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Short and long-term structural effects of terahertz radiation on cryo-cooled bovine trypsin crystals

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The use of non-ionizing Terahertz (THz) radiation in technology is a new growing field. New methods utilise THz radiation in for instance security screening at airports and border controls, and in medical diagnostics [1]. In contrast to x-rays, the THz radiation can non-destructively screen medical patients. In addition, the radiation generates a high contrast in soft tissue due to water absorption [2], and has higher spatial resolution compared to MRI and ultrasound [3]. Albeit non-ionizing, research have shown that THz radiation might still induce changes biomolecules due to collective oscillations [4]. In addition, a report from the U.S. National Toxicology Program states that non-ionizing radio waves from cell phones might have a cancerous effect [5]. Therefore, it is imperative to study this protein-radiation interaction, not only for the sake of potential adverse effects biological systems, but also for the new insight this information provide to protein - light interactions.

To research the effect of THz interactions, bovine trypsin was crystallized, flash-cooled to 100 K, and studied in a pump-probe x-ray crystallography experiment. During the experiment, the crystals were pumped with 0.5 THz radiation, for 22.5 ms, and simultaneously probed with 14 KeV x-rays during 25 ms, with no THz radiation at half of the duty cycle. The data (diffraction up to 1.15 Å) show differences in the isotropic and anisotropic contribution of the atomic displacement parameter (ADP) tensor, for individual atoms. The differences are detected during both short term timescales (differences between odd and even frames), and long term timescales (differences between THz-radiated and reference crystals). Additionally, the ADP tensor forms functional links to seemingly unrelated parts of the protein. In general, these data indicate a structural order which is induced by the THz radiation, in contrast to the general disorder, expected from an increased thermal energy.

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Mechanisms of methyl-dependent signalling in chromatin regulation

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Elucidating the functional details of the human mitochondrial HtrA2 serine protease

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HtrA2 (High temperature requirement protein A2) or Omi is a mitochondrial serine protease of the HtrA family found in all kingdoms of life[1]. HtrA2 resides in the inner mitochondrial membrane and is released from the membrane and into the cytosol through cleavage of the transmembrane domain upon apoptotic stimuli, where it works a as a pro-apoptotic factor[2]. In addition, HtrA2 has been implicated in a number of neurodegenerative diseases such as Parkinson's and Alzheimer's disease as well as several cancer types[1,3–6]. Due to its importance in protein quality control and its connection to several severe human diseases, HtrA2 is an important target of study. However, so far, its functional cycle, regulations as well as interaction partners of HtrA2 remain mostly elusive. To decipher the functional details of the HtrA2-protease we initiated a study of the structure and dynamics of human HtrA2 by using high-resolution nuclear magnetic resonance (NMR) spectroscopy techniques. To initially characterize the secondary structure elements in solution, we have created a mutant of HtrA2 which renders the functional active trimer monomeric. This monomeric construct enables a first analysis of the structural adaptions and dynamics underlying HtrA2 function. We will proceed to transfer these initial observations to the active trimeric form in order to delineate a working model for the HtrA2-protease cycle.

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Towards time-resolved structural studies of oxygen activation in μ-oxo-bridged dinuclear metalloproteins

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Studying high-oxidation-state metal sites in proteins by X-ray crystallography is challenged by X-ray induced photoreduction of such sites. This problem is further highlighted when attempting to capture more or less short-lived intermediate states in systems undergoing chemical change.

The effect of photoreduction can be minimised by turning to femtosecond X-ray crystallography where X-ray free-electron lasers are applied to deliver X-ray pulses short enough for "diffraction before destruction"-styled serial crystallography and thereby preserving structural data of such high-oxidation-state metal sites.

For the work presented here, the sample delivery system makes use of a conveyor-belt-style drop-on-tape sample delivery system. Keeping the experimental setup enclosed in a helium environment with the reduced metal site sample passing through an oxygen chamber prior to the X-ray exposures provides the possibility to control the length of time that the sample is exposed to oxygen prior to diffraction data collection.

The main model systems for these studies are members of the ribonucleotide reductase (RNR) family in which oxygen activation to generate a μ-oxo-bridged metal site is required for forming radicals to be shuttled to a catalytic subunit for ribonucleotide reduction. Being able to control the time of oxygen exposure provides a chance to capture structural data on the higher-oxidation-state intermediates of the dinuclear metal site [(III)/(IV) or (IV)/(IV)] during oxygen activation.

Early results from data collected at LCLS (Linac Coherent Light Source), Stanford, USA and SACLA (SPring-8 Angstrom Compact free electron LAser), Sayo-cho, Japan will be presented.

