using Phaser after data expansion to $P1$ symmetry, with as many as 56 Hyp-1 molecules in the unit cell. Analysis of their arrangement revealed the true $C2$ space group with 28 independent protein molecules, arranged in 14 dimers around a non-crystallographic 2_1 screw along c with a pitch of $1/7$. The monstrous crystal structure has been successfully refined to an R-factor of 21.8%, revealing the presence of three ANS binding sites in each Hyp-1 molecule, two of which are located within the internal cavity, and one in a surface pocket. There are also numerous interstitial ANS molecules. From the crystallographic point of view, this is a rare example of a commensurately modulated protein structure, with seven-fold repetition of the basic unit cell in the c direction. This crystal structure holds a record in the PDB from the point of view of the complexity of the asymmetric unit contents.

L3.5

Structural and functional analysis of EcoG65I restriction-modification system from clinical isolate *Escherichia coli* G65

Martyna Franczuk, Anna-Karina Kaczorowska, Tadeusz Kaczorowski

University of Gdańsk, Department of Microbiology, Gdańsk, Poland
e-mail: Martyna.Franczuk <martyna.franczuk@gmail.com>

Mobile genetic elements play a crucial role in horizontal gene transfer (HGT) by facilitating spread of genes coding for antibiotic resistance, toxins or other virulence factors. Comparative genomes analysis revealed that HGT is one of the most important mechanism shaping genetic variability among microorganisms. Restriction-modification (RM) systems studied in our laboratory affect HGT by modulating the flow of genes among bacteria. There is increasing number of evidences suggesting that this function is as much important as involvement of the RM systems in protection of microorganisms against phage infection.

The aim of our study is structural and functional analysis a RM system EcoG65I of pathogenic strain of *Escherichia coli* G65. This clinical isolate was analyzed in our laboratory using PCR as a serotype O26, positive for virulence determinants *eaeA* (intimin) and *hlyA* (hemolysin A) characteristic for verotoxigenic strains of *E. coli*. The EcoG65I RM system consists of two enzymatic entities. The first is restriction endonuclease (R.EcoG65I) which recognizes specific nucleotide sequence 5'-CTGCAG-3' and cleaves it best at 37°C between adenine and guanine residue leaving 3' four-nucleotide protruding ends. The second constituent is DNA methyltransferase (M.EcoG65I) which modifies target sequence and thus protects bacterial DNA against cleavage by the cognate endonuclease. In this respect EcoG65I enzymes are isospecific to PstI RM system of *Providencia stuartii*. The genes coding for EcoG65I RM system have been cloned, overexpressed and their nucleotide sequence was determined. The genes are transcribed in the same orientation, with the gene coding for M.EcoG65I (*ecoG65IM*) preceding the gene coding for R.EcoG65I (*ecoG65IR*). The two genes overlap by four nt. M.EcoG65I is predicted to be 489 aa in length (54990,9 Da); the R.EcoG65I is predicted to be 317 aa (35062,9 Da). The overall G+C content of these genes is 52,5% (51,6% for *ecoG65IM* and 53,4% for *ecoG65IR*) which is close to *E. coli* genomic DNA (50,7%). In order to determine how codon usage pattern of the EcoG65I RM genes differs from that of *E. coli*, the codon adaptation index (CAI) was calculated. The CAI values obtained (0,27 and 0,25 for the *ecoG65IM* and *ecoG65IR*, respectively) indicate that both genes belong to the subset of *E. coli* genes that were most probably acquired by horizontal gene transfer.

Amino-acid sequence of R.EcoG65I shows similarity to isospecific enzymes: R.BsuBI (69%), R.BbrUIII (44%), and R.PstI (42%). Whereas aa sequence of M.EcoG65I shows significant similarity to Eco57I RM enzyme (83%), M.BsuBI (52%) and M.PstI (42%). The presence and distribution of highly conserved nine aa sequence motifs and a putative target recognition domain in the enzyme structure suggest that M.EcoG65I belongs to the exocycling DNA methyltransferases that modify adenine residues at N6 position.

L3.6

A novel highly thermostable lysozyme, a PGRP-like amidase from *Thermus bacteriophage* Ph2119

Magdalena Plotka¹, Aleksandra Stefanska¹, Anna-Karina Kaczorowska¹, Gudmundur O. Hreggvidsson², Sławomir Dabrowski³, Janusz M. Bujnicki⁴, Tadeusz Kaczorowski¹

¹University of Gdańsk, Department of Microbiology, Gdańsk, Poland, ²Matis, Icelandic Food and Biotech R&D, Iceland, ³A&A Biotechnology, Poland, ⁴International Institute of Molecular and Cell Biology, Laboratory of Bioinformatics and Protein Engineering, Warsaw, Poland
e-mail: Magdalena.Plotka <mplotka@biotech.ug.gda.pl>

Nucleic acids based technologies now underpin a large and fast-growing industry, including research, diagnostics and pharmaceutical production. Thermostable enzymes have played a key role in this development and polymerases (DNA and RNA), ligases, nucleases and lysozymes are of great importance in the research industry today.

Here, we present characterization of highly thermostable lysozyme from bacteriophage Ph2119 infecting *Thermus scotoductus*. The phage strain Ph2119 and its host, *Th. scotoductus* strain 2119 was isolated from a sample taken at Hrafninnuskur, which is located in the highlands of Iceland, near the volcano Katla. Its optimum growth temperature is 65°C. The phage 2119 sequencing resulted in 212 contigs and the bioinformatics analysis revealed the existence of a 468-bp open reading frame (ORF) encoding a protein which shows similarity to eukaryotic Peptidoglycan Recognition Proteins (PGRPs), a family of proteins homologous to T3 and T7 bacteriophages lysozymes.

The tertiary structure of the Ph2119 protein is similar to *Drosophila melanogaster* Peptidoglycan Recognition Proteins (PGRPs): PGRP-Lb, PGRP-Le and PGRP-Sa which all recognize bacterial peptidoglycan. Additionally, PGRP-Lb has the ability to directly destroy bacterial peptidoglycan by hydrolysing the peptidoglycan amide bond. Hence, we decided to explore the possibility that the novel ORF product possesses the peptidoglycan lytic activity.

The Ph2119 lysozyme gene was synthesized and the recombinant protein was purified from *E. coli* TUNER (DE3) harbouring plasmid pET15b [Ph2119lysozyme] using Ni²⁺ affinity chromatography, yielding 34 mg per 1 litre of culture. On SDS-PAGE, the purified recombinant protein migrated as a single band with an apparent molecular mass of 19.7 kDa.

Ph2119 lysozyme bacterial lytic activity was tested. Ph2119 enzyme was mainly active against Gram-negative bacteria including: extremophile *Thermus thermophilus* HB8 (100%), *Escherichia coli* (34%), *Salmonella panama* (10%) and *Pseudomonas fluorescense* (13%). Ph2119 lysozyme showed no activity against Gram-positive bacteria with the exception of *Bacillus cereus* (15%). It is noteworthy that Ph2119 lysozyme can lyse Gram-negative bacteria from without, what is very unique among lysozymes and perhaps is associated with the presence of amphipathic characteristics at the C-terminal end of the protein. Ph2119 lysozyme was found to be highly thermostable, it maintained approx. 87% of enzyme activity after 6 h incubation at 95°C. Optimum working pH was determined as 8.0.

As Ph2119 lysozyme effectively lyses the *Thermus thermophilus* sp. without the necessity of adding outer membrane permeabilization agents, it can be used in spheroplasts preparation, for effective genomic/plasmid DNA isolation and, most importantly, in enzymes preparations from thermophilic bacteria.

Oral presentations

03.1

Nuclear transport receptor CRM1

Anna Kozakiewicz¹, Thomas Monecke²,
Piotr Neumann², Ralf Ficner²

¹Faculty of Chemistry, N. Copernicus University, Poland; ²Institute of Microbiology and Genetics (GZMB), Georg-August-University Göttingen, Germany
e-mail: Anna Kozakiewicz <akoza@chem.umk.pl>

The exchange of macromolecules between the cell nucleus and the cytoplasm is tightly restricted by the nuclear pore complexes (NPCs). Macromolecules such as RNA and proteins require association with karyopherins called importins to enter the nucleus and exportins to exit. Proteins that must be imported into the nucleus from the cytoplasm carry nuclear localization signals (NLSs) that are recognized by importins. Proteins, transfer RNA, and assembled ribosomal subunits are exported from the nucleus due to association with exportins, which bind signaling sequences called nuclear export signals (NESs). The ability of both, importins and exportins, to transport their cargoes is regulated by the small GTPase Ran. In its GTP bound state, Ran is capable of binding karyopherins. Importins release cargo upon binding to RanGTP, while exportins must bind RanGTP to form a ternary complex with their export cargo. The dominant nucleotide binding state of Ran depends on whether it is located in the nucleus (RanGTP) or the cytoplasm (RanGDP) [1].

CRM1 is a member of the importin β superfamily of nuclear transport receptors. It mediates nuclear export of numerous cargoes through the recognition of the leucine-rich nuclear export signal (LR-NES). CRM1 is unusual among karyopherins in that it has a cargo-binding site on its outer surface, but this is important for CRM1 to carry a broad range of cargoes that vary greatly in size and shape, including huge cargoes such as ribosomal subunits. In contrast to the cargoes that bind to the outer surface of CRM1, RanGTP binds to the inner surface of CRM1 [2]. CRM1 binds cooperatively to RanGTP and its export cargo, leading to the formation of a trimeric transport complex in the nucleus [1]. Many amino acids of Ran participate in the interaction with the CRM1, among others K37 and K38. To check whether these amino acids are important, firstly Ran was acetylated on these lysine residues (Ran-Q69LK37/K38Ac) and then trimeric complexes (CRM1-RanQ69LK37/K38Ac-SPN1) were generated.

References:

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03.2

A novel *in silico* simulation approach to identify selective STAT1 inhibitory compounds to treat atherosclerosis

Małgorzata Szeląg¹, David Schaller¹, Anna Czerwonec²,
Krzysztof Sikorski¹, Joanna Wesoły³, Hans A.R. Bluysen¹

¹Department of Human Molecular Genetics, ²Bioinformatics Laboratory, ³Laboratory of High Throughput Technologies, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University in Poznań, Poznań, Poland
e-mail: Małgorzata Szeląg <grete@amu.edu.pl>

Signal transducers and activators of transcription (STATs) comprise a family of transcription factors that are structurally related and which participate in signaling pathways activated by cytokines, growth factors and pathogens. Crucial for STAT activation is the Src homology 2 (SH2) domain, which is highly conserved among the STAT proteins and interacts with phosphorylated tyrosine motifs for specific contacts between STATs and receptors and for STAT dimerization. Ligand-induced receptor pTyr binding by the SH2 domain of STATs allows for phosphorylation of the protein at the cytoplasmic domain of the receptor and subsequent formation of transcriptionally active STAT-homodimers that move into the nucleus and activate transcription.

STAT1 has been implicated in a number of inflammatory and autoimmune diseases. We and others previously identified STAT1 as a point of convergence for cross-talk between different pro-inflammatory stimuli in immune and vascular cells, implicating this transcription factor in development of cardiovascular diseases.

Therefore we decided to search for STAT1-targeting compounds based on computational docking studies exploring the phosphotyrosine 701/SH2 interaction area of STAT1. For this purpose, based on existing crystal structures of human STAT1 and mouse STAT3, we generated new hSTAT1, hSTAT2 and hSTAT3 protein models. Accordingly three functional cavities could be recognized in the SH2 domain: pY+0, pY+1 and pY-X that can be targeted by inhibitors. Our studies of Stattic, known STAT3 inhibitor and Fludarabine, known STAT1 inhibitor revealed that compounds targeting only pY+0 and pY-X demonstrate cross-binding specificity between STAT1, STAT2 and STAT3.

In search of novel specific STAT1-SH2 inhibitors, we performed comparative *in silico* screens of small-compound libraries and discovered three lead compounds, which in ECs *in vitro* inhibited phosphorylation of hSTAT1 more effectively than phosphorylation of hSTAT3. Subsequent rescreen of drugable compound libraries, using these leads as templates identified six potential candidates, targeting pY+0, pY-X and pY+1 cavities of hSTAT1 and not of hSTAT2 and hSTAT3, making them ideal candidates for specific STAT1 inhibition.

We developed a novel comparative virtual screening approach to identify specific hSTAT1-SH2 domain inhibitors, that can be used as a novel strategy in treatment of atherosclerosis.

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03.3

Conversion of 5'-O-monothiophosphate nucleosides to 5'-O-monophosphate nucleosides under cellular conditions — interaction of NMPS with Hint1 enzyme

Agnieszka Krakowiak, Róża Pawłowska,
Beata Rębowska, Wojciech J. Stec

Centre of Molecular and Macromolecular Studies PAS, Department of
Bioorganic Chemistry, Poznań, Poland
e-mail: Agnieszka.Krakowiak <akrakow@bio.cbmm.lodz.pl>

Histidine triad nucleotide binding proteins (Hints) are the most ancient members of the histidine triad protein superfamily (HIT proteins) of nucleotidyl transferases and hydrolases. *In vitro* Hint-1 enzyme exhibits substrate specificity towards many compounds containing different type of phosphate linkages such as: phosphoramidates (P-N), phosphates (P-O), phosphorothioates (P-S) and phosphorfluoridates (P-F). We have found that Hint1 can hydrolyze the P-S bond in adenosine 5'-O-monophosphorothioate (AMPS) at the rate of 0.2 nmol/min/μg, what is about 40-times faster than the Fhit enzyme does and, moreover, it is also able to convert other (d)NMPS to (d)NMP under *in vitro* conditions by mechanism similar to the P-N bond cleavage in phosphoramidate substrates. [1], [2] Recently, phosphorothioate modification of DNA has been identified in bacteria so, the ability to hydrolyze P-S bond in different (d)NMPS by this protein has become more significant. Furthermore, synthetic oligonucleotide phosphorothioates have been developed as antisense probes for genomic research and medicinal applications. Their hydrolysis in plasma, kidney, and liver proceeds mainly from the 3' end, resulting in the appearance of the nucleoside 5'-monophosphorothioates. The (d)NMPS may exert cytotoxic effects affecting cell proliferation, DNA or RNA synthesis, and other so far unknown processes, so the knowledge about their metabolism seems to be of more wide interest.

Because *Hint-1* orthologs were found from prokaryotes to eukaryotes, we hypothesize that this protein could be the enzyme responsible for the (d)NMPS metabolism *in vivo* and that this process undergoes *via* their conversion to (d)NMP.

To prove this assumption, we synthesized non-hydrolysable phosphorothioate dinucleoside derivative Br5dUO-3'-PS-OCH₂CH₂CH₂O-PS5'-OA, which contains 5-bromo-2'-deoxyuridine (Br5dU), the residue able to make a covalent bond in nucleic acid-protein complexes by means of photocrosslinking (at 300 nm). The compound consists of four diastereoisomers due to chirality at both phosphorus atoms. We have found that isomer R_pR_p forms complex with the recombinant Hint1 the most efficiently. Similar complexes (probably with Hint1 enzyme) are formed with proteins present in cellular extracts obtained from A549 cells (Hint1⁺, Fhit⁻). In the next step, we have generated siRNAs directed towards HINT1 mRNA and used them to transfect A549 cells. Lysates of these cells were used in experiment with AMPS and the level of desulfuration was measured. The decreased level of the AMPS desulfuration was observed with cell lysate in which Hint1 was silenced as compared to the extent of the above reaction under control condition (cell lysates not transfected with HINT1-specific siRNAs).

Above results allow for conclusion that intracellular Hint1 enzyme can be the protein responsible for the desulfuration process of nucleoside- 5'-monophosphothioate *in vivo*. Undoubtedly, it participates in metabolism of (d)NMPS.