Tetrameric Transthyretin co-crystal structures with amyloid probes

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Amyloidosis is characterized by the extracellular deposition of insoluble protein fibrils and occurs in serious medical disorders such as Alzheimer's disease and transmissible spongiform encephalopathies. Currently, more than 30 structurally and functionally different proteins are known to cause amyloidosis where one of them is TTR. Transthyretin is a 55 kDa homotetrameric protein composed of 127-residue *β*-sheet-rich subunits. Transthyretin (TTR) is a homotetrameric protein in plasma and cerebrospinal fluid (CSF) transporting the thyroid hormone thyroxine (T4) and retinol-binding protein bound to retinol. This ligand binding helps stabilize the tetramer and prevent dissociation and misfolding of the monomer, which is important as TTR is an amyloid protein. While the wildtype protein sporadically misfolds and forms amyloids, TTR amyloid diseases are predominantly inherited and caused by a wide range of missense mutations: Familial amyloid polyneuropathy (FAP) is e.g. associated with the TTR V30M mutation, while TTR V122I is commonly the underlying mutation causing Familial amyloid cardiomyopathy (FAC).

TTR amyloids in various diseases show structural polymorphism, which is attributed to diversity in disease severity (Fändrich et al. 2018). We have been developing small molecule, fluorescent probes which target and specifically distinguish these different states spectroscopically (Campos et al., 2015; Zhang et al., 2017). These probes are based on a trans-stilbene scaffold originating from the natural compound resveratrol. Interestingly the same probes also bind the native tetramer conformation at the thyroxine (T4)-binding site.

This binding modality allows high resolution work using X-ray crystallography as the native TTR tetramer is readily crystalized. We have co-crystallized TTR with a variety of these probes with the aim to obtain high resolution structures of probes in the bound state to elucidate the molecular basis for diverse spectral output when bound to TTR. This will enhance our understanding of amyloid probe design.

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Serial Femtosecond Crystallography of Reaction Center from *Blastochloris Viridis*

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Photosynthesis in plants and photosynthetic bacteria, to transform sunlight into energy, is one of the fundamental biological processes for life on Earth. The basis of this system is the excitation of an electron by a photon that lets it travel between several different cofactors and proteins. This process eventually leads to the build-up of a proton gradient across the membrane which in turn can be used to synthesize ATP, the main energy source for the bacteria.[1]

We have studied the first protein in this chain of events; the Reaction Center from *Blastochloris Viridis*. Through the use of serial femtosecond crystallography at an X-ray Free Electron Laser (XFEL) the structural changes that follow the first few picoseconds after photon absorption can be seen. The work includes optimizing crystals for the liquid injection system at the beamline which requires crystals to be smaller than ~20-25µm in size.[2] Data was then acquired in a pump-probe manner with a laser at 960nm collecting snapshots of the protein at 5 and 300ps following excitation of the special pair of bacteriochlorophylls revealing the movement of the cofactors and surrounding sidechains on an ultrafast timescale.

Recently a new Lipidic Cubic Phase (LCP) crystallization protocol has been developed. A dataset diffracting to 2.2Å was collected at SACLA, Japan. Solving the structure showed that the mobile quinone could be modelled into its binding pocket making it possible to collect data on longer time-points in the future.

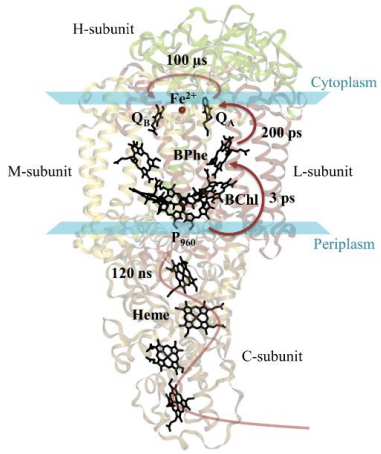

Figure1. The different timepoints of electron transfer in Photosynthetic Reaction Center following photoexcitation. The electrons are transferred from the special pair to the mobile quinone Q_B that diffuses into the membrane after reduction.

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High Electric Field Induced Structural Changes in Protein Complexes

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Microtubules are extended biological nanotubes that are critical for maintaining cell integrity and controlling cell division. Time-resolved crystallography has shown that electric fields may drive functionally relevant conformational changes in proteins, and the growth kinetics of microtubules show non-thermal effects when exposed to microwaves.

The project aims to further investigate these phenomena by applying electric fields of increasing amplitude and alternating frequencies to a solution of microtubules. Changes to the microtubules structure, stability, and assembly/disassembly kinetics shall be investigated using preliminarily spectrophotometer and XFEL based approaches.

This work will be carried out in collaboration with Chalmers University of Technology in order to safely and adequately make use of high voltage electric fields.

The Swedish National Facility for Cryo-EM

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Abstract: With the explosion of cryo-EM to get high-to-medium resolution structures of proteins and protein complexes, request for data collected on high-end microscopes is rising. The costs of the equipment as well as the need for expertise on both microscope operation and image processing, is pushing institutions to gather forces for the creation of national centres for cryo-EM. In Sweden, funding from the Knut and Alice Wallenberg, Family Erling Persson, and Kempe Foundations, SciLifeLab, Stockholm University and Umeå University allowed the creation of the Cryo-EM Swedish National Facility, which provides the scientific community with state-of-the-art equipment and expertise in single particle cryo-EM and cryo-electron tomography (cryo-ET). The talk will present the building of the Facility, the equipment available and the way projects are handled in both screening and data acquisition.

Structural biology as a key tool for fragment-based drug design at Sprint Bioscience

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Our mission is to create small molecule anticancer drugs, targeting proteins involved in tumor metabolism, tumor microenvironment and immune-oncology. The starting points of our drug discovery projects are small molecular fragments, binding weakly to the target proteins. Structure-based drug design is used to grow the fragments, thus improving target affinity - sometimes as much as 10.000 times. Finally, the selectivity and ADME-properties are optimized, to confer the compounds pharmacological properties suitable for clinical trials.

Structural biology is a key expertise in the drug discovery process at Sprint Bioscience. Improving properties such as affinity of the fragment hits towards their target proteins relies on a very accurate knowledge of the interactions between the protein and the fragments. Sprint Bioscience relies on X-ray crystallographic structures of fragment-protein complexes to acquire this knowledge.

From an initial fragment hit to a suitable drug candidate, usually many dozens (sometimes hundreds!) of X-ray crystallographic structures are generated to help guide computational chemistry with the design of our inhibitors. Improving docking models or understanding unexpected gain or loss of potency are just a few points among many others where information provided by structural biology appears essential in our drug discovery process.

Here we will present how structural biology is integrated into Sprint Bioscience drug discovery pipeline and, through some examples, how we transform challenges into opportunities.

A decade of GPCR Structural Biology

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G Protein-Coupled Receptors (GPCRs) are cellular gatekeepers that regulate a variety of physiological processes in the human body and serve as attractive pharmaceutical drug targets. Structure-function studies of this superfamily have been enabled in 2007 by multiple breakthroughs in technology that included receptor stabilization, crystallization in a membrane environment, and microcrystallography. This talk will summarize key advancements in our understanding of the mechanisms of ligand recognition, allosteric modulation and signal transduction across the membrane, contributed by the last 10 years of structural studies of GPCRs. Recent advancements in X-ray free electron lasers and cryo-electron microscopy open up new opportunities and promise to further accelerate structure-function studies of the whole GPCR superfamily.

Cryo-ET of miotochondria: The role of ATP synthases in shaping cristae

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Free energy of β2-Adrenergic Receptor activation using the String of Swarms Method

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G-protein coupled receptors (GPCR) are membrane proteins able to trigger diverse cell signalling pathways by binding specific ligands. They constitute thus a major drug target family. Ligand binding occurs at the extracellular side and allosterically propagates conformational changes on the intracellular side. Binding of different ligands modifies the overall conformational landscape that is accessible to the receptor, and this can be quantified by characterizing the free energy along the activation pathway.

Characterizing the free energy of complex, non-linear conformational changes is in general a difficult problem. Molecular dynamics simulations provide an atomistic resolution picture but are limited in the time scales they allow to observe. Advanced schemes called enhanced sampling molecular dynamics can then in principle be used but how to best use those for such applications is still an active area of research with many open questions.

Here, we show that we can adapt the string of swarms method to characterize the minimum free energy pathway of activation of a class A GPCR, the β2 adrenergic receptor. We validate our approach by comparing the results to available experimental data. We further characterize the conformational ensemble and the activation pathway, highlighting the molecular degrees of freedom implicated in the process. This computationally affordable method is easily transferable to other GPCRs and will be useful to further our understanding of activation processes, of the molecular basis of their regulation and may open new avenues for drug design. This method is also anticipated to be adaptable to study conformational changes in other biomolecules.