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03.4

Improving PCR specificity by use of RadA protein from *Pyrococcus woesei*

Aleksandra Stefańska¹, Sławomir Dąbrowski², Tadeusz Kaczorowski¹

¹University of Gdansk, Department of Microbiology, Gdańsk, Poland, ²A&A Biotechnology, Gdynia, Poland
e-mail: Aleksandra.Stefanska <stefanska@biotech.ugda.pl>

Background: Strand exchange catalysts play a pivotal role in homologous recombination — universal reaction catalyzed by RecA homologues in all domains of life[1]. Our present studies involve cloning, purification and characterization of a series of such proteins; one of them is RadA from hyperthermophilic archaeon *Pyrococcus woesei*. The protein of 38.4 kDa was reported to exhibit DNA-dependent ATPase, D-loop formation and strand displacement activities[2]. Characteristic ability of pairing homologous DNA sequences with high specificity together with great thermostability of the enzyme encouraged us to test its influence on PCR specificity.

Materials and Methods: Gene encoding RadA was amplified by PCR and cloned into pET15b vector under strong T7 promoter resulting in a fusion of RadA with N-terminal His-tag. Expression in BL21(DE3) pRARELys strain provided high yield of soluble protein. Purification was carried on TALON[®] affinity resin. Different amounts of purified RadA together with optimized ATP concentration of 400 μ M were tested in model PCR, where a 650 bp long fragment of Human DNA was chosen as a target for amplification. Major problems with efficiency and specificity in case of standard reaction enabled testing potential positive effect of RadA activity.

Results and Conclusion: 0.1 μ g of RadA per 25 μ l of reaction volume resulted in almost total loss of unspecific products while the target sequence of 650 bp was amplified with efficiency much higher than in case of the standard reaction. ATP itself had no positive effect on the specificity, nor the efficiency of target amplification. Providing positive results of subsequent RadA tests in multiplex PCR, the protein could be used to solve problems with diagnostic PCR, where specificity is a crucial issue.

Acknowledgements:

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References:

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Posters

P3.1

Protective effect of Trolox against storage lesions in red blood cells for transfusion

Adam Antosik, Natalia Cichon, Magdalena Szejka, Michał Bijak, Paweł Nowak, Halina M. Zbikowska

Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Łódź, Łódź, Poland
e-mail: Adam.Antosik <adama@biol.uni.lodz.pl>

The quality of stored red blood cells (RBCs) has improved with the use of anticoagulant/additive solutions. However, these storage conditions do not fully preserve viability and function of RBCs. There is evidence that oxidative damage to lipids and proteins can contribute to RBC injury during storage which can be manifested by, i.e. enhanced lipid peroxidation, reduced glutathione (GSH) and haemoglobin (Hb) concentrations, increased activity of extracellular lactate dehydrogenase (LDH) and haemolysis. Vitamin E is an essential nutrient for humans and a powerful hydrophobic antioxidant. It is a scavenger of peroxy radical that protects membrane lipids and other vital cellular molecules.

The aim of the study was to investigate the effects of Trolox (a water soluble analogue of vitamin E) on changes generated in the stored RBCs. The cells were stored at 4°C in the presence of Trolox (1–125 μ M) or without the antioxidant (control). At the 1-, 10- and 20-day of storage the level of lipid peroxidation (expressed as a concentration of thiobarbituric acid reactive substances; TBARS), the activity of extracellular LDH, the concentration of GSH in RBCs as well as % hemolysis in RBC supernatant were measured.

It was shown that addition of Trolox to the RBCs did not prevent lipid peroxidation during storage. However, Trolox (125 μ M) at the day 20 of storage significantly reduced extracellular LDH activity and hemolysis as well as it increased the concentration of GSH (by approx. 15% and 20% as well as by above 35% *vs.* the 20-day control without the antioxidant, respectively).

The results suggest that Trolox would be beneficial as a cytoprotective and antioxidant agent but other protective substances are necessary to decrease lipid peroxidation in stored RBCs.

P3.2

Flavonoids as a direct coagulation factor Xa inhibitors

Michał Bijak, Adam Antosik, Michał B. Ponczek, Halina M. Żbikowska, Paweł Nowak

Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Łódź, Łódź, Poland
e-mail: Michał Bijak <mbijak@biol.uni.lodz.pl>

Coagulation process consists of a series of zymogens that can be converted by limited proteolysis to active enzymes leading to the generation of thrombin, which in turn converts soluble fibrinogen into insoluble fibrin clot. Activated FX (FXa) interacts with its cofactor FVa and forms on the TF-bearing cell prothrombinase complex which is responsible for conversion of prothrombin to thrombin. FXa is a member of S1 family of serine proteases called chymotrypsin-like with typical for this family active center (His57, Asp102 and Ser195). Researches carried out in the last years provided evidence that polyphenol compounds are able to inhibit activity of many enzymes including serine proteases.

The aim of our study was to examine the effects of the polyphenol compounds on amidolytic activity of activated coagulation factor X and to explain this interaction using bioinformatic analyses.

We observed in our study that from all tested polyphenolic compounds only polyphenols belonging to flavonoids group such as: cyanidin, quercetin, procyanidin B2 and silybin had inhibitory effect on FXa amidolytic activity. Bioinformatic analyses performed using Autodock Vina 1.0 showed that cyanidin, quercetin, procyanidin B2 and silybin bound in the pocket located in vicinity of the FXa active site and blocked access of substrates to Ser195 and His57. Similar docking result was obtained for rivaroxaban, FXa inhibitor using in pharmacotherapy.

The results presented here showed that flavonoids have potential anticoagulant effect. Controlling of coagulation factor X activity may prevent uncontrolled thrombin generation process, one of major factors responsible for the thrombus formation in the blood vessels.

P3.3

Mutational analysis of FGF1 and FGF2 to identify binding sites involved in interaction with new binding partners

Joanna Bober¹, Michał Kostaś², Justyna Tomala², Małgorzata Zakrzewska¹, Daniel Krowarsch², Jacek Otlewski¹

¹University of Wrocław, Department of Protein Engineering, Wrocław, Poland; ²University of Wrocław, Department of Protein Biotechnology, Wrocław, Poland

e-mail: Joanna Bober <joanna.bober@uni.wroc.pl>

Fibroblast growth factor 1 (FGF1) and 2 (FGF2) are the members of fibroblast growth factor family. They are involved in several biological processes - proliferation, cell growth, cell survival and morphogenesis by binding to and activating specific cell surface receptor. Additionally they possess the ability to translocate across cellular membrane into the cytosol and nucleus. However, their intracellular functions remain unknown despite many years of research. To understand the role of FGF1 and FGF2 inside the cell, "pull down" experiments followed by mass spectrometry analysis were performed to identify potential binding partners of these fibroblast growth factors. Based on proteomic approach we identified several proteins, i.e. HSP90, nucleolin, PCAF and p53. To determine residues of FGF1 and FGF2 involved in the interactions with these proteins, we designed 18 surface mutations for both growth factors. These mutations, which disturb putative binding sites, were selected based on structural information from complexes of FGFs deposited in PDB and predicted using bioinformatics tools. We used web servers including meta-PPISP (which predicts the residues that are likely to form the binding sites), ConSurf (which analyses evolutionary conserved residues) and SWAKK (which detects amino acid sites that may be under positive selection). Site-directed mutagenesis, expression and purification of 36 mutational variants followed by surface plasmon resonance (SPR) experiments were performed. SPR analysis of interactions indicated that some variants exhibit a significant decrease or total reduction in binding response, suggesting the involvement of selected residues of FGF1 and FGF2 in binding to cellular partners. Such approach leads from structural analysis of protein to exploration of its function. Identification of specific binding regions within FGF1 and FGF2 interacting with intracellular partners sheds the light on the potential role of growth factors in cytoplasm and nucleus.

P3.4

Expression and purification of Fibroblast Growth Factor Receptors 1 and 2 (FGFR1 and FGFR2)

Aleksandra Borek, Aleksandra Sokołowska-Wędzina, Jacek Otlewski

University of Wrocław, Department of Protein Engineering, Wrocław, Poland

e-mail: Aleksandra Borek <korkus@protein.pl>

The FGFR family contains five closely related genes. Four of them (FGFR1–4) are transmembrane tyrosine kinase receptors. The family of fibroblast growth factor receptors (FGFRs) plays an important role in cell growth, survival, differentiation and angiogenesis. Deregulation of FGFR signaling has been associated with several developmental syndromes as well as with many types of cancer.

The first phase of the project comprised expression and purification of recombinant proteins, which will then serve as molecular targets for the selection and isolation of antibodies. We expressed extracellular domains of Fibroblast Growth Factor Receptors: FGFR1 (isoform IIIc) and FGFR2 (isoform IIIc). FGFR constructs were consisted of 3 immunoglobulin (Ig)-like domains (D1, D2, D3). Coding sequences were cloned into vector pLEV113 and produced by transient gene expression in CHO cell line to obtain fully glycosylated variants of FGFR1 and FGFR2. Signal peptide sequences were introduced into the protein sequences, allowing secretion of proteins into the culture medium. Proteins were tagged with Fc fragment to allow both an efficient purification of proteins using affinity chromatography on Protein A Sepharose and increase their stability.

FGFR1 and FGFR2 were expressed in CHO cells and analyzed using gel electrophoresis, Western Blotting and mass spectrometry. Analysis showed that both receptors were fully glycosylated. To verify molecular mass of purified proteins we used PNGase F enzyme to deglycosylate both receptors. Gel electrophoresis and mass spectrometry confirmed masses of deglycosylated proteins.

These results show that expression in CHO cells and purification using Protein A Sepharose allow to obtain fully glycosylated variants of FGFR1 and FGFR2 that are required to further studies.

P3.5

Lyn kinase differently regulates MyD88- and TRIF-dependent signaling pathways of TLR4 activated by LPS

Kinga Borzęcka, Agnieszka Płóciennikowska, Katarzyna Kwiatkowska

Nencki Institute of Experimental Biology, Department of Cell Biology, Warsaw, Poland

e-mail: Kinga Borzęcka <k.borzecka@nencki.gov.pl>

Toll-like receptor 4 (TLR4) is localized in the plasma membrane of macrophages and is activated by lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria. Under the influence of LPS, TLR4 binds MyD88/TIRAP adaptor proteins triggering a signaling pathway that leads to production of pro-inflammatory cytokines. After internalization, TLR4 in endosomes binds second set of adaptor proteins, TRIF/TRAM, and induces synthesis of type I interferons and chemokines. Activation of TLR4 by LPS can be dependent on an involvement of lipid domains, rafts, of the plasma membrane. Therefore, we examined an involvement of raft-residing Lyn kinase in the two signaling pathways of TLR4. We found that LPS induced dose- and time-dependent activation of Lyn kinase, as indicated by phosphorylation of tyrosine residue 396 of the catalytic domain of Lyn and simultaneous dephosphorylation of inhibitory tyrosine residue 507. Integrity of rafts was crucial for the activity of Lyn kinase. To interfere with the activity of Lyn, we obtained a series of mutated forms of the kinase fused with GFP which were next overexpressed in RAW264 macrophage-like cells. The transfection efficiency reached 40% of cell population. An expression of K275R kinase-dead Lyn increased TRIF-dependent production of chemokine RANTES by 60–75% in cells stimulated with 1–1000 ng/ml LPS. On the other hand, this mutant form of Lyn did not affect substantially MyD88-dependent production of TNF α . Similar effects were exerted by R135A Lyn kinase mutated in its SH2 domain and by doubly mutated Lyn at K275R and R135A. However, when only SH4 domain of Lyn kinase was expressed in cells, both TNF α and RANTES production were reduced by 40–50%. The inhibitory effect of the expression of Lyn-SH4 on TLR4 signaling can be related to exceptionally high transfection efficiency with this construct, affecting up to 80% of all cells, leading to perturbations in the plasma membrane organization by this plasma-membrane anchored protein. Taken together our data indicate that Lyn kinase serves a negative regulator of TRIF-dependent signaling pathway of TLR4, as interference with the activity of the endogenous kinase increased RANTES production.

P3.6

TrfA structural motifs required for double stranded DNA binding

Elżbieta Zabrocka¹, Katarzyna Bury¹, Sławomir Kubik¹, Anna Karłowicz¹, Urszula Walkow¹, Maria Moreno², Rafael Giraldo², Igor Konieczny¹

¹University of Gdansk, Department of Molecular and Cellular Biology, Gdańsk, Poland; ²Centro de Investigaciones Biológicas, Department of Chemical and Physical Biology, Spain
e-mail: Katarzyna Bury <igor@biotech.ug.edu.pl>

TrfA protein forms specific complexes with DNA in order to initiate RK2 plasmid DNA replication. First, TrfA monomers bind to direct repeats (iterons) located within the plasmid origin sequence (*oriV*) [1]. This event results in origin opening, melting of dsDNA structure within *oriV* AT-rich region [2]. Structure of TrfA nucleoprotein complex is not yet fully known. Due to high instability of replication initiators and therefore difficulties with crystallographic analysis, the knowledge about structure of plasmid Rep proteins is limited. Homology modeling revealed that TrfA (like other plasmid replication initiators) has two winged-helix domains WH1WH2 at the C-terminal region. The N-terminal region is disordered in solution and its structure is not known [3]. The aim of this study was to identify structural motifs of TrfA responsible for binding with DNA.

By using *in vivo* binding assay and SPR analysis we tested how substitutions located in different regions of TrfA protein will affect its interaction with dsDNA. The obtained results showed that specific mutations in different protein regions affect the TrfA nucleoprotein complex formation. Moreover, we observed compensatory effects of the analyzed substitutions, which were located outside of WH1WH2 domains. We concluded that apart from two WH1WH2 domains, which are typical for plasmid Rep proteins, there is a third DNA binding domain namely DBD3. We purified both DBD3 and WH1WH2 domains in fusion with SUMO protein and we tested their biochemical properties. Our results demonstrate that for effective DNA binding all three domains of TrfA are needed. Using approach that combines cross-linking, proteolysis and mass spectrometry we identify residues in TrfA that are in contact with dsDNA. We found that motif ³³⁹LMCGSD-STRVK₃₄₉ located in the WH2 domain is directly involved in iteron binding through the Lysine₃₄₉.

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P3.7

Levels of total and High-Molecular Weight adiponectin in serum of matched maternal-cord pairs: relations to fetal anthropometric parameters

Magdalena Chelchowska¹, Joanna Gajewska¹, Jadwiga Ambroszkiewicz¹, Tomasz M. Maciejewski², Teresa Laskowska-Klita¹

¹Institute of Mother and Child, Screening Test Department, Warsaw, Poland; ²Institute of Mother and Child Department of Obstetrics and Gynecology, Warsaw, Poland
e-mail: Magdalena Chelchowska <magdalena.chelchowska@imid.med.pl>

Objective. Adiponectin as a key regulator of insulin sensitivity could thus be expected to have significant effects on fetal growth and development. Therefore the aim of this study was to examine serum High-Molecular Weight adiponectin (HMW) and total adiponectin concentrations in pregnant women and umbilical cord blood and their correlations with fetal anthropometric parameters.

Methods. Forty healthy pregnant women, attending the Institute of Mother and Child in Warsaw were asked to cooperate in this study. All pregnant volunteers signed a written informed consent form, approved by the Institute's Ethical Committee. Venous blood samples were collected from women in I (median: 12 wk) and III trimester (median: 39 wk). Cord blood was collected immediately post partum from the umbilical vein after clamping of the cord. Serum HMW and total adiponectin concentrations were determined by immunoenzymatic method using a commercial available kits. Statistical analysis was done using the STATISTICA 8.0 and the significance level was set at $p < 0.05$.

Results. Concentrations of adiponectin and HMW isoform in serum of mothers increased during pregnancy and were 20% higher in third than in the first trimester. Umbilical serum total and HMW adiponectin levels were significantly higher compared with maternal levels at birth (total: 17.98 ± 6.61 $\mu\text{g/ml}$ vs 7.79 ± 3.36 $\mu\text{g/ml}$; HMW: 13.89 ± 6.67 $\mu\text{g/ml}$ vs 5.12 ± 3.29 $\mu\text{g/ml}$; $p = 0.0001$), and no correlation was found between cord and maternal adiponectin levels. In contrast to the findings in adults cord blood values were significantly correlated with both birth weight ($r = 0.62$; $p < 0.01$) and birth length ($r = 0.5$; $p = 0.01$).

Conclusion. The levels of total and HMW adiponectin were higher in umbilical cord serum than in maternal serum. The positive correlations between cord blood values of this protein and their isoform and birth weight and length suggest, that rather fetal adiponectin, not maternal serum adiponectin, may be involved in fetal development during pregnancy.

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P3.8

Direct determination of phospholipase D activity by infrared spectroscopy

Le Duy Do^{1,2}, René Buchet¹, Slawomir Pikula²,
Abdelkarim Abousalham¹, Saida Mebarek¹

¹Université de Lyon, France; Université Lyon 1, France; INSA-Lyon, France; CPE Lyon, France; ICBMS CNRS UMR 5246, France; ²Department of Biochemistry, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Poland
e-mail: Le Duy Do <ldo@nencki.gov.pl>

To determine phospholipase D (PLD) activity an infrared spectroscopy assay was developed, based on the phosphate vibrational mode of the phospholipid substrates of the enzyme such as dimyristoylphosphatidylcholine (DMPC), lysophosphatidylglycerol (lysoPG), dimyristoyl ethanolamine (DMPE), and lysophosphatidylserine (lysoPS). Characteristic vibrational bands were located at 1230, 1226, 1221 and 1218 cm⁻¹, respectively, and served to monitor the hydrolysis of phospholipids. The appearance of the phosphate vibrational band of phosphatidate at 1130 cm⁻¹, served to monitor the amount of byproduct of the hydrolytic cleavage of phospholipids by PLD. *In situ* measurements could be performed within less than 20 min, using 2–40 mM DMPC and at least 5–10 ng of *S. chromofuscus* PLD having specific activity of 30 nmol min⁻¹ μg⁻¹ (corresponding to 150–300 pmol hydrolysed DMPC per minute) at pH 8.0 in the presence of 10 mM Ca²⁺. The feasibility of the infrared assay using lysoPG, DMPE and lysoPS was also demonstrated, indicating that various natural phospholipids could be employed as substrates to measure the PLD activity. Reproducible apparent maximum velocities (V_{max}) were also determined. The direct infrared assay could be used as a possible screening tool to find specific PLD inhibitors.

P3.9

Homoisocitrate dehydrogenase from *Candida albicans*: functional and structural analysis

Iwona Gabriel, Marek Wojciechowski, Slawomir Milewski

Gdansk University of Technology, Department of Pharmaceutical Technology and Biochemistry, Gdańsk, Poland
e-mail: Iwona Gabriel <iwogabri@pg.gda.pl>

Identification and characterization of enzymes which are essential for fungal survival and/or pathogenicity is crucial for the rational design of antifungal drugs. In the present communication we report results of our studies on *Candida albicans* enzyme — homoisocitrate dehydrogenase (CaHicDH) — catalyzing biosynthetic reaction from α-aminoadipate pathway of L-lysine biosynthesis present only in fungal cells and having no counterparts in mammalian cells.

CaHicDH encoded by the *LYS12* gene was overexpressed in *E. coli* cells transformed by pET15b-*LYS12* expression plasmid. It catalyzes the Mg²⁺ and K⁺ dependent oxidative decarboxylation of homoisocitrate (Hic) to α-ketoadipate, using NAD⁺ as a oxidative substrate. The wild-type enzyme was purified and characterized for its physicochemical properties. k_{cat} was 0.40 s⁻¹, k_{cat}/K_M = 0.44 × 10³ M⁻¹s⁻¹ and k_{cat}/K_M (NAD⁺) = 8.75 × 10³ M⁻¹s⁻¹. Substrate spectrum analysis demonstrated strict specificity for Hic and no activity with isocitrate and 3-isopropylmalate. It was shown that the possibility of using Ic as a substrate by *T. thermophilus* HicDH was strongly dependent on presence of Arg85 and replacing this residue with Val prevented isocitrate binding and enhanced activity with Hic as a substrate. According to the multiple sequence alignment, Arg85 of TtHicDH corresponds to Val109 in the CaHicDH sequence, therefore the strict substrate specificity of the latter enzyme version is not surprising. Results obtained by SDS-PAGE, Native-PAGE and size-exclusion chromatography indicate that the native enzyme may be composed of four subunits and this assumption was supported by the results of the homology modelling of the enzyme molecule. Looking for the molecular basis of a tetrameric structure by native CaHicDH — never reported previously for HicDH from fungal sources — we identified a characteristic loop, relatively rich in charged residues (¹⁵⁵KKED¹⁵⁸) which may participate in intersubunit interaction. Multiple sequence alignment, amino acid sequence analysis and homology modelling of 3D CaHicDH structure revealed the presence of a sequence known as a isocitrate/isopropylmalate dehydrogenase conserved site: IPRO19818 (²⁶⁸NLYGDILSDGAAALVGSLGV²⁸⁷) and the catalytic Lys-Tyr pair (Lys211 and Tyr147), corresponding to Lys206 and Tyr150, shown previously in *S*HicDH to catalyze the acid-base chemistry of the enzymatic reaction.

P3.10

Aggregation test for fragment of human cystatin C Ala52–Asp65

Emilia Iłowska¹, Jakub Barciszewski², Mariusz Jaskólski², Aneta Szymańska¹

¹Department of Medicinal Chemistry, Faculty of Chemistry, University of Gdańsk, Gdańsk, Poland; ²Center for Biocrystallographic Research, Institute of Bioorganic Chemistry, Polish Academy of Science, Poznań, Poland
e-mail: Emilia.Iłowska <emiliagorna@gmail.com>

Research carried out on fibrilization process leads to some new hypotheses concerning the mechanism of this process, as well as the factors responsible for triggering and progress of fibril formation by a particular protein. One of them is hypothesis about existence of a specific amino acid sequences and clearly defined structure which promote these processes. The presence in proteins structures with very high propensity for formation of the β -stand conformation, located near the surface and have high ability for self-association and fibrilization called “steric zipper” motif [1, 2]. This motif was suggested by Americans scientists from prof. Eisenberg's group. They proved that in amyloidogenic proteins are sequences that promote aggregation and fibrilization process [1–3].

The aim of this project is to check the applicability of the steric zipper hypothesis for the analysis and understanding the amyloidogenic propensities of human cystatin C (hCC). Due to its physical and chemical parameters (good solubility in aqueous buffers, low molecular mass), cystatin C may be considered as a good model protein in studies aimed at verifying the Eisenberg's hypothesis. The 3D profile method and calculated Rosetta algorithm values for hCC show that in this protein there are two fragments for which high fibrilization propensity can be expected: the first N-terminal hairpin fragment encompassing the loop L1 structure (residues from Ala⁵² to Asp⁶⁵) and C-terminal part of the molecule between Arg⁹³ and Thr¹¹⁶. In this project we want to do experimental studies to prove this hypothesis.

Confirmation of existence of the steric zipper motif in the hCC sequence may have a significant impact on better understanding of the mechanism of self-association, aggregation and fibrillation of this protein.

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P3.11

MOR receptor gene polymorphism (A118G) in patients with acute pancreatitis

M. Jacuński¹, Ł. Jaśkiewicz¹, A. Janicka¹, K. Patyra¹, M. Matysiewicz², B. Kocbach², A. Cieślińska², J. Snarska², E. Kostyra²

¹The University of Warmia and Mazury in Olsztyn, Faculty of Medical Sciences, Olsztyn, Poland; ²The University of Warmia and Mazury in Olsztyn, Faculty of Biology and Biotechnology, Olsztyn, Poland
e-mail: Mateusz.Jacuński <anna.cieslinska@uwm.edu.pl>

Opioid receptors are classified as: μ (μ), κ (κ), δ (δ) and σ (σ). They belong to a large family called G protein — coupled receptors (GPCR) and they have different significance and physiological functions. μ — receptor (MOR) is important target place for opioid drugs. In humans, opioid compounds acts on central nervous system showing their wide range of analgesic action, causing euphoria, sedation, respiratory depression and cough suppression. They also have peripheral effects like constipation. In the process of inflammation, opioids are transported from ganglion cells of dorsal roots to peripheral sensory nerve fibers. At the same time immunological response cells, which contain endogenous opioid peptides, gather themselves within inflammatory altered tissue. Environmental stimulation such as stress, endogenous factors (e.g. corticotropin-releasing factor, cytokines), can release this opioids peptides allowing interaction with neuronal opioid receptors and causing local analgesia.

A large number of point mutations (SNP) have been identified in MOR gene. The most common is A118G polymorphism in exon I — it comes to replacement from adenine (A) to guanine (G) in position 118. This results in replacing Asn to Asp in amino acid chain. Nowadays exact molecular consequences of A118G MOR polymorphism, which may explain noticed clinical effects, remain unknown. However it is commonly known that appearance of G allele in polymorphic place of MOR results in decreasing capacity of the receptors binding. It was shown that people with this type of polymorphism feel more intensively intoxicating properties of ethanol. Furthermore people with G allele confirmed ethanol abuse among their ancestors. Ethanol is one of the most important risk factors of acute pancreatitis episode, thus it is possible relationship between MOR gene polymorphism with acute pancreatitis.

The aim of this work was to determine the frequency of A118G polymorphism of MOR gene in population of healthy people and people who suffer from pancreatitis as well as investigation of correlation between this polymorphism and falling for acute pancreatitis.

DNA was isolated from epithelium and blood of healthy people (control group) and from people with acute pancreatitis (test group). The polymorphism of MOR gene was determined with PCR — RFLP.

In the whole examined population the frequency of A allele was 0.94 and G allele 0.06. The results indicate the need for continuation further research on the A118G polymorphism in MOR gene including a wider group of people.

P3.12

Characterization of leucine-rich repeat domain containing protein, LRRC45 in ciliate *Tetrahymena thermophila* and in mammalian cell lines

Katarzyna Jedynak¹, Ewa Joachimiak^{1,2}

¹Department of Animal Physiology, University of Warsaw, Warsaw, Poland; ²Laboratory of Physiology of Cell Movements, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland
e-mail: Katarzyna Jedynak <kjedynak@biol.uw.edu.pl>

Leucine-rich repeat (LRR) domain is found in a variety of proteins and is suggested to be involved in protein-protein interactions. LRR-containing proteins participate in a wide range of biological processes, such as DNA repair, transcription, signal transduction, apoptosis, cell adhesion and immune response. Furthermore, many mutations of proteins from LRR-containing family were shown to be associated with malignant diseases.

The aim of this studies was determination of function and localization of LRRC45 protein in *Tetrahymena thermophila* and in mammalian cell lines.

We choosed model organisms *Tetrahymena thermophila*, murine cell line NIH 3T3 and human cell line A431 to investigate the impact of overexpression and silencing of protein LRRC45 on ultrastructure, and did biochemical and phenotypic studies.

We analyzed *Tetrahymena thermophila* genome and available proteomes and identified pellicular LRR-containing protein. Our initial studies showed that the identified protein is a novel cytoskeletal proteins. In *T. thermophila*, these LRR protein localize to the cortical cytoskeleton near the basal bodies but the function and linking with one of the cell compartments remain unknown. When the overexpression is on high level protein fused with GFP forms structures resembling vesicles of unknown origin. Overexpression of GFP-LRRC45 causes acceleration of *Tetrahymena thermophila* cell division rate as opposed to overexpression of LRRC45 without tag that reduces multiplication rate. Control and mutant cells were grown in medium supplemented with CdCl₂. The cells density was determined every three hours.

Tetrahymena LRRC45 protein has single human homologue, HsLRRC45. We demonstrated that in cells overexpressing HsLRRC45 fused with GFP on C- or N- terminus, the recombinant protein localized to juxtanuclear vesicular-like structures. The intracellular localization resembles localization of activated Rab5, caveolin-1 or the early endosome marker, EEA-1. The origin of these vesicles was determined by testing their putative colocalization with markers specific for different membranous structures like transport vesicles, lysosomes, autophagosomes. We demonstrated that there was incomplete colocalization with cis-Golgi marker GM130 and overexpressed and nativ HsLRRC45 protein. Furthermore the level of HsLRRC45 protein depends on cell line and is inversely proportional to the GM130 protein level. Finally, we identified the potential protein partners of the studied *Tetrahymena* protein and it's mammalian homologue using immunoprecipitation, among which both basal bodies proteins, vesicular trafficking and microtubular cytoskeleton associated proteins were present.

Overall, our results suggest that LRR domain containing novel protein probably participate in vesicular trafficking, probably between Golgi apparatus and endoplasmic reticulum.

P3.13

Calorimetric evidence of anandamide — albumin interactions

Marek Kaczynski¹, Krzysztof Bilmin², Magdalena Przybyło¹, Paweł Grieb², Marek Langner¹

¹Institute of Biomedical Engineering and Instrumentation, Wrocław University of Technology, Wrocław, Poland; ²Department of Experimental Pharmacology, Polish Academy of Sciences Medical Research Center, Poland
e-mail: Marek Kaczynski <marek.kaczynski@pwr.wroc.pl>

Anandamide, (arachidonylethanolamide, AEA) is an endogenous cannabinoid neurotransmitter. Since its discovery in 1992, several other functions have been reported and especially interesting is its anti-cancer activity. However if one would like to introduce the molecule into the organism, several problems arise. Because of its chemical structure, AEA is poorly soluble in water and susceptible to enzymatic degradation. It is also highly possible that arachidonic residue of anandamide will associate with proteins that have the ability to bind fatty acids, e.g. albumins, therefore its pharmacokinetics may be altered.

Albumin is the most abundant plasma protein (35–50 g/L human serum). It is a monomeric, multi-domain, nonglycosylated, negatively charged plasma protein, with numerous functions important for maintaining the body homeostasis. HSA is the main determinant of plasma oncotic pressure and the main modulator of fluid distribution between body compartments. It is characterized by an extraordinary ligand-binding capacity, being a carrier for many endogenous and exogenous compounds. Due to its high affinity to long-chain fatty acids, albumin plays an important role in their transport and in the modulation of their metabolism. The question of possible anandamide – albumin interactions arisen in the course of the *in vitro* studies in which the antiproliferative properties of AEA were studied. The HeLa cells growth have been halted only when anandamide had been introduced in serum free medium. In order to verify the hypothesis that the studied compound interacts with albumin, the isothermal titration calorimetry studies have been performed to determine thermodynamic parameters of anandamide-albumin interactions.

Because of the compound low solubility the liposomal formulation of AEA has been designed so the binding experiment could be successfully performed.

Results obtained with the isothermal titration calorimetry show distinct difference between the binding parameters of anandamide interacting with albumin when the fatty acids have been or have not been present. The obtained results and developed methodology can be used in the process of the developing new formulations for anti-cancer cannabinoid drugs.