MraY – an essential membrane-bound enzyme involved in peptidoglycan synthesis

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Antibiotic resistance, is a current serious threat for human health and the environment. The rapid growth of antibiotic-resistant bacterial infections require that new novel targets are characterized as a step in the development of new antibacterial drugs. A promising target is the prokaryotic membrane-bound enzyme MraY, an essential protein for bacterial cell wall synthesis not present in eukaryotes. MraY belongs to the PNPT-superfamily of enzymes and catalyzes the synthesis of C_{55}-PP-MurNAc-pentapeptide, also called Lipid I, using UDP-MurNAc-pentapeptide as the donor substrate.

MraY has been notoriously difficult to overexpress and purify to homogeneity in high amounts. This has indeed hampered the efforts to characterize the enzyme structurally. In 2013 the first crystal structure of MraY was published, namely MraY from the Gram negative bacterium *Aquifex aeolicus* (3.3 Å, PDB ID: 4J72)[1]. The structure was followed by MraY from *Aquifex aeolicus* in complex with muraymycin D2 (2.95 Å, PDB ID: 5CKR)[2] and MraY from the pathogenic Gram positive bacterium *Clostridium bolteae* in complex with tunicamycin (2.6 Å, PDB ID: 5JNQ)[3].

Our work focus on further characterize MraY using biophysical methods and determine the binding of potential compounds and hopefully will we in the future also be able to present new crystal structures which will aid in the development of new antibacterial drugs.

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Purification and co-crystallization of Asparaginyl tRNA synthetase from *Brugia malayi, with inhibitors*

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The infamous condition elephantiasis is caused by the neglected tropical disease lymphatic filariasis [1]. In Asia the disease is spread by mosquitoes carrying a small nematode called *Brugia malayi.* These nematodes occupy the lymphatic system of infected humans which can cause limbs to swell. Currently there are no efficient drug to kill adult worms. However, a new drug target called Asparaginyl tRNA synthetase (AsnRS) has been recognized in *Brugia malayi* [2]. AsnRS is one of several aminoacyl tRNA synthetases which recycles aminoacyl-tRNA in the cell which are essential for protein production. The goal of this project was to express, purify and co-crystallize both full length and truncated recombinant AsnRS from *Brugia malayi* with both identified inhibitors and potential inhibitors identified by collaborators [3-4]. Furthermore, an activity assay was used to validate that purified protein was active and that inhibitors lower the activity of AsnRS [5]. Out of three tested constructs only two could be purified while the third was almost completely expressed into inclusion bodies. The activity assay revealed that only one of the purified proteins were active. Activity assay additionally showed that the purified truncated protein could be inhibited by some of the compounds. Future work includes determining the IC_{50} values of the compounds of interest. Finally, screening co-crystallization conditions as none of the crystals produced diffracted at satisfactory level.

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Bioinformatic discovery and biochemical characterization of proteolytically active bacterial flagella

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Bacterial flagellins are the major building block of the flagellar filament, which play a key role in cell motility, adhesion and virulence, but also functions as sensory organelle.

Here, we present the discovery of the first family of enzymatically active flagellin variants that occur naturally in more than 100 bacterial species spanning >35 phylogenetically diverse genera. These flagellins have acquired a unique peptidase domain within their central hypervariable region (HVR). The evolutionary origination of the peptidase domain likely occurred by an ancestral insertion into the flagellin hypervariable region, followed by widespread lateral gene transfer within and between various phyla. In-depth sequence analysis revealed a conserved HExxH motif crucial for proteolytic activity, a potential structural Ca^{2+} binding site, and a remote sequence homology to clostridial collagenases.

We recombinantly expressed and purified a representative proteolytic flagellin (flagellinolysins) HVR from the animal pathogen *Clostridium haemolyticum*, and demonstrate it to be an active peptidase. Specificity profiling using a proteome-derived peptide library (PICS proteomics) and quenched fluorescent peptides revealed a substrate preference similar to human matrix metalloproteases (MMPs), suggesting a role in the degradation of extracellular matrix and/or bacterial biofilm proteins. Importantly, enzymatic characterization of intact flagellar filament verified *bona fide* proteolytic activity in the flagellar context, however, with a slightly altered sequence specificity profile.

In conclusion, these flagellinolysins copolymerize with non-enzymatic structural flagellins and hence transform the bacterial flagellum into a gigantic proteolytic machinery with approximately 1000 enzymatically active sites (Eckhard. 2017, Nat. Commun. 8(1):521).