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P3.14

Influence of polysiloxanes on the stability and activity of collagen

Leszek Kadziński¹, Zyta Banecka-Majkutewicz², Magdalena Prokopowicz³, Jerzy Łukasiak⁴, Bogdan Banecki¹

¹Intercollegiate Faculty of Biotechnology University of Gdańsk - Medical University of Gdańsk, Department of Molecular and Cellular Biology, Gdańsk, Poland; ²Medical University of Gdańsk, Department of Neurology, Gdańsk, Poland; ³Medical University of Gdańsk, Department of Physical Chemistry, Gdańsk, Poland; ⁴Gdańsk Management College, Gdańsk, Poland
e-mail: Leszek Kadziński <banecki@biotech.ug.edu.pl>

Silicone polymers are assumed to be chemically stable in living systems. Therefore they have been used in the medical field for many years as biocompatible biomaterials ranging from intraocular lenses to breast implants. Silicone fluids are widely used as a surgical tamponade in severe cases of vitreoretinal pathology and as compounds of bioactive organic-inorganic materials and drug delivery systems. An inherent problem associated with implanted prostheses is their propensity to be coated in host proteins shortly after implantation. Some of research works, specifically those that focused on the implanted silicone prostheses, suggest that silicone is adjuvant to the human immune system and may induce conformational changes and aggregation of the proteins. Furthermore patients with silicone prostheses display a variety of nonspecific syndromes that usually disappear after the removal of the implant. In this work we investigated the effect of cyclic and linear siloxanes on the structure and function of collagen — the major structural protein in human body. Using the FITC-labelled collagen we have shown protein-siloxanes complexes. We have demonstrated the influence of the protein-siloxanes interactions on the collagen fibril formation — the main function of collagen molecules. We have also determined that collagen-siloxanes interactions do not change the conformation of triple helical collagen molecules.

P3.15

Lon protease — mechanism of proteins degradation on DNA

Katarzyna Wegrzyn, Marta Gross, Anna Karłowicz, Igor Konieczny

Intercollegiate Faculty of Biotechnology UG-MUG, Department of Molecular and Cellular Biology, Gdańsk, Poland
e-mail: Anna Karłowicz <anna.karlowicz@wp.pl>

Lon belongs to the family of ATP-dependent proteases involved in protein quality control. Bacterial Lon is considered to be the most important factor involved in the degradation of misfolded proteins. This protease consists of three domains: the highly variable N-terminal domain, the central AAA+ ATPase domain containing nucleotide binding site, and the C-terminal peptidase domain. It forms barrel-like shaped oligomers with a central cavity within the macromolecular structure. To date, crystallographic data are available for full length Lon from *Bacillus subtilis* and *Thermococcus onnurineus* (Duman & Lowe, 2010; Cha *et al.*, 2010) and only fragments of enzymes from a few other organisms (including *Escherichia coli*) (Li *et al.*, 2010; Botos *et al.*, 2004; Botos *et al.*, 2005). It has been shown that Lon protease possesses the ability to bind DNA however, no requirements considering protein structure have been described so far (Zehnbaauer *et al.*, 1981). Moreover, our data suggest that nucleoprotein formation by Lon is required for its proteolytic activity.

We propose quaternary structure of the hexameric *E. coli* Lon protease predicted on the basis of data on crystallized fragments of Lon and homology modeling of the remaining parts of the protein. The analysis of our Lon structure prediction model revealed potential regions and residues possibly involved in DNA interaction. Utilizing site-directed mutagenesis we introduced uncharged or negatively charged substitutions and created series of Lon protein mutants. After purification step we tested them using *in vitro* methods for DNA binding and their activities towards substrates that specifically bind or do not bind DNA. Our results allowed us to indicate amino acid residues of Lon engaged in DNA interaction. Moreover, Lon mutants lacking DNA-binding ability were not able to degrade substrates which specifically bind nucleic acid. Our results confirm crucial role of nucleoprotein complex formation by Lon for its proteolysis of substrates interacting with DNA.

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P3.16

Desensitization of glucosamine-6-phosphate to inhibition by UDP-GlcNAc

Karolina Kwiatkowska, Agnieszka Napiorkowska, Sławomir Milewski

Gdansk University of Technology, Department of Pharmaceutical Technology & Biochemistry 11/12 Narutowicza St., 80-233 Gdansk, Poland

e-mail: Karolina.Kwiatkowska <karolina.kwiatkowska85@gmail.com>

Glucosamine-6-phosphate (GlcN-6-P) synthase, known also as L-Glutamine: D-fructose-6-phosphate amidotransferase, catalyzes the first committed step in the pathway leading to the ultimate formation of UDP-GlcNAc. The final product of this pathway is an activated precursor of numerous macromolecules containing amino sugars, including chitin and mannoproteins in fungi, peptidoglycan and lipopolysaccharides in bacteria, and glycoproteins in mammals. The reaction catalyzed by the enzyme is conversion of fructose-6-phosphate into glucosamine-6-phosphate with L-glutamine as a nitrogen donor.

The important differences between prokaryotic and eukaryotic forms of the enzyme include a substantially larger size of the latter and sensitivity of the latter but not the former to the feedback inhibition by uridine 5'-diphospho-N-acetyl-D-glucosamine (UDP-GlcNAc). The enzyme has been proposed as a promising target in antifungal chemotherapy as well as for the treatment of insulin-independent diabetes mellitus.

Our studies have been focused on GlcN-6-P synthase from human pathogenic fungi *Candida albicans* coded by the *GFA1* gene. We were able to demonstrate a crystal structure of the isomerase domain (ISOM) of *C. albicans* GlcN-6-P synthase in complex with UDP-GlcNAc [2]. Now we report results of our further studies involving the site-directed mutagenesis of *GFA1*, leading to the following substitutions of amino acid residues located at the putative UDP-GlcNAc binding site in the gene products: Gly474Leu, Thr487Ile, Gly490Leu and His492Phe. Muteins obtained at consecutive steps of mutagenesis were isolated and characterized. The final product of mutagenesis was completely insensitive to inhibition by UDP-GlcNAc, while sensitivities of the intermediate products were diminished. Conclusions concerning the probable role of UDP-GlcNAc binding for enzyme structure and catalytic properties are presented.

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P3.17

Analysis of A β 1–40 peptide oligomers using ion mobility separation coupled with MS and factors influencing the peptide structure compaction

Marcin Kloniecki¹, Agnieszka Jabłonowska¹, Jarosław Poznański¹, James Langridge², Kevin Giles², Nick Tomczyk², Chris Hughes², Michał Dadlez^{1,3}

¹Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Department of Biophysics, Warsaw, Poland; ²Waters Corporation, Atlas Park, Simonsway, Manchester, UK; ³Institute of Genetics and Biotechnology, Faculty of Biology, University of Warsaw, Warsaw, Poland
e-mail: Marcin.Kloniecki <marcinkloniecki@wp.pl>

Alzheimer's disease (AD) poses a serious challenge on social care systems worldwide and this justifies intense studies on mechanisms underlying AD pathology and on the nature of the neurotoxic agent. Several pieces of evidence point to a causative link between the soluble and non-monomeric forms of A β peptide, derived by proteolysis of APP protein, and AD, founding the basis for the revised amyloid cascade hypothesis. It has been argued that the level of cognitive disability correlates better with the pool of A β oligomers, rather than with aggregated amyloid deposits. It became clear that different A β species, also extracted from AD patient's tissues, exert neuro/synaptotoxicity in a wide range of experimental setups. Thus, "oligomer hypothesis" is central for AD studies.

To gain insight into the A β 1–40 oligomer structure we have applied Ion Mobility separation (IMS) coupled with MS. It allowed us to determine the mobility of A β 1–40 oligomer ions and, on the basis of the mobility, calculate the relative collisional cross-section for each oligomeric form observed. For several oligomers, at least two different species of different Ω values were detected, indicating the presence of at least two families of conformers: compact and extended. When incubated at room temperature for 24 h the drift time profile of several oligomeric forms changes in general with the shift towards more compact forms. The strongest changes were observed from trimers to pentamers showing that a compact structure becomes dominant with time. Similar shift was observed for higher oligomers, hexamers and heptamers. This, however, does not hold for dimeric signals in which the mutual population of both forms barely changes after 24 h. In general, whenever in a given charge state of oligomers larger than dimers both compact and extended forms are present in the spectra of freshly dissolved peptide, the fraction of compact species increases with time. These oligomers equilibrate towards the domination of the most compact species at longer times. Interestingly similar effect can be obtained just after dissolution of the A β peptide if metals are added. An addition of copper or zinc caused that the drift time profile of several metal-oligomeric complexes shifts towards more compact forms. Experimentally observed behavior of higher A β species by IMS in gas phase allowed us to construct a set of molecular models of A β oligomer structure that provided a very good correlation between the experimental and theoretical Ω values for both compact and extended forms. Moreover, in case of the open form, the model explains the flexibility that enables the formation of potentially neurotoxic channel-like structures that are able to dysregulate calcium ion flux at the synapses.

P3.18

The structural studies of β -lactoglobulins from the whey fraction of milk

Joanna Loch, Mateusz Czub, Mira Ludwikowska,
Sylwia Świątek, Krzysztof Lewiński

Faculty of Chemistry, Jagiellonian University, Kraków, Poland
e-mail: Joanna Loch <loch@chemia.uj.edu.pl>

β -Lactoglobulin (LG) is a protein present in milk of several species as e.g. cow, goat, sheep, and pig. The core element of LG structure is an antiparallel β -barrel surrounded by flexible loops that is a primary binding site for ligands. The binding of ligand in β -barrel requires conformational changes of EF loop in the process called Tanford transition. The most widely studied bovine lactoglobulin (BLG) can bind hydrophobic ligands but the selectivity of binding is very low. To increase affinity to selected compounds, we plan to re-engineer the protein. Such modification will allow to utilize it in the future as a transporter of bioactive compounds with potential applications in medicine.

To characterize the mechanism of ligand binding, structural studies on bovine and goat (GLG) protein have been performed. BLG and GLG have different amino acids at six positions L11, D53N, D130K, S150A, E158G and I162V. Such substitutions seem to affect not only the physico-chemical properties of protein but also its allergenicity and ability of ligand binding, although all substitutions are outside the binding site. BLG and GLG have been isolated from milk by salting-out and ion-exchange chromatography. Proteins have been crystallized using vapour-diffusion method in hanging drop set-up. Structures have been solved by molecular replacement.

Three crystal forms (P3₁2₁, P2₁2₁2 and P3₂2₁) of GLG have been obtained, the BLG was crystallized only in P3₂2₁ and C222₁ space group. The comparison of BLG and GLG structures suggests that differences in amino acids composition do not affect the conformation of main chain. The GLG structures revealed that conformational changes accompanying Tanford transition include not only EF loop but also the GH loop. The interactions between Asn90 - Ser110 and Glu89 - Ser116 seem to play main role in this phenomenon. Comparison of BLG and GLG complexes with ligands revealed the position of the ligand in the binding site is slightly different in BLG and GLG. Crystallization experiments showed that crystals of BLG complexes of with ligands can be obtained at pH 7.5 and higher but crystallization of similar GLG complexes requires the pH 8.5. In the GLG structure secondary ligand binding site has been identified in the region of GH loop.

P3.19

The molecular characteristics of the nuclease from extreme psychrophilic bacterium *Psychromonas ingrahamii* 37

Natalia Maciejewska^{1,2}, Aneta Szymańska¹,
Emilia Iłowska¹, Marcin Olszewski²

¹Department of Medicinal Chemistry, University of Gdańsk, Gdańsk, Poland; ²Department of Microbiology, Gdańsk University of Technology, Gdańsk, Poland
e-mail: Natalia Maciejewska <n.maciejewska@gmail.com>

Information regarding psychrophilic organism proteins is exceptionally limited, particularly given their proliferation; approximately 85% of the earth's biosphere is psychrosphere, with a temperature of below 5°C. This is, in part, owing to the difficulties involved in their acquisition. Scarcely more than a dozen protein structures of psychrophilic origin are known at present. Such proteins display high activity at low temperatures. One of the most desirable psychrophilic microbiological products are enzymes, in other words, nucleases.

Nucleases are important molecular tools belonging to the group of hydrolase-degrading nucleic acids. Nucleases are important molecular tools belonging to the group of hydrolase-degrading nucleic acids. Depending on the kind of substrate digested, it is possible to distinguish deoxyribonucleases, which hydrolyze deoxyribonucleic acids, and ribonucleases, which degrade ribonucleic acids. By the same token, depending on the activity site, we are able to distinguish endonucleases, which hydrolyze intermolecular phosphodiester bonds in a polynucleotide chain, as well as exonucleases, which cleave to the terminal nucleotides of the chain. We report the identification and characterization of the nuclease from extreme psychrophilic bacterium *Psychromonas ingrahamii* 37 that grows exponentially at -12°C and may well grow at even lower temperatures. Psychrophilic microorganisms, living in extremely cold environments, produce enzymes which are adapted to performing reactions at a low temperature. This is extremely important in the case of nucleases, as it will allow their utilization in the reaction for the elimination of nucleic acids from thermosensitive preparations.

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P3.20

Identification and characterization of class III UvrA protein from *Stigmatella aurantiaca*

Marta Marszałkowska¹, Magdalena Bil²,
Łukasz Kreft¹, Marcin Olszewski¹

¹Gdańsk University of Technology, Department of Microbiology, Chemical Faculty, Gdańsk, Poland; ²Katholieke Universiteit Leuven, Department of Biology, Research group of Insect Physiology and Molecular Ethology, Leuven, Belgium
e-mail: Marta Marszałkowska <mm.marszalkowska@gmail.com>

UvrA protein is a part of UvrABC repair system, which catalyzes the recognition and processing of DNA lesions. This protein is one of the key components of NER repair system pathway in prokaryotes. In last years, several works have been published about discovering additional *uvrA* genes in chromosomes of some prokaryotic species (*Xanthomonas axonopodis*, *Pseudomonas putida* or *Deinococcus radiodurans*).

We investigated sequences of over 2 400 bacterial genomes and as a result we found 130 examples of bacteria containing *uvrA* genes. Sequence analyses conducted on these homologues revealed that previously established division of *uvrA* genes was incorrect. Therefore we created a new division based on our *uvrA* sequence's base and current knowledge about UvrA proteins. This division consist of four main *uvrA* classes and five subclasses, among which only class I genes are well characterized.

In order to investigate the problem of UvrA homologues and their role in bacteria, we decided to conduct expression and characterize *uvrA* gene product from mesophilic, aerobic bacteria — *Stigmatella aurantiaca*. As a next step we are planning to obtain its crystal structures and examined its function using mutagenesis techniques.

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P3.21

Renalase levels and activity in blood and urine of healthy adult volunteers

Natalia Matoszka¹, Katarzyna Siewierska²,
Magda Wiśniewska³, Barbara Dołęgowska¹

¹Department of Medical Analytics, Pomeranian Medical University, Szczecin, Poland; ²Students Learned Society, Department of Medical Analytics, Pomeranian Medical University, Szczecin, Poland; ³Clinic of Nephrology, Transplantology and Internal Diseases, Pomeranian Medical University, Szczecin, Poland
e-mail: Natalia Matoszka <n.matoszka@gmail.com>

Background: Renalase is a novel, soluble flavoenzyme with potential ability to oxidize biogenic amines. It is expressed mainly in kidney and heart, but also, in a lesser extent, in skeletal muscles, liver, small intestine, brain or gonads, and is secreted into the blood and urine. When discovered (2005), it was described as third monoaminooxidase – MAO-C; the most recent direct research of renalase activity (2012), however, revealed a different way of action than known monoaminooxidases, but did not exclude the potential catecholamines-degrading properties, and cardioprotective effects confirmed in many other studies. Here, we strongly suggest that renalase metabolizes biogenic amines — especially adrenaline — to corresponding aminochromes, what is a confirmation of the mentioned above research made by the renalase discoverers and the leading renalase researchers — G.V. Desir and co-workers. Nevertheless, there is still a huge problem to resolve — how renalase contributes to lowering blood pressure and preventing many cardiovascular disorders, while producing toxic aminochromes?

Aim of the study: Aminochromes are natural products of catecholamines oxidation, including autooxidation, with potential toxic properties. They are present at very low concentrations in blood and urine, and quickly neutralised. Our aim was to test, develop, and in further perspective - standardize, the method of measuring renalases activity in blood and urine of healthy volunteers, assuming that renalase indeed oxidizes catecholamines to aminochromes.

Materials and methods: Concentration of renalase in urine and blood serum of healthy volunteers was measured using commercial ELISA kit. All the kinetic parameters, including NADH+H⁺ oxidation, production of hydrogen peroxide and formation of adrenochrome from adrenaline were measured using spectrophotometric methods. General blood and urine biochemical analyses were carried out.

Results: Amount of adrenochrome, produced in one minute from 1ng of renalase was almost the same in blood and urine and depended mainly on the time lapse of reaction. Concentration, and therefore activity of renalase – measured by the amount of formed adrenochrome, was about two times higher in urine than in blood serum.

Conclusions: Obtained results suggest, that adrenochrome is in fact the product of renalase enzymatic activity in presence of adrenaline and depends on renalase concentration, therefore amount of adrenochrome formed in presence of renalase in certain conditions can be considered as constant parameter.

P3.22

Multi-method approach to structure and function of the mRNA 5' cap-binding proteins: eIF4E and PARN

Anna Niedźwiecka^{1,2}, Małgorzata Lekka³, Remigiusz Worch¹, Per Nilsson⁴, Edward Darzynkiewicz², Anders Virtanen⁴

¹Institute of Physics PAS, Laboratory of Biological Physics, Warsaw, Poland; ²University of Warsaw, Faculty of Physics, Division of Biophysics, Warsaw, Poland; ³Institute of Nuclear Physics PAS, Kraków, Poland; ⁴Uppsala University, Department of Cell and Molecular Biology, Uppsala, Sweden
e-mail: Anna Niedźwiecka <annan@ifpan.edu.pl>

A biophysical bases of molecular mechanisms underlying the recognition of the mRNA 5' terminal structure called "cap" by proteins is crucial both for understanding of the complex process of regulation of eukaryotic gene expression at the levels of translation and mRNA surveillance, as well as for putative drug design.

Recognition of the 5' cap by the eukaryotic initiation factor 4E (eIF4E) is the rate limiting step of protein biosynthesis, while poly(A)-specific ribonuclease (PARN) is a 5' cap-dependent enzyme that plays a key role in 3' deadenylation, is involved in nonsense-mediated mRNA decay, and also in regulation of cytoplasmic polyadenylation. The goal of the studies was to find structural requirements for the affinity of the cap-binding proteins to the cap [1], thermodynamic driving forces [2, 3] and kinetic characteristics of the intermolecular recognition, as well as to gain an insight into the structure and structural dynamics, that are biologically relevant.

We have established a precise method of the protein-ligand binding constants determination [4] and found that eIF4E exploits conformational changes to provide tight binding of the cap and the synergy of interactions with eIF4G/4E-BP1 [1-3], while PARN is the only one among 3' exoribonucleases which interacts with the 5' mRNA terminal structure [5] to provide the processivity of deadenylation. PARN is thus the minimal protein context to bind two mRNA termini concurrently [6]. eIF4E and PARN share similar structural cap-binding motif (Trp-m⁷G) but they have different thermodynamic and kinetic binding properties that correlate with the biological functions of these proteins. We have also visualized single PARN molecules by Atomic Force Microscopy in liquid [7] that provided mesoscopic structural description complementary to the protein fragments known from crystallography.

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P3.23

Specific interactions between the amyloid precursor and cholesterol in a lipid bilayer by means of molecular dynamics simulations

Łukasz Nierzwicki, Jacek Czub

Gdansk University of Technology, Department of Physical Chemistry, Gdańsk, Poland
e-mail: Łukasz Nierzwicki <nierzwicki.l@gmail.com>

C99 is a transmembrane protein, which was identified as the precursor of β -amyloid. It is generated from the amyloid precursor protein (APP) by β -secretase. Cleaving of C99 by γ -secretase in a cell membrane releases β -amyloid polypeptides, which are associated with Alzheimer disease (AD). According to the work by Barrett *et al.* [1], C99 when present in a lipid environment, can specifically bind a cholesterol (CHL) molecule. This might induce cleaving by γ -secretase either by increasing the affinity of C99 to lipid rafts in which γ -secretase occurs or by direct participation of cholesterol in the cleaving process. Investigating such interactions may reveal a new potential ways of drugs design, as it was proposed that preventing of the binding between C99 and cholesterol may interfere with. Here we used molecular dynamics (MD) simulations to investigate the association between C99 and cholesterol in a lipid bilayer at molecular level. The overall orientation of C99 in a lipid bilayer of different cholesterol content as well as the ordering effect of the protein on membrane lipids was investigated by means of equilibrium MD simulations. The Gibbs free energy profiles for binding cholesterol to the C99 protein was determined by the umbrella sampling method. The obtained profiles confirmed the specific binding between C99 and cholesterol and allowed us to propose the structure of the complexes. Two minima found in the free energy profiles correspond to the two possible low-energetic structures of the CHL-C99 complex. Using the acquired MD data, we analyzed molecular driving forces governing the specific interactions between C99 and cholesterol. To validate our results, MD simulations were also performed for the E693A mutant which was found to bind cholesterol much less effectively. Based on these simulations, we propose the possible molecular mechanism explaining the observed decrease in affinity of C99 to cholesterol.

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P3.24

Characterization of single-stranded DNA-binding proteins from the psychrophilic bacteria

Marcin Olszewski, Marta Nowak, Marta Śpibida

Gdańsk University of Technology, Department of Microbiology, Gdańsk, Poland
e-mail: Marcin Olszewski <molsza@wp.pl>

Single-stranded DNA-binding proteins (SSBs) are indispensable elements in the cells of all living organisms. SSB proteins interact with ssDNA in sequence in an independent manner, preventing them from forming secondary structures and from degradation by nucleases. In this way, SSB-binding proteins participate in all processes involving ssDNA, such as replication, repair and recombination. Although there are differences in amino acid sequences, SSBs have a high-conservative domain, the oligonucleotide/oligosaccharide-binding fold, referred to as the OB-fold, which is responsible for binding with ssDNA. In the single-stranded DNA-binding proteins described so far, four OB-fold domains form an active protein. These proteins have also the ability to bind RNA and are present in all three branches of life organisms and in viruses. The cooperative binding of single-strand DNA and RNA which is a property of SSBs has led to their being used as tools in molecular biology methods and analytics. Thermostable proteins are particularly useful in this respect. To date, only a few thermostable SSB proteins with these valuable applications have been identified and, as yet, no studies have been undertaken into single-strand binding proteins from cold-adapted organisms and their applications.

We report the characterization of single-stranded DNA-binding proteins from the psychrophilic bacteria *Desulfotalea psychrophila* (DpsSSB), *Flavobacterium psychrophilum* (FpsSSB), *Psychrobacter arcticus* (ParSSB), *Psychrobacter cryohalophilus* (PcrSSB), *Psychromonas ingrahamii* (PinSSB), *Photobacterium profundum* (PprSSB) and *Psychroflexus torquus* (PtoSSB). The proteins show a high differential within molecular mass of their monomers and length of amino acid sequences. The high level of identity and homology in respect of *Eco*SSB relates to the OB-fold and some of the last amino acid residues. The fluorescence titrations indicated that the ssDNA-binding site size is about 32 nucleotides long and is salt independent. The DpsSSB, FpsSSB, ParSSB, PcrSSB, PinSSB, PprSSB and PtoSSB cause the destabilization of duplex DNA, as expected. The greatest decrease of duplex DNA melting temperature was observed in the presence of PtoSSB (17°C). The investigated SSBs possess relatively high thermostability for the proteins derived from cold-adapted bacteria.

P3.25

Effect of various protein receptors on the catalytic activity of rattlesnake phospholipase A₂: spectrofluorimetric studies

Maciej Ostrowski^{1,2}, Pierre-Jean Corringer¹, Grazyna Faure¹

¹Institut Pasteur, Department of Neuroscience, Unite Recepteurs-Canaux, France; ²Nicolaus Copernicus University, Department of Biochemistry, Poland
e-mail: Maciej Ostrowski <maciejost@umk.pl>

The secreted phospholipases A₂ of IIA group (PLA₂) are small, disulfide bridge-rich proteins which were found in human synovial fluid and snake venoms. These enzymes catalyze hydrolysis of glycerophospholipids at *sn*-2 position, releasing fatty acids and lysophospholipids. The group IIA PLA₂s interact with various protein targets, in agreement with their multifunctional nature (Faure & Saul, 2012). The biological effects of PLA₂ are determined by their enzymatic activity (regulation of plasma membrane lipid composition, production of an arachidonic acid for eicosanoids biosynthesis, etc.) or/and by specific high-affinity interaction with various pre- and post-synaptic receptors and human coagulation factor Xa. Two novel specific targets of PLA₂ were recently identified: the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) (Faure et al, Edelman, submitted) and bacterial pentameric proton-gated ion channel (GLIC) (Faure et al., Corringer, manuscript in preparation). To better understand the mechanism of interaction between protein targets and PLA₂s, in the present study we investigated the effect of direct binding of three different proteins (human coagulation factor Xa, nucleotide binding domain 1 of CFTR, and GLIC) on the enzymatic activity of PLA₂. PLA₂ activity was determined by spectrofluorimetric method using pyrene-labelled glycerophospholipid as substrate. Kinetic parameters (K_m , V_{max} , k_{cat} , k_{cat}/K_m) of PLA₂ were determined, showing different regulatory effects of FXa, NBD1-CFTR, and GLIC on PLA₂ activity. To identify the structural domain of PLA₂ involved in interaction with these protein targets, we studied the inhibitory effect of a specific rattlesnake PLA₂ inhibitor (an oxidiazolone derivative PMS 1062), and determined the kinetic parameters of inhibition (K_i , K_m^{app}). Kinetic characterization of the enzymatic activity of PLA₂-receptor/channel complexes opens interesting perspectives to investigate the physiological relevance of these interactions. Co-crystallization studies will also be performed to determine the binding interface between the protein partners and detect conformational changes after complex formation.

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P3.26

The link between A118G polymorphism in the μ -opioid receptor (MOR) gene and predisposition to the occurrence of addictions

Konrad Patyra, Katarzyna Regin, Ewa Fiedorowicz, Michał Matysiewicz, Anna Cieślińska, Elżbieta Kostyra

University of Warmia and Mazury, Department of Biochemistry, Faculty of Biology and Biotechnology, Olsztyn, Poland
e-mail: Konrad.Patyra <anna.cieslinska@uwm.edu.pl>

The opioid receptors belong to G-protein coupled family. There are four types of opioid receptors: MOR, NOR, DOR, KOR. These receptors are mainly located in the nervous system. Furthermore, they can also be found in other body tissues such as smooth muscle. They send signals associated with pain perception, motility of the organism or mood control. The opioid receptors also play a role in the organism's addiction to opiates, cocaine or alcohol, especially the μ -opioid receptor (MOR). A high number of single nucleotide polymorphisms (SNP) has been detected in the μ -opioid receptor (MOR) gene. One of the most extensively studied SNP is a substitution adenine to guanine at 118 position which results in the substitution of asparagine to aspartate at 40 position. These mutation occur with the allele frequency of 10–32% in different ethnics groups. Some of A118G polymorphism studies suggest that there is a contribution of the G allele at the increased risk of addiction to heroin and alcohol. Moreover, the occurrence of G allele may decrease receptor binding ability in smokers. The aim of the study was to determine the relationship between A118G polymorphism occurrence in the μ -opioid receptor (MOR) gene and predisposition to addiction to different substances (alcohol, energy drink, chocolate, coffee, cigarette) among students. Furthermore, the percentage of genotypes AA, AG and GG in the study population was estimated.

The questionnaire was distributed among tested students. Based on the survey, the frequency of drug use was estimated. In addition, the DNA was isolated from students' blood. The A118G polymorphism of MOR gene was determined using the PCR-RFLP method. The percentage of examined genotypes was estimated, the obtained results were correlated with the questionnaire answers.

On the basis of the results, the percentage of genotype AA was 82%, AG — 14% and GG — 4% in tested population. Due to the low share of one of the genotypes, there was no association between the occurrence of the genotype and predispositions to addiction, but one can notice some relations. The obtained results indicate on the necessity to continue working on the μ -opioid receptor (MOR) gene and its susceptibility to addictions.

P3.27

Autogenous activation-repression role of C protein in Csp231I restriction-modification system

Anna Petrusiewicz, Kinga Ruszkiewicz, Monika Rezulak, Iwona Mruk

Department of Microbiology, University of Gdansk, Gdańsk, Poland
e-mail: Anna.Petrusewicz <anna.petrusewicz@gmail.com>

Many bacteria produce restriction-modification (R-M) systems, in part to protect against invading DNA such as phage genomes. Most type II R-M systems include independently-active restriction endonuclease (REase) and protective DNA methyltransferase (MTase) proteins. After R-M genes enter a new cell, MTase activity must appear before REase or the chromosome will be cleaved. These R-M systems thus represent temporally-controlled genes, where the genes have a long-term relationship with their new hosts, and where the consequences of misregulation can be lethal.

Some R-M systems achieve delayed REase expression by co-transcribing the REase gene with the gene for an autogenous transcription regulator (the controlling or “C” protein). R-M systems associated with C proteins fall into several groups based on their sequences and target DNA sequence specificities. The archetypes of three currently-recognized groups are the best studied for C.PvuII and C.EcoRV (names after first example discovered). The third group — C.EcoO109I comprising two members: C.EcoO109I and C.Csp231I is unexplored. The purpose of our research is to investigate C.Csp231I protein and its R-M system located on chromosome of *Citrobacter* sp.RFL231I. Though, the crystal structure of C protein has been solved, there is no information on biological activity of C protein *in vivo*.

C proteins bind to conserved operator sequences called “C-boxes” located upstream of their genes overlapping DNA promoter region. We tested *in vivo* effect of C.Csp231I protein on DNA fragments carrying C-boxes fused to reporter gene when C gene is delivered *in trans*. The C gene was cloned under arabinose promoter to obtain a gradient of C expression after arabinose induction. We observed the transcription activation in very low C concentration and subsequent repression in higher C level. The effect was not present when C protein was mutated to prevent C-box binding. Same mutation introduced into R-M system background did not abolish the restriction activity suggesting different mode of regulation of gene expression. In addition, the entire R-M system seems to be toxic to *E. coli* cells to such extent the R-M system gene transfer is possible only if the host cells are premethylated. The host cells show the distinct filamentation as upon SOS response. Interestingly, the filament formation is not correlated with the restriction level, which is unexpected and not clear.

P3.28

Preeclampsia-associated changes in signal transduction in umbilical cord arteries

Lech Romanowicz, Zofia Galewska, Tomasz Gogiel, Krzysztof Sobolewski

Department of Medical Biochemistry, Medical University of Białystok, Białystok, Poland

e-mail: Lech Romanowicz <lech.romanowicz@umb.edu.pl>

Preeclampsia, the most common pregnancy-associated pathological syndrome, is accompanied by significant remodelling of the extracellular matrix and alteration in lipid composition of the umbilical cord artery. We evaluate phosphatidylinositol composition, TGF- β , MAP-kinase expression, MT-MMP and COX-1, COX-2 activity in umbilical cord artery and their alteration in preeclampsia. Solid phase extraction, thin layer chromatography and high-performance liquid chromatography were employed for phosphatidylinositol characterization. Western immunoblot and ELISA technique were used for others. The umbilical cord artery wall is abundant in phosphatidylinositols. Preeclampsia is associated with significant increases in MT1-MMP and active form of ERK 1/2 content and proportional activity of COX-2. An increase in MT1-MMP content may intensify TGF- β conversion to the active form in preeclamptic umbilical cord arteries. Higher amount of active form of MAP-kinase may indicate faster signal transduction in comparison to control material. This may cause a deregulation of cell metabolism, resulting in accumulation of some protein in arterial wall in preeclampsia.