MetalJet Technology and Applications in Protein- and Small Molecule Diffraction

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High-end x-ray diffraction techniques such as small molecule crystallography,macromolecular crystallography and non-ambient crystallography rely heavily on the x-ray source brightness for resolution and exposure time. As boundaries of technology are pushed forward samples are becoming smaller, weaker diffracting and less stable which put additional requirements on ever brighter sources. With bright enough compact sources, time resolved studies can be achieved even in the home laboratory. Traditional solid or rotating anode x-ray tubes are typically limited in brightness by when the e-beam power density melts the anode. The liquid-metal-jet technology (MetalJet) has overcome this limitation by using an anode that is already in the molten state thus e-beam power loading above several megawatts per mm are now feasible.

Over a decade ago the first prototypes of liquid-metal-jet x-ray sources were demonstrated. These immediately demonstrated unprecedented brightness in the range of one order of magnitude above current state-of-the art sources [1-3]. Over the last years, the liquid-metal-jet technology has developed from prototypes into fully operational and stable X-ray tubes running in more than 50 labs over the world. X-ray crystallography has been identified as a key application for the x-ray tube technology, since this application benefits greatly from small spot-sizes, high-brightness in combination with a need for stable output. To achieve a single-crystal-diffraction (SCD) platform addressing the needs of the most demanding crystallographers, multiple users and system manufacturers has since installed the MetalJet x-ray source into their SCD set-ups with successful results [4].

This contribution reviews the evolvement of the MetalJet technology specifically in terms of stability, lifetime, flux and brightness and its applicability for pushing boundaries of high end SCD supported by recent user data. We also present recent possibilities to achieve cost effective solutions attainable for a wider application range. Finally, we discuss details of the technology with a focus on the fundamental limitations and its possibilities and capabilities in home lab based SCD and XRD.

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Structural dynamics of a redox- linked proton pump by advanced X-ray methods

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Membrane proteins perform numerous functions within the cell including energy transduction, transport and sensory perception. Cytochrome c oxidase (CcO) is an integral membrane protein that participates in aerobic respiration in mitochondria and many bacteria. It receives electrons from upstream biological transporters that reduces molecular oxygen to water. The redox energy derived from this process is used to pump protons up-hill across a biological membrane against a trans-membrane proton concentration gradient. The free energy stored in this electrochemical proton gradient can then be used for transmembrane transport and ATP synthesis. Although a large amount of structural and biochemical data is available to elucidate coupling of oxygen reduction to proton pumping in CcO, however, the mechanism of this redox-linked proton translocation still remains elusive.

In order to understand the mechanistic details of chemistry and biological function, structural information is essential. The aim of our projectis to probe the structural dynamics of redox-driven proton pump in ba3-type CcO from Thermus thermophilus using time-resolved wide-angle X-ray scattering (Tr-WAXS). To address the actual biological question of oxygen binding, we have performed studies with oxygen mimic (CO) and caged-oxygen that can be released into photochemically into the enzyme in order to see the conformational changes during a whole reaction cycle of O_2 reduction and proton pumping across the membrane. Our studies reveal fluctuations in conformational transitions in the enzyme during the time scales of an enzymatic reaction. This can further provide an entirely new approach to study intermediate states of terminal oxidases during cellular respiration. Success in these experiments will have major scientific impact that will bridge the huge gap in our understanding of structural dynamics of important biochemical reactions and addressing fundamental questions in bioenergetics of cells.

Protein mediated transition metal flux visualized in giant unilamellar vesicles

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Transition metals are important micronutrients that are involved in a range of cellular processes, both in prokaryotic and eukaryotic organisms. Since metal ions in high concentrations are toxic, metal homeostasis is tightly regulated. Correct levels are maintained by a number of proteins that are responsible for acquisition and extrusion of metals. Studies of the function of these proteins are usually limited to *in vivo* experiments, which are difficult due to the complexity of living cells. Here, we present a method for measuring metal transport in artificial membranes, bypassing the issues encountered when performing *in vivo* assays.

Proteins facilitating metal flux were overexpressed, purified and successfully reconstituted into giant unilamellar vesicles (GUV, diameter > 10 μm), with a metal-sensitive fluorescent dye trapped inside (1). The vesicles were then washed and fixed on a glass slide, and visualized with a fluorescent microscope. Well-behaved vesicles were subsequently subjected to a flow of high concentrations of metal ions, and fluorescence changes reflecting metal influx were recorded. The results were quantified in ImageJ and normalized by vesicle size. Correct membrane protein incorporation into the vesicles was demonstrated by fluorescent labelling of the proteins and visualization under an appropriate filter.

Our GUV based method shows a number of improvements compared to usage of smaller liposomes (diameter < 0.5 μm). Multilamellar and disrupted vesicles are easily distinguished and excluded from analysis. Moreover, visualization of transport in real time removes the need for harvesting and washing of vesicles (or liposomes) at specific time-points. The presented method thus increases both the sensitivity and accuracy of the results, and can be exploited for demonstrating flux of a number of transition metals (Cu, Zn, Ni, Co, Fe, and Cd).

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Targeting biosynthesis of the mycobacterial cell wall by structural biochemistry

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Mycobacterium tuberculosis (*Mtb*) is the most medically important pathogen of its genus, killing nearly two million people around the globe annually. A tough, highly complex mycobacterial cell envelope protects the pathogen during the dormancy and also forms the first defense barrier protecting the pathogen against pharmaceutical compounds targeting it during the active disease stages. Therefore it is important to identify and selectively target the *Mtb* cell wall biosynthesis machinery.

We have determined the high-resolution crystal structure of one of the *Mtb* enzymes involved in biosynthesis of a lipid important for maintaining structural integrity of the mycobacterial cell wall. Molecular docking data together with serendipitous binding of crystallization mixture components evident in the crystal structure allowed us to identify the binding site for one of the enzyme substrates. Additionally, co-crystallization with the second substrate provided us with interesting details on substrate binding and the structural basis for a mechanistic proposal.

Importantly, the human biosynthetic pathway in differs from the prokaryotic analogue, making this enzyme a very attractive drug target. Together with the Chemical Biology Consortium Sweden (CBCS) we are currently preparing for inhibitory compound screening.

Structural and functional characterization of metalloregulators in an actinomycete model organism

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Metal ions are essential for life, as structural components of the cellular machinery and catalysts of chemical reactions, yet are toxic at high concentrations. Metal homeostasis is therefore tightly controlled in order to prevent deficits, while avoiding equally harmful excess (1). Iron and manganese are two of the most commonly used metal ions in protein cofactors. While iron readily reacts with reactive oxygen species, damaging cellular components, manganese protects cells against oxidative damage (2). Consequently manganese and iron homeostasis are closely integrated. This regulatory network has a profound influence on cellular metabolism and plays an important role in virulence of bacterial pathogens, but the underlying mechanisms are poorly understood.

In this project we will characterize the transcription factors regulating the intracellular concentrations of manganese and iron in the actinomycete *Saccharopolyspora erythraea* using biochemical, biophysical and molecular genetic methods in order to achieve a comprehensive understanding of their function on a molecular and systemic level. The results from this project will form the basis for unraveling the metalloregulatory network in this organism, while also shedding more light on the fundamental question of how proteins differentiate between manganese and iron.

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Crystal structure of the emerging cancer target MTHFD2 in complex with a substrate-based inhibitor

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To sustain their proliferation, cancer cells become dependent on one-carbon metabolism to support purine and thymidylate synthesis. Indeed, one of the most highly upregulated enzymes during neoplastic transformation is MTHFD2, a mitochondrial methylenetetrahydrofolate dehydrogenase and cyclohydrolase involved in one-carbon metabolism. Because MTHFD2 is expressed normally only during embryonic development, it offers a disease-selective therapeutic target for eradicating cancer cells while sparing healthy cells. Here we report the synthesis and preclinical characterization of the first inhibitor of human MTHFD2. We also disclose the first crystal structure of MTHFD2 in complex with a substrate-based inhibitor and the enzyme cofactors NAD^+ and inorganic phosphate. Our work provides a rationale for continued development of a structural framework for the generation of potent and selective MTHFD2 inhibitors for cancer treatment.

Reference: Gustafsson, R., et al. (2017) Cancer Research; 77(4); 937-948.

Structural characterization of the activity-regulated cytoskeleton-associated protein (ARC) in solution and its binding features

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The activity-regulated cytoskeleton-associated protein (ARC) is involved in synaptic plasticity and the formation of long-term memory. ARC is expressed in neurons during neural activity and has shown to promote the endocytosis of AMPA receptors. How ARC is involved in function remains unknown. However, many proteins connected to various cellular processes, such as endocytic trafficking, actin regulation and transcription, have been reported as interaction partners to ARC (Nikolaienko et al. 2017). The ARC protein consists of multiple domains, a predicted helical N-terminal domain followed by a linker region and then two globular domains that are homologues to the HIV gag capsid protein and the only part of the protein with known structure (Zhang et al. 2015). Some indications of a possible capsid assembly of the ARC protein have been reported (Myrum et al. 2015) and recently it was shown that ARC can transport RNA from one neuron to another through the formation of extracellular vesicles (Ashley et al. 2018, Pastuzyn et al. 2018).