P3.29

Measurement of oxidant-antioxidant balance with the use of total antioxidant capacity and total oxidant status

Ewa Romuk¹, Bronisława Skrzep-Poloczek¹, Celina Wojciechowska², Wojciech Jacheć², Alina Ostałowska¹, Ewa Birkner¹

¹Silesian Medical University, Department of Biochemistry, ²Silesian Medical University, Department of Cardiology, Zabrze, Poland

e-mail: Ewa Romuk <eromuk@gmail.com>

Background: Oxidative stress is induced by a wide range of environmental factors including UV stress, pathogen invasion and oxygen shortage. Oxygen deprivation stress is distinguished by three physiologically different states: transient hypoxia, anoxia and reoxygenation. Generation of reactive oxygen species (ROS) is characteristic for hypoxia and especially for reoxygenation. The main cellular components susceptible to damage by free radicals are lipids (peroxidation of unsaturated fatty acids in membranes), proteins (denaturation), carbohydrates and nucleic acids. The formation of ROS is prevented by an antioxidant system: low molecular mass antioxidants (ascorbic acid, glutathione, tocopherols), enzymes regenerating the reduced forms of antioxidants, and ROS-interacting enzymes such as SOD, peroxidases and catalases. Antioxidants act as a cooperative network, employing a series of redox reactions. The measure of total antioxidant capacity (TAC) considers the cumulative action of all the antioxidants present in plasma and body fluids. The capacity of known and unknown antioxidants and their synergistic interaction is therefore assessed, thus giving an insight into the delicate balance in vivo between oxidants and antioxidants. Measuring plasma TAC may help in the evaluation of physiological, environmental, and nutritional factors of the redox status in humans. Determining plasma TAC may help to identify conditions affecting oxidative status in vivo (e.g., exposure to reactive oxygen species and antioxidant supplementation).

Total oxidant status (TOS) expresses the total level of all peroxides produced in the body under the influence of ROS. It appears to be a more sensitive parameter and much more accurate than simply marking only the products of lipid peroxidation.

The aim of this study was estimation and comparison of TAC and TOS between group of different patients and searching the index which is the exponent of the reaction occurring for maintaining the oxidation-antioxidant balance.

Materials and methods: We have analysed results of TAC and TOS in 100 patients with osteoarthritis and 190 patients with heart failure and in the control group of 20 healthy volunteers. Total antioxidant capacity (TAC) and total oxidant status (TOS) was measured with the colorimetric method by Ozcan Erel.

Results: TAC in the control group was 1.25 ± 0.22 . TAC in osteoarthritis group was 0.86 ± 0.14 ($p < 0.0001$, comparing to the control) and in the heart failure group was 1.25 ± 0.25 . TOS in the control group was 2.24 ± 1.72 . TOS in osteoarthritis group was 28.53 ± 0.14 ($p < 0.0001$, comparing to the control) and in the heart failure group was 147.81 ± 0.2 ($p < 0.0001$, comparing to the control).

Conclusion: Obtained results indicate on the commitment of oxidation reaction in pathogenesis of osteoarthritis and heart failure. There was statistically significant increase in TOS in studied group of patients comparing to the control group. There was statistically significant decrease of TAC activity in osteoarthritis patients comparing to the control

and there was no differences between TAC in the heart failure patients and control. It seems that antioxidant capacity system is inefficient and isn't sufficient to protect organism against ROS.

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P3.30

Structural investigations of equine and bovine serum albumin binding properties of 3,5-diiodosalicylic acid

Bartosz Sekula, Kamil Zielinski, Anna Bujacz

Institute of Technical Biochemistry, Lodz University of Technology, Łódź, Poland

e-mail: Bartosz Sekula <bartosz.s87@gmail.com>

Transport of different substances within circulatory system is a very intriguing issue especially in a context of metabolites, waste products, toxins and drugs. All these compounds are transported by the most abundant protein of vertebrates' plasma — serum albumin. Although all mammalian serum albumins have quite high sequence similarity, their binding abilities are different.

The heart-shaped and highly helical structure of serum albumin possesses a number of binding pockets. Each of the three homologous domains has evolved specific binding properties. Although serum albumin domains exhibit extensive structural homology, the distribution of their binding sites is not the same.

Salicylic acid and its derivatives are used as components of pharmaceutical products, dyes, flavors, and preservatives. The compound, 3,5-diiodosalicylic acid (DIS), is used as an intermediate for veterinary anthelmintic agents (*closantel* and *rafoxanide*). Due to the presence of iodine atoms, DIS is also used as a source of heavy atom phases in crystallographic investigations of the new crystal structures of proteins and, at the same time, is a good ligand for analyzing protein binding properties.

Here we characterize the binding abilities of equine and bovine serum albumins based on their crystal structures in complexes with 3,5-diiodosalicylic acid at resolution 2.10 Å and 2.65 Å, respectively. The comparison of these structures with an analogous complex of human serum albumin (HSA) reveals numerous differences in the mode of binding of DIS.

Three DIS binding locations are common for this ligand in BSA and ESA and one site differs. In the case of HSA there are reports indicating two binding sites of DIS without fatty acids and only one DIS site in the presence of fatty acids. HSA is unable to bind the 3,5-diiodosalicylic acid in the locations corresponding to DIS3 and DIS4 in BSA and ESA, although other aromatic ligands, such as: iophenoxic acid, indomethacin, iodipamide are capable to bind in these pockets.

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P3.31

scFv anti-integrin $\alpha_v\beta_3$ — auristatin E conjugates in anticancer therapy

Anna M. Serwotka, Jacek Otlewski

Department of Protein Engineering, Faculty of Biotechnology,
University of Wrocław, Wrocław, Poland
e-mail: Anna Maria Serwotka <anna.serwotka@uni.wroc.pl>

Despite decades of research, cancer remains a leading cause of human deaths all over the world. Currently, there are many recognized cancer markers that can be used both in diagnostics and targeted therapy. Integrin $\alpha_v\beta_3$, receptor for vitronectin, belongs to promising cancer markers, mainly in glioblastoma and melanoma.

Monoclonal antibodies are the most common recombinant proteins that are applied to cure cancer via recognizing the cell surface markers of cancer markers. However, full format antibodies are large proteins (~150 kDa) that are rather difficult and expensive to produce. Instead, smaller antibody fragments, like scFvs (single-chain variable fragments), can be used. scFvs are the smallest antibody fragments that are capable of antigen recognition. An additional advantage of scFvs is the possibility of production in bacterial systems. The ease of production of scFv proteins is also reflected in the much lower cost of production.

In this study, scFv anti-integrin $\alpha_v\beta_3$ was designed based on sequence of humanised lapin monoclonal antibody anti-integrin $\alpha_v\beta_3$. scFv protein was produced in mammalian CHO-S cell line and purified on Ni-NTA agarose. scFv was properly folded, as revealed by circular dichroism spectra. The specificity of scFv – integrin $\alpha_v\beta_3$ interaction was confirmed by surface plasmon resonance, flow cytometry analysis and fluorescence microscopy. Experiments with U-87 MG glioblastoma cell line overexpressing integrin $\alpha_v\beta_3$ showed that scFv specifically recognizes integrin $\alpha_v\beta_3$. No interaction was observed in negative control experiments on SK-BR-3 adenocarcinoma cell line. scFv anti-integrin $\alpha_v\beta_3$ — MMAE (auristatin E) conjugate was synthesized and its cytotoxicity will be evaluated in the forthcoming experiments.

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P3.32

Selection of scFv targeting FGFR1 and FGFR2

Aleksandra Sokołowska-Wędzina,
Aleksandra Borek, Jacek Otlewski

University of Wrocław, Department of Protein Engineering, Wrocław, Poland
e-mail: Aleksandra Sokołowska-Wędzina <ola@protein.pl>

Fibroblast Growth Factor Receptors 1 and 2 (FGFR1 and FGFR2) overexpression has been found in different types of human cancer, including breast, bladder, prostate, endometrial, lung and hematologic cancers. Thus, FGFRs are promising candidates for potential cancer targeting molecule. To selectively target FGFR 1 and 2 overexpressed in cancer we used the strategy based on antibody targeting. Antibodies are the most specific and common protein binders, which provide high affinity and specificity to the interaction with the target. To generate FGFR 1 and 2 specific antibodies we used phage display technology. Due to the large size of immunoglobulin, we chose Tomlinson library based on antibody fragments: scFv scaffold (*scFv* — *single chain Fragment variable*).

Using Tomlinson I and J library, we selected a panel of antibody fragments specific for FGFR1 and FGFR2. After 3 or 4 rounds of selection we performed monoclonal ELISA of selected clones. At this stage we obtained 41 positive clones interacting with FGFR1, and 25 positive clones interacting with FGFR2. We further verified their potential interaction with receptors using another technique: SPR (*Surface Plasmon Resonance*) screening. We confirmed positive interaction for 20 (anti-FGFR1) and 15 (anti-FGFR2) ELISA-positive clones with their target receptors. Sequence analysis of SPR-positive clones has identified 18 different sequences of scFv selected against FGFR1, and 5 unique sequences selected against FGFR2. We found specific amino acid motifs within CDR3 loop of heavy chain, which is a region particularly important in antigen binding. Finally we chose 5 representative scFv variants for FGFR1, and 5 scFv variants for FGFR2, which were then expressed in HB2151 cells and purified using Protein A Sepharose. SPR measurements were performed with different concentrations of scFv monomeric fraction. Two strongest FGFR1 — specific binders, and one FGFR2 — specific binder were chose for further affinity maturation.

P3.33

CMP-sialic acid/UDP-N-acetylglucosamine chimeric protein partially improves glycosylation in mammalian cells deficient in UDP-galactose and CMP-sialic acid transporters

Paulina Sosicka, Dorota Maszczak-Seneczko, Mariusz Olczak

Laboratory of Biochemistry, Faculty of Biotechnology, University of Wrocław, Wrocław, Poland

e-mail: Paulina Sosicka <paulinka604@gmail.com>

Glycosylation is one of the most important posttranslational modification which occurs in the endoplasmic reticulum and Golgi apparatus. Plenty of enzymes are involved in this process whilst the most important are transferases, which deliver sugars to the glycan structure and nucleotide sugar transporters (NSTs), which provide substrates for them. Studies on the function and substrate specificity of NSTs includes structural modifications leading to the development of chimeric proteins, composed of portions derived from different transporters. In literature two such NSTs have already been described. Both CMP-sialic acid (CST; SCL35A1)/UDP-galactose (UGT; SLC35A2) and UDP-galactose/UDP-N-acetylglucosamine (NGT; SLC35A3) chimeric proteins were constructed to determine part of the transporter crucial for its substrate specificity. In our present study we investigated a novel chimeric transporter which is composed of the N-terminal portion of human CST and the C-terminal part of human NGT. CST/NGT was overexpressed in three cell lines, which are well known from their glycosylation defects — MDCK-RCA⁺, CHO-Lec8 (cell lines deficient in UGT) and CHO-Lec2 (cell line deficient in CST). We used UGT deficient cells because in our previous study we have found that NGT overexpression in these cell lines partially restore galactosylation of N-glycans. Using immunohistochemical staining we observed that CST/NGT chimeric protein was localized in the Golgi apparatus of stably transfected cells. Further experiments in CHO-Lec2 cells performed by application of highly specific lectins have shown that this transporter partially rescues sialylation defect. Moreover, there is slight improvement on galactosylation and sialylation in UGT deficient cells. This data suggest that CST/NGT chimeric protein might be able to transport UDP-galactose and CMP-sialic acid in some extent but they still need to be confirmed *via* structural glycans analysis and investigation of UDP-galactose and CMP-sialic acid uptake into the Golgi apparatus.

P3.34

ApoLp-III-derived polypeptides from *Galleria mellonella* hemolymph

Aneta Sowa-Jasilek¹, Paweł Mak², Sylwia Stączek¹, Agnieszka Zdybicka-Barabas¹, Małgorzata Cytryńska¹

¹Institute of Biology and Biochemistry, Department of Immunobiology, Maria Curie-Skłodowska University, Lublin, Poland; ²Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland

e-mail: Aneta Sowa-Jasilek <kese8@wp.pl>

Apolipoprotein III (apoLp-III) is an abundant hemolymph protein involved in immune response and lipid transport in insects. This protein is able to associate with bacterial and fungal cells through binding components of their cell walls, e.g. lipopolysaccharides, lipoteichoic acids and β -1,3-glucan. Moreover, upon bacterial challenge, apoLp-III interacts with lipids and joins to lipophorin particles forming low density lipophorins, which are absorbed by insect blood cells — hemocytes. ApoLp-III is involved in pathogen recognition, in detoxification of microbial cell wall components and can be considered as a signaling molecule informing the insect immune system about infection. It was demonstrated that apoLp-III can exist in hemolymph in several forms differing in isoelectric point.

Our previous research revealed that apoLp-III (18 kDa) occurs in two main forms in the hemolymph of naive *Galleria mellonella* larvae, but after immune challenge additional three or four forms (depending on the immunogen used) differing in pI and molecular mass were detected. As apoLp-III is synthesized in a fat body and was also detected in the hemocytes, we analyzed if apoLp-III-derived polypeptides are present in these tissues of naive individuals and larvae immunized by Gram-negative bacteria (*Escherichia coli*), Gram-positive bacteria (*Micrococcus luteus*), yeasts (*Candida albicans*) and filamentous fungi (*Fusarium oxysporum*). Immunoblotting performed with anti-apoLp-III antibodies after IEF/SDS-PAGE revealed one form of apoLp-III in the fat body of naive as well as immune-challenged insects, whereas in the hemocytes usually two forms of the protein differing in isoelectric point were detected.

G. mellonella apoLp-III-derived polypeptides present in hemolymph of larvae challenged by different pathogens were separated using RP-HPLC chromatography. Two fractions were received: the first fraction contained 14 kDa and 7 kDa polypeptides, while the second fraction contained differing in pI apoLp-III isoforms of ca. 18 kDa. The percentage ratio of the first to the second fraction in the hemolymph varied depending on the microorganism used to immune-challenge of the larvae. Our findings may indicate that apoLp-III is synthesized in the fat body as one of the basic form of the protein, but in the hemocytes and mainly in the hemolymph after infection various forms of apoLp-III appear in response to attack of different pathogens. The detected apoLp-III-derived polypeptides and isoforms probably are generated from apoLp-III by limited proteolysis, which occurs in the hemolymph and the hemocytes after infection. It is possible that in response to particular class of pathogen characteristic pattern of apoLp-III-derived polypeptides are produced.

P3.35

Novel primosomal protein B from *Clostridium pasteurianum*

Marta Śpibida, Marta Marszałkowska, Marcin Olszewski

Gdańsk University of Technology, Department of Microbiology, Gdańsk, Poland

e-mail: Marta Śpibida <spibida.marta@gmail.com>

PriB is a primosomal protein that catalyzes DNA replication in Prokaryota. The replication pathway starts with PriA protein — the initiator protein that binds to a DNA replication fork, unwinds double-stranded DNA and role of PriB is to stabilize PriA on the DNA. However there are many biochemical differences in replication mechanism in bacteria and only some of them use PriB proteins. A few of PriB proteins were published and only three structures of them were resolved (*Escherichia coli*, *Klebsiella pneumoniae* and *Neisseria gonorrhoeae*). All up-to-date known PriB proteins have one OB domain per monomer and they are homodimers in solution.

Recently, we have published the crystal structure of PriB protein from *Thermoanaerobacter tengcongensis* that represents new class of PriB with two oligonucleotide/oligosaccharide-binding domain (OB) per monomer that means it exists as monomer in solution.