Earlier structural studies of the protein are few due to aggregation or possible formation of capsid-like assemblies. We have studied the structural properties of the monomeric ARC protein using SAXS, MALS and SRCD to determine the domain organization and secondary structure composition of the protein in solution. We also investigated structural effects of the ARC protein upon binding to peptides from known interacting proteins and characterized the properties of membrane binding, which could be important features in the formation of the extracellular vesicles and the transport of information from neuron to neuron.

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Microwaves and Microtubules

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Modern technology surrounds us with electrical fields of varying kinds. Cellular phones and WiFi-routers are prevalent examples of electronic devices that emit radiation in the microwave range. It is of societal importance to study the biological effects of this radiation. The present study aims to investigate possible structural perturbations of biological macromolecules induced by electrical fields.

The protein Tubulin is ubiquitous in life and can polymerize into microtubules and as such constitute an integral part of each cells structure, the cytoskeleton (**Figure 1**).

Microwaves heat biologicals, primarily by heating water. For this reason, we need to be able to distinguish between the thermal effect of microwaves and other possible effects.

Recently performed Small and Wide Angle X-ray Scattering (SWAXS) measurements at the Swiss Light Source (SLS) gives a first insight into the structural perturbations of an applied field while at the same time measuring the thermal response in the sample.

Difference scattering curves (measurements with applied microwaves minus the control) for two different collection protocols shows a different thermal response (heating and cooling respectively) at high scattering angles while preserving similar structural changes at low angles (insert in **Figure 2**).

Modelling of structural changes that corresponds to the measured differences is currently ongoing.

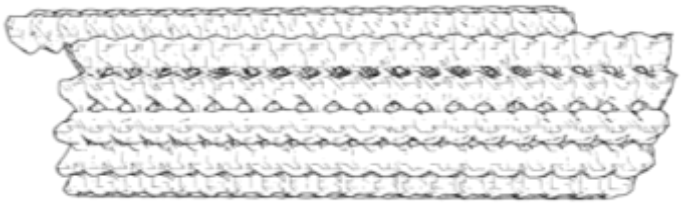

Figure 1: Tubulin dimers assembled into a microtubule

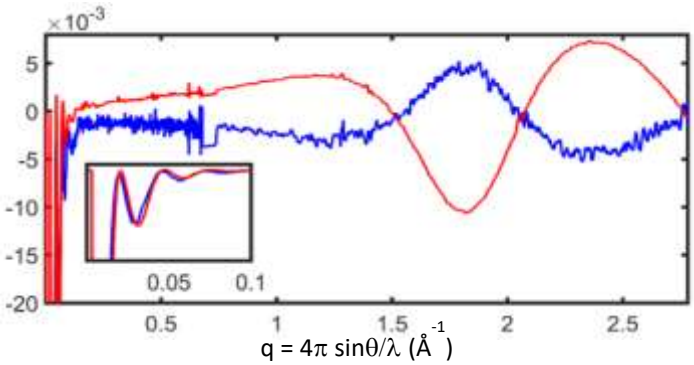

Figure 2: SWAXS difference scattering curves

Can microwaves affect the kinetics of microtubule polymerization?

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Abstract:

Microtubules are extended tubular polymers of tubulin that are a component of the cytoskeleton present throughout the cytoplasm. These microtubules are highly dynamic and provide a platform for intracellular transport and are also involved in a variety of cellular processes. They are formed by the non-covalent polymerization of α and β tubulin dimers that require energy input in the form of GTP. Microtubules have a distinct polarity with one end having the α-subunits exposed and the other end having the β subunits exposed, and these are termed the (-) and (+) ends, respectively. Elongation of a microtubule typically occurs at the (+) end. The (+) end of a microtubule is the region where assembly and disassembly of dimers take place which results in dynamic instability. During polymerization both the subunits of the dimer are bound to a molecule of GTP which is the stable state.

We have developed tools to investigate if microwaves in the kHz to GHz domain can influence the kinetics of microtubule formation. We have developed a setup which applies microwaves across a sample containing tubulin, and an ultraviolet light scattering probe monitors the formation of microtubules. The kinetics of microtubule polymerization is followed as a function of the frequency and energy of the applied electric field. We show that microwaves increase the rate of polymerization the results of which are presented here.

Lipid Directed Perilipin-1 Segregation in Human Primary Adipocytes

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Perilipin-1 is a lipid droplet adherent phosphoprotein known to regulate lipid metabolism in adipocytes. It serves as a physical barrier and recruitment site for lipases and other relevant lipolytic proteins. Phosphorylation of perilipin-1 by protein kinase A rapidly initiates lipolysis, but the detailed mechanism on how perilipin-1 controls lipolysis is unknown. We previously identified the glycerol channel AQP7, as a novel interaction partner of perilipin-1 in human primary adipocytes.[1] We also found that perilipin-1 forms topologically distinct micro domains on the lipid droplet surface during insulin stimulation, and that this segregation is abolished under lipolytic conditions.[1] Further investigation of this phenomenon revealed a dynamic and hormonally regulated lipid-directed segregation of perilipin-1.[2] We find that perilipin-1 interacts specifically with certain lipids in the cell, such as triacylglycerol, cholesteryl esters and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, a gel phase phospholipid known to support lipid micro domains.[2] Our findings show that these protein-lipid interactions are likely crucial for the formation of the perilipin-1-rich dynamic micro domains, which in turn may contribute to the regulation of the lipolytic rate in human adipocytes.[2] These results are very exciting, as they point to perilipin-1 as a potential important new target for treatment of obesity, insulin resistance and type 2 diabetes.

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Revealing the spatial arrangement of Type V pili from *P. gingivalis*

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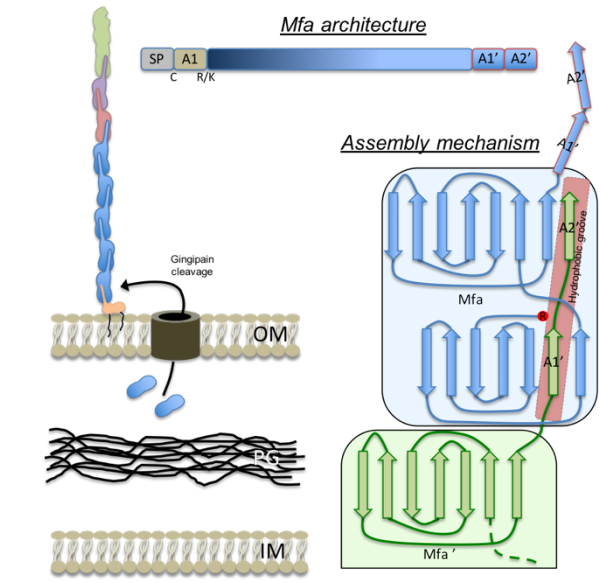
P. gingivalis belongs to the obligate anaerobic Gram-negative bacteria discussed as the main cause of periodontitis and gingivitis but is also involved in other diseases like rheumatoid arthritis[1], diabetes, pancreatic cancer[2–4] and cardiovascular disease[5]. Its pathogenicity is associated with different virulence factors, like Arg- and Lys-specific cysteine proteases (gingipains), lipopolysaccharides and fimbriae[6]. Fimbria are hair-like structures many bacteria build up on their surface to facilitate binding to host cells, matrix proteins and other bacteria. In *P. gingivalis* two types[7] of fimbriae are known, the "long" FimA- and the "short" Mfa1-fimbriae, both named after their main stalk building protein. The Mfa1 fimbriae, transcribed from a five gene polycistronic genecluster[8], has a size of 7x100 nm in average[9]. Studies on Mfa1 fimbriae revealed their association with cell-cell auto aggregation[10], community forming with oral commensals, like Strep gordonii[9], as well as survival in human myeloid dendritic cells[11]. In recent publications the structures of Mfa1-4 were published[12/13], which suggested the assembly mechanism to be similar to the N-terminal strand exchange mechanism[14] used in the E. coli type I pilus (see figure). This suggestion was extended by a second publication, which indicates that the C-terminal Beta-strand is essential as well[15]. To verify these results and receive the first structural view of type V pili, a combination of X-ray crystallography and cryo-electron microscopy is applied.



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Cotranslational protein folding

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Allosteric modulation via an intersubunit transmembrane site in pentameric ligand-gated ion channels

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Small-molecule modulators, including general anesthetics and insecticides, have been shown to bind an intersubunit transmembrane site in pentameric ligand-gated ion channels. However, due in part to limited crystallographic data, the structural basis and mechanistic impact of these interactions remain unclear. Using the structurally accessible model system GLIC, we applied a combination of X-ray crystallography, oocyte electrophysiology, and molecular dynamics simulations to characterize interactions stabilized in either open or closed states by allosteric modulators. Mutations at the intersubunit site associated with anesthetic *potentiation* stabilized solvent-mediated interfacial contacts specific to the apparent open state, a mechanism distinct from mutations