The aim of this study is identification and characterization of the primosomal protein B (PriB) from bacterium *Clostridium pasteurianum* (CpaPriB). It is the largest known bacterial PriB protein consisting 234 amino acid residues with a calculated molecular mass of 30 kDa. Surprisingly, it is functional as monomer containing two single-stranded DNA binding domain (OB-fold) and it is the completely new kind structure of SSB protein like PriB protein from thermophilic bacterium *Thermoanaerobacter tengcongensis*. Therefore, our studies suggest that we discovered new classes of PriB from mesophilic bacteria.

P3.36

Characterization of the novel cysteine peptidase Tpr from the pathogenic bacterium *Porphyromonas gingivalis* W83 and its interaction with the cystatins — human host protease inhibitors

Dominika Staniec^{1,2}, Magnus Abrahamson², Jan Potempa^{1,3}

¹Department of Microbiology, Faculty of Biochemistry, Biophysics, and Biotechnology, Jagiellonian University, Kraków, Poland; ²Department of Laboratory Medicine, Division of Clinical Chemistry and Pharmacology, Lund University, Sweden; ³University of Louisville Dental School, Center for Oral Health and Systemic Diseases, Louisville, USA

e-mail: Dominika Staniec <dominikastan@gmail.com>

The relation between the pathogenic potential of *P. gingivalis*, an etiologic agent of periodontitis, and other serious illnesses like cardiovascular and lung diseases or rheumatoid arthritis is confirmed. Peptidases produced by *P. gingivalis* are recognized as essential virulence factors. Accordingly, bioinformatics analysis of the *P. gingivalis* genome revealed the presence of several genes encoding putative peptidases. One of these genes (PG1055) encodes a protein ("Tpr", thiol protease) with sequence similarity to cysteine peptidases of the calpain family. We have already created a large-scale expression and purification system, which allows to obtain recombinant protein Tpr. The peptidase showed low latency and underwent autocatalytic processing. Examination of proteolytic activity of Tpr released that enzyme possess Ca²⁺-dependent activity against gelatin, Z-Phe-Arg-NHMec, and Suc-Leu-Leu-Val-Tyr-NHMec, keeping with calcium-dependent autoproteolysis. These results confirmed that enzyme recognizes and digests amino acid sequences typical for collagen and might play role in host destructive events in periodontitis. To investigate the possibility that human-derived natural cysteine peptidase inhibitors can inhibit bacterial calpain-like peptidase, we determined the effect of cystatin A and C on the Tpr activity, and found that cystatins are potent inhibitors. As the peptidases can be obvious target for drug design in novel therapy strategies in order to dealing with periodontal disease, this results could aid in development of synthetic inhibitors, potential therapeutic agents. Moreover, to our knowledge Tpr is first bacterial cysteine protease which is inhibited by cystatins. This project would enable also further progress into investigation of more complex systems, e.g. peptidases — inhibitors models.

P3.37

Application of fluorescent Bocillin for testing penicillin binding properties of recombinant PBP 3, PBP 4 and PBP 5 proteins from *Neisseria meningitidis*

Karol Stawski¹, Anna Skoczyńska², Anna Goc¹

¹Department of Genetics, Nicolaus Copernicus University, Toruń, Poland; ²Department of Epidemiology and Clinical Microbiology, National Medicines Institute, Warsaw, Poland
e-mail: Karol Stawski <stawski@umk.pl>

Neisseria meningitidis is a Gram-negative human pathogen and a leading cause of bacterial meningitis and septicemia worldwide. A reduced susceptibility or resistance to penicillin is connected in some bacteria with alterations in penicillin binding proteins (PBPs), a group of enzymes responsible for the final periplasmic steps in the synthesis and maturation of peptidoglycan. The varied PBPs occur in different numbers per bacterial cell and have variable affinities for penicillin. For example a gram-positive bacteria *Bacillus subtilis* have 16 PBPs while gram-negative *Neisseria meningitidis* only 5. The differences between sequences of low molecular mass PBP 3, PBP 4 and PBP 5 of *N. meningitidis* may affect their enzymatic properties such as DD-carboxypeptidase or endopeptidase activity. We have amplified selected genes encoding PBPs of *N. meningitidis* using oligonucleotide primers and PCR products were cloned into pEt28a plasmid. The recombinant soluble PBP 3, PBP 4 and PBP 5 proteins do not include signal peptides, additionally PBP 3 and PBP5 are lacking extreme 22 and 105 amino acids of carboxyl terminus, respectively. Plasmids pEt28a encoding recombinant PBPs were transformed into *E. coli* B121(DE3)pLysS and their expression was induced with 1 mM IPTG for 4 hrs. Next, cells were pelleted and PBPs containing polyhistidine-tag were extracted using Ni²⁺ affinity chromatography columns. The interaction of beta-lactam with a recombinant PBPs was assessed by incubating the proteins for 0,5, 1, 2, 4 and 8 min with Bocillin, a fluorescent penicillin. The fluorescence emission from Bocillin in SDS gel of PBP 3, PBP 4 and PBP 5 have revealed their high affinity to beta-lactam antibiotics. However the rate of Bocillin acylation by recombinant PBPs was very fast and saturation of the enzymes occurred in the first 30 seconds.

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P3.38

RNA chaperone activity of proteins essential for the life cycle of HIV-2: implications for the design of the retroviral inhibitors.

Katarzyna Pachulska-Wieczorek, Agnieszka K. Stefaniak, Katarzyna J. Purzycka, Ryszard W. Adamiak

RNA Structure and Function Laboratory, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznań, Poland
e-mail: Agnieszka Katarzyna Stefaniak <astef@ibch.poznan.pl>

While preventive vaccine against AIDS is still not available, conventional therapies including highly active retroviral therapy (HAART), are based primarily on the HIV reverse transcriptase and protease inhibitors. This approach is associated with a number of side effects due to its high toxicity. Identification of the molecular mechanisms of HIV retroviruses, with particular emphasis on the structure and properties of macromolecules involved, is an indispensable prerequisite for the design of effective therapies against HIV/AIDS.

Nucleocapsid proteins (NC) are crucial for the HIV genomic RNA dimerization, packaging and reverse transcription, making these proteins new targets for the antiretroviral therapy. However, such an approach has not yet been exploited because of the lack of sufficient knowledge concerning both, specific and non-specific interactions of NC with viral RNA. Released from Gag, during viron maturation, nucleocapsid proteins (NC) are small basic proteins that possess two copies of highly conserved CCHC zinc fingers. The NC functions in HIV replication are correlated with their ability to act as nucleic acids chaperones (NAC). In contrast to NCp7 protein (HIV-1) little is known about the NAC activity of NCp8 (HIV-2).

Our research is focused on identifying interactions of HIV-2 RNA with regulatory proteins Tat-2, NCp8 and Gag polyprotein, that are crucial for the HIV replication. We examined the ability of NCp8 to chaperone annealing of DNA with complementary strand as well as DNA and RNA strand exchange in duplex nucleic acids *in vitro*. Furthermore, we investigated whether NCp8 is capable of enhancing ribozyme-directed cleavage of an RNA substrate *in vitro*. We found that NCp8 possesses nucleic acids chaperone activity similar to but somewhat different than NCp7 protein.

P3.39

The influence of resveratrol and its derivatives on the structure and function of glyceraldehyde 3-phosphate dehydrogenase under oxidative stress conditions

Joanna Strumiłło, Aleksandra Rodacka, Mieczysław Puchała

Institute of Biophysics, Department of Molecular Biophysics, Division of Radiobiology, University of Łódź, Łódź, Poland
e-mail: Joanna.Strumillo<joastr@biol.uni.lodz.pl>

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is glycolytic enzyme ubiquitous both in eukaryotic and prokaryotic proteomes. It is responsible for the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. GAPDH is also one of the classical examples of multifunctional proteins called "moonlighting" proteins. It plays a key role in such processes as transcription, apoptosis and vesicle shuttling. Furthermore, oxidatively modified GAPDH is found to be involved in the protein aggregation processes and pathogenesis of neurodegenerative diseases. Resveratrol and its derivatives are naturally occurring polyphenolic compounds with anti-oxidative properties. It is believed that they have anticancer, cardioprotective properties and prevents from neurodegeneration.

The objective of the presented research was to examine the influence of natural polyphenols on the structure and function of glyceraldehyde-3-phosphate dehydrogenase in the presence or absence of hydrogen peroxide. The compounds used in this study were: resveratrol and its analog (trans-3,3,5,5-tetrahydroksy-4-metoksystilbene), yuccaol A and yuccaol C.

We investigated the influence of the above mentioned compounds on the activity, secondary structure and content of thiol groups in the presence or absence of hydrogen peroxide. Furthermore, we checked the rate of 1,8-ANS (1-anilinonaphthalene-8-sulfonic acid) fluorescence after binding to protein exposed to polyphenols both in the presence and absence of hydrogen peroxide. There was no significant influence of the used compounds on the activity and thiols content in the absence of hydrogen peroxide. On the other hand, during oxidative stress induced by hydrogen peroxide and in the presence of used polyphenols we observed inactivation of the enzyme, changes in the content of free thiol groups and secondary structure of GAPDH. However, no significant increase of 1,8-ANS fluorescence was observed. The most significant changes in the secondary structure were observed in the presence of hydrogen peroxide and resveratrol analog and with both yuccaols.

The presented data clearly indicate that resveratrol, its analog and yuccaols A and C have pro-oxidative properties toward glyceraldehyde-3-phosphate dehydrogenase.

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P3.40

Structural and thermodynamic characteristics of avidin-biotinylruthenocene complex

Paweł Strzelczyk¹, Anna Bujacz¹, Damian Płażuk², Janusz Zakrzewski², Grzegorz Bujacz¹

¹Institute of Technical Biochemistry, Lodz University of Technology, Łódź, Poland; ²Department of Organic Chemistry, University of Lodz, Łódź, Poland
e-mail: Paweł.Strzelczyk<pawol.strzelczyk@gmail.com>

Avidin is a glycosylated protein from eggs of amphibians, reptiles and birds. Homotetramer of avidin binds four molecules of biotin with the highest known in Nature affinity ($K_d \approx 10^{-13}$ - 10^{-15} M). This interaction is several orders of magnitude higher than in the typical antigen-antibody complexes [1]. The biotin-avidin system, based on the extremely high affinity is widely used in bioanalysis, drug delivery and nanotechnology.

This study is focused on the interaction of avidin from hen egg white with biotinylruthenocene (BR) based on crystallographic research and isothermal titration calorimetry. We present crystal structure of avidin-BR complex (PDB ID: 4I60) and thermodynamic parameters of interaction between these two molecules.

Avidin is organized in eight-stranded antiparallel β -barrel with right-handed twist. Crystal of the avidin-BR complex belongs to a tetragonal system in the space group $P4_22_2$. The tetrameric assembly is located at the crossing of the three two-fold axes and is created by crystal symmetry. Each monomer is stabilized in the tetramer by interactions of extended loops and the external surfaces of the β -barrels. The crystal structure of the avidin-BR complex shows that most of the interaction of biotin moiety is maintained in comparison to avidin-biotin complex and the ruthenocene moiety is capable of interacting with protein via hydrogen bonds and π -stacking contacts. The larger ligand practically does not influence the avidin conformation with the exception of a small movement of the loop L3-4. The biotin conjugation with ruthenocene reduced ligand affinity, which was expected. The replacement of the strong hydrogen bonds created between the carboxyl group from biotin with Ser73 and Ser75 by weaker contacts with a cyclopentadienyl ring from the biotinylruthenocene moiety could account for the lower affinity of that ligand to avidin. Smaller affinity of the biotinylruthenocene to avidin may be beneficial when such complex will be used as a drug delivery system [2].

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P3.41

Effect of ferulic acid on fungal 26S proteasome activities *in vitro*

Anita Swatek, Anna Golecka, Magdalena Staszczak

Department of Biochemistry, Institute of Biology and Biochemistry,
Maria Curie-Skłodowska University, Lublin, Poland
e-mail: Anita Swatek <anitaswatek89@gmail.com>

The 26S proteasome, a large multicatalytic protease complex is involved in highly selective ATP-dependent and ubiquitin-mediated degradation of intracellular proteins in eukaryotes. Its presence was found in animals, plants and yeast [1, 2]. In the kingdom of fungi proteasomes were also reported in ligninolytic *Basidiomycetes* [3, 4]. The proteasome-mediated proteolysis plays a key role in the regulation of critical cellular processes such as transcriptional control, cell cycle progression, oncogenesis, and stress response. Previous studies from our laboratory demonstrated involvement of ubiquitin/proteasome pathway in the regulation of ligninolytic activities in the wood-decaying fungi. The 26S proteasome is assembled from a core particle (20S proteasome) and one or two regulatory complexes 19S. The core particle of the 26S proteasome houses proteolytically active sites, identified based on their specificity towards short synthetic peptides as: “chymotrypsin-like” (CHT-L), “trypsin-like” (T-L), and “caspase-like” (C-L). In the present work we studied effect of ferulic acid (4-hydroxy-5-methoxycinnamic acid), known as an inducer of some ligninolytic enzymes, on peptidase activities of the 26S proteasomes isolated from mycelia of the white-rot fungus *Trametes versicolor*. Proteasomes were separated from other low molecular proteases using Biomax PBVK polyether-sulfone ultrafiltration membranes (NMWL 500 kDa) [3]. Peptidase activities were detected by monitoring cleavage of fluorogenic substrates: Suc-LLVY-AMC, Z-GGR-AMC and Z-LLE-βNA for CHT-L, T-L, and C-L activity, respectively. Moreover, the ability of ferulic acid (in concentrations up to 2 mM) to affect degradation of a model protein substrate (β-casein) by the isolated 26S proteasomes was also evaluated using spectrofluorimetric and electrophoretic analyses. The absolute rate of protein degradation was spectrofluorimetrically quantified by the modified fluorescamine assay [5] for release of free α-amino groups generated upon the cleavage of peptide bonds in β-casein.

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P3.42

Proline-rich polypeptide complex (PRP) and its nonapeptide fragment (NP) protect neuronal cells from toxic effect of amyloid beta 1-42

Agnieszka Zabłocka¹, Anna Urbaniak¹,
Marianna Kuropatwa², Maria Janusz¹

Ludwik Hirsztfeld Institute of Immunology and Experimental Therapy,
Polish Academy of Sciences, ¹Laboratory of Immunochemistry,
²Laboratory of Signaling Proteins, Wrocław, Poland
e-mail: Anna Urbaniak <zablocka@iitd.pan.wroc.pl>

Introduction: Deposits of amyloid β protein are pathological hallmark of AD. They are toxic for neural cells both *in vivo* and *in vitro*. Aggregates and fibrillar forms of amyloid β are closely associated with inflammation. It was shown in several reports that uncontrolled production of nitric oxide could be involved in the amyloid β42 toxicity.

Aim: The ability of proline-rich polypeptide complex (PRP) isolated from ovine colostrum and its nonapeptide fragment (NP) to protect neuronal cell line PC12 from toxic effects of aggregated amyloid β42 was studied.

Methods: Rat pheochromocytoma PC12 cell line was treated with PRP/NP and aggregated amyloid β42. PRP/NP was applied to the cells simultaneously, 24 hours before and 24 hours after amyloid β42 treatment. Cell viability was checked by MTT assay. The level of neuronal NOS was determined by Western Blotting. The intracellular level of nitric oxide was measured by Griess method after reduction of nitrates to nitrites using NADPH – dependent nitrate reductase. The level of cGMP was detected by ELISA.

Results: A colostrum proline-rich polypeptide complex PRP and its nonapeptide fragment (NP) can modulate the toxic effect of amyloid β42 on neuronal PC 12 cell line. It was observed that PRP and NP significantly lowered the toxicity of amyloid β42. The regulatory effect of PRP/NP on NO production induced by amyloid β42 was also shown. The neuronal response to amyloid β42 was cGMP-dependent only for PRP when used at low doses.

Conclusion: The results obtained indicate that both PRP and NP can modulate the amyloid β42 cytotoxicity.

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P3.43

Construction and selection of phage display libraries based on variable lymphocyte receptor sequences

Magdalena Wezner-Ptasinska, Jacek Otlewski

University of Wrocław, Department of Protein Engineering, Wrocław, Poland

e-mail: Magdalena Wezner-Ptasinska <magdalena.wezner-ptasinska@uni.wroc.pl>

Macromolecules specifically recognizing certain antigens are invaluable tools in research and clinical applications. The most common natural binders are antibody molecules providing high affinity and specificity towards the target. Recently, the alternative binders, usually small and stable proteins, have challenged the immunoglobulin applications. Usually, binding molecules can be selected from combinatorial libraries based on display technologies. Phage display is a powerful methodology that allows the selection of a particular phenotype (e.g., binding to a defined antigen) from repertoires of proteins displayed on phage surface. Over the years it has been established as a robust and reliable technology for specific binders selection.

In the project the alternative scaffold based phage display libraries were tested as a source of proteins specifically recognizing biomarkers for cancer therapies. Two different phage display libraries were constructed on the previously designed dVLR (variable lymphocyte receptor) template based on receptor sequences that mediate adaptive immunity in sea lamprey. dVLR contains six LRR (leucine-rich repeat) modules forming horseshoe shape structure whose concave surface is responsible for target binding. Crystal structure analysis of VLR-ligand complexes indicated the positions responsible for interaction with antigens. For these positions different schemes of randomization have been chosen. Two different phage display libraries has been designed: P16 — 16 randomized positions and Sm11S — 11 randomized positions, resulting in functional library size of 2×10^{10} and 1×10^8 amino acid sequences respectively. In both libraries SRP protein translocation pathway has been applied. In next step phage display libraries were used for isolation of binders. Cancer biomarkers S100A7 and HER2 were chosen as targets for panning. A wide panel of selection protocols was tested, both on plastic surface and in solution. Preliminary surface plasmon resonance experiments confirmed interaction between selected variants and desired antigen.

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P3.44

VEGF in intraluminal thrombus of abdominal aorta

Małgorzata Wolańska¹, Emilia Bańkowska-Guszczyńska¹, Radosław Kowalewski², Krzysztof Sobolewski¹

¹Department of Medical Biochemistry and ²Department of Vascular Surgery & Transplantation, Medical University of Białystok, Białystok, Poland

e-mail: Małgorzata Wolańska <ma.wolanska@interia.pl>

The pathogenesis of aorta aneurism is characterized by a breakdown of extracellular matrix due to an excessive proteolytic activity. First of all elastin is degraded and replaced by collagen. The remodelling of arterial wall is controlled by the action of some peptide growth factors, especially by VEGFs. In most cases the aorta aneurisms are accompanied by intraluminal thrombi (ILT). The functions of cytokines, especially those of VEGFs, in the pathobiology of arterial wall are relatively well known. On the other hand the peptide growth factors in the intraluminal thrombi were not studied till now. For this reason it was decided to search for the expression of VEGFs, their receptors and mitogen-induced kinases (MAP)-kinases in intraluminal thrombi taken from patients submitted to surgical treatment of abdominal aneurism aorta.

Studies were performed on fragments of aneurism walls taken from 24 patients (20 men and 4 women) of the age 62–80 (average 67.9 ± 7.2) operated at the Department of Vascular Surgery and Transplantation. The diameter of the aneurism was 72 ± 14 mm. The parietal thrombi were separated from aneurism wall and divided into luminal and abluminal layers. The control material (aorta wall) was the fragments of abdominal aorta taken from 12 organ donors (10 men and 2 women) of the age 47 ± 5 .

The most important finding of our study was demonstration the high expression of VEGFs in both layers of intraluminal thrombus. No doubt the blood-borne cells are engaged in their biosynthesis. Also the receptors of VEGFs were detected. Furthermore, the activity of phosphorylated MAP-kinases: ERK1 and ERK-2 was found in intraluminal thrombus. In some cases the expression of VEGFs and their receptors in the thrombus was even higher than in aorta wall and in aneurism wall. It allows to conclude that some blood cells may synthesize VEGFs which stimulate these cells to produce extracellular enzymes which degrade the fibrin. Furthermore, the VEGF may penetrate from the thrombus to aneurism wall and to enhance the neovascularisation process. Furthermore it may stimulate the remodelling of extracellular matrix. It may result in enhanced degradation of elastin and its replacement by collagen.

P3.45

Studies on specificity of DNA methyltransferase M.BstZ1II and its deletion mutant

Ewa Wons-Karczyńska, Iwona Mruk, Tadeusz Kaczorowski

Department of Microbiology, University of Gdańsk, Gdańsk, Poland
e-mail: Ewa Wons-Karczyńska <ewawons@gmail.com>

DNA methylation is a major form of epigenetic modification. The process is provided by DNA methyltransferases (MTases), which catalyze a transfer of methyl group from the donor S-adenosyl-L-methionine to the adenine or cytosine residue within recognized nucleotide sequence. MTases fall into two groups depending on methylated atom — endocyclic MTases (C-MTases) modify carbon C5 of cytosine, while exocyclic MTases (N-MTases) methylate nitrogen from amino group of adenine (N6) or cytosine (N4).

MTases contain two domains — the larger with a set of nine/ten well defined amino acids motifs creating the AdoMet binding pocket and the active site, and the smaller — responsible for recognizing the specific DNA sequence (TRD — target recognition domain). While larger domains of all MTases share a common core structure referred as AdoMet-dependent MTase fold, smaller domains of different proteins are dissimilar in amino acids sequence, size and structure.

We have been focusing on the group of five MTases, which despite origin in distinct bacteria species recognize the same nucleotide sequence 5'-AAGCTT-3' and modify the first adenine residue at N6. These enzymes are: M.HindIII, M.EcoVIII, M.Csp23II, M.LlaCI and M.BstZ1II. Comparison of their amino acid sequences revealed 68-aa region located before TRD in M.BstZ1II, which was neither present in the rest of studied proteins, nor was similar to any other sequences in the GenBank. Therefore the mutant protein devoid the region was constructed and named M.BstZ1IIΔ. Since the plasmid DNA carrying shorter variant of M.BstZ1II was fully protected against digestion by restriction endonuclease of the same specificity, the mutant protein was active. To determine the possible role of the region, the specificity towards different substrate DNA of two purified variants (mutant and WT) was assessed. Both forms showed relaxed specificity when reaction mixture was supplemented with 40% DMSO and were able to modify sequences with single substitutions in regard to the canonical AAGCTT. M.BstZ1IIΔ exhibited slightly stronger preference towards the 2nd position of the specific sequence than the WT. Moreover, it appeared that the 68-aa region may be responsible for interacting with the DNA backbone, as M.BstZ1IIΔ modified with lower efficiency all substrates lacking internucleotide linkage within the canonical sequence than the WT as well as in contrast to the WT was unable to methylate canonical sequence located directly at the end of the oligonucleotide.

P3.46

Major urinary protein levels in cystathionine beta synthase deficient mice

Jacek Wróblewski¹, Joanna Perla-Kaján², Hieronim Jakubowski^{1,2,3}

¹Poznań University of Life Sciences, Department of Biochemistry and Biotechnology, Poznań, Poland; ²UMDNJ-New Jersey Medical School, International Center for Public Health, Department of Microbiology and Molecular Genetics, Newark, NJ 07101, USA; ³Institute of Bioorganic Chemistry, Poznań, Poland
e-mail: Jacek Wróblewski <jacek.wroblewski.jr@gmail.com>

Background: Major Urinary Proteins (MUP) are excreted in mouse urine and play an important role in sexual signaling by binding volatile pheromones and prolonging their persistence in scent marks. MUPs expression decreases in senescent males. Female mice tend to avoid senescent, older mates, which are unable to retain the ability to produce costly sexual signals. Factors affecting MUPs expression are largely unknown. Here, we examined how hyperhomocysteinemia due to cystathionine β-synthase (Cbs) deficiency impacts the expression of MUPs in the mouse.

Methods: We used the C57BL/6J *Tg-1278T Cbs*^{-/-} mouse model (Jakubowski H *et al.*, 2009, *FASEB J* **23**:1721–177). MUPs concentrations and urinary creatinine in *Cbs*^{-/-} mice (n=7) and *Cbs*^{+/+} littermates (n=5) were determined using Coomassie and picric acid, respectively. Patterns of MUP expression were analyzed on SDS/PAGE gels. Urinary homocysteine (Hcy), Hcy-thiolactone, and N-Hcy-protein levels were measured using HPLC-based assays (Jakubowski H, 2008, *Anal Biochem* **380**: 257–261) on 1260 Infinity UPLC instrument (Agilent). Site-specific N-homocysteinylolation of MUPs lysine residues was analyzed using LC-MS/MS.

Results: Urinary Hcy, Hcy-thiolactone, and N-Hcy-protein levels were extremely elevated in *Cbs*^{-/-} mice, compared to *Cbs*^{+/+} animals. *Cbs*^{-/-} male mice had significantly lower urinary MUP (protein/creatinine ratio was 0.38±0.13 in *Cbs*^{-/-} mice and 0.93±0.08 in *Cbs*^{+/+} animals), seen also on SDS-PAGE gels. Patterns of MUPs separated on SDS-PAGE gels differed between *Cbs*^{-/-} and *Cbs*^{+/+} mice. LC-MS/MS analysis of tryptic digests of MUPs from male and female *Cbs*^{-/-} mice identified a MUP11 peptide, ⁹⁵AG(N-Hcy-K) YSVTYDGFNTF¹⁰⁸, containing Hcy N-linked to lysine residue K⁹⁷.

Conclusions: Hyperhomocysteinemia increases N-homocysteinylolation and decreases expression of MUPs in mice. These findings identify MUPs as a new target for N-homocysteinylolation *in vivo* and suggest that hyperhomocysteinemic males are impaired in their ability to produce scent marks, and thus may be less preferred by their females mates.

P3.47

Lithium chloride protects neurons from toxicity of cytosolic PrP

Tomasz Zajkowski, Hanna Nieznanska, Krzysztof Nieznanski

Department of Biochemistry, Nencki Institute of Experimental Biology, Warsaw, Poland

e-mail: Tomasz.Zajkowski <t.zajkowski@nencki.gov.pl>

Prion protein (PrP) is mostly extracellular glycoprotein anchored in plasma membrane, constitutively expressed in the nervous system. In misfolded state this protein is prone to form aggregates, which are found in diseases called transmissible spongiform encephalopathies (TSE). In some of these diseases PrP abnormally localizes in cytosol (cytoPrP) interacting with proteins, which are not its physiological partners. In previous studies we have demonstrated that PrP is causing tubulin oligomerization, inhibits microtubule formation and disassembles microtubular cytoskeleton of epithelial cells. We have also showed that microtubule associated proteins (MAPs) that regulate microtubule stability can prevent above-mentioned effect of PrP. According to our study at least two proteins of this group: Tau and MAP2 were able to prevent deleterious effect of PrP but not in phosphorylated state. Interestingly, hyperphosphorylated Tau has been frequently detected in TSE. Extending the application of the above one should be able to modulate effect of cytoPrP on microtubular cytoskeleton by influencing level of phosphorylation of MAPs. In our study we used synthetic peptide encompassing first 30 amino acids of PrP (PrP1-30) as a model of cellular prion protein. The peptide consists of N-terminal signal sequence responsible for penetration of the molecule through cell membrane and major tubulin-binding site (23–30). To reduce level of phosphorylation of MAPs we chose inhibitors of GSK-3 a kinase known to be responsible for modification of these proteins. Specific inhibitor of the kinase — CT98014 and less selective LiCl were employed. By means of confocal microscopy we demonstrated disassembly of microtubular cytoskeleton and loss of neurites of primary neurons exposed to PrP1-30. In contrast, the cells treated with PrP1-30 in the presence of either LiCl or CT98014 did not differ from control cells. In cytotoxicity tests (MTT, LDH) we confirmed protection of neurons from toxic effect of PrP1-30 by both inhibitors of GSK-3. Our observations may help in understanding the molecular mechanism of neurotoxicity of cytoPrP. Since LiCl is well-known pharmaceutical used in the treatment of several mental disorders we believe that our study may be useful in design of therapy of TSE.

P3.48

Crystallographic studies of serum albumins in complexes with ligands

Kamil Zielinski, Bartosz Sekula, Anna Bujacz

Institute of Technical Biochemistry, Faculty of Biotechnology and Food Sciences, Lodz University of Technology, Łódź, Poland

e-mail: Kamil.Zielinski <kamilzielinski@poczta.onet.pl>

Serum albumins are highly soluble proteins naturally present in plasma. Due to their unique properties and high ability to bind various ligands, mammalian serum albumins are used in many laboratory procedures. They are known as versatile transport proteins that carry physiological ligands such as: fatty acids, hormones, bilirubin and others [1].

Most of the serum albumin binding sites have affinity to more than one group of ligands, therefore determination of the protein-ligand interactions are essential for understanding the complex character of this protein transporter. Albumin is a heart-shaped molecule composed of three helical domains consisting of two subdomains each, showing a different binding specificity.

The topology of serum albumin binding pockets has been well characterized based on the human serum albumin (HSA) crystal structures and it has been explored in other species [2].

The inspiration for the presented research was the observation that drug affinity could be dependant on the presence of other small molecules that bound to albumin.

We present the crystal structures of bovine, equine and leporine serum albumin complexes with an iodine derivative of salicylic acid and naproxen. The first ligand is used as an intermediate for veterinary anthelmintic agents, a medication capable of causing the evacuation of parasitic intestinal worms, while the second is a non-steroidal anti-inflammatory drug, commonly used for the reduction of pain, fever and inflammations.

Comparison of the investigated complexes will allow to determine the differences in ligand binding properties, which result from different sequence and spatial specificity of individual albumins. The structural investigations of these complexes provide the information about their surface shape and potential that can be utilized as specific epitopes for antibodies.

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P3.49

Yeast prion protein Rbs1

Marta Żurańska

Institute of Biochemistry and Biophysics PAS, Department of Genetics,
Warsaw, Poland

e-mail: Marta Żurańska <zuranska.marta@gmail.com>

Prions (protein infectious particles) are unusual proteins that exist stably in many functionally distinct conformations at least one of which is self-replicating. Prions are responsible for several severe mammalian illnesses like Creutzfeldt–Jakob disease or bovine spongiform encephalopathy. On the contrary in yeasts they do not cause diseases, but act as non-Mendelian inheritance elements that have important role in such processes as fidelity of translation termination or transcription regulation. Yeast prions are thought to be mechanism for generating fast phenotypic diversity that helps to survive in changing environment. Rbs1 has been identified among the candidates selected in a bioinformatic proteome-wide survey for prionogenic proteins in *Saccharomyces cerevisiae* (Alberti *et al.*, 2009, *Cell* **137**: 146–158).

N-terminal part of Rbs1 protein of ordered structure contains R3H domain which potentially binds single stranded nucleic acids. In contrast, C-terminal part of Rbs1 forms a molten globule.

Prion features of Rbs1 are supported by our current studies. We found that yeast Rbs1 protein shuttles between the cytoplasm and the nucleus. When localised in cytoplasm, Rbs1 forms foci. The amount of foci is increased in the older the cells. Inactivation of R3H domain by mutagenesis decreases efficiency of foci formation. Rbs1 is not essential protein and its exact function is still unknown but our work indicates its influence on biogenesis of RNA polymerase III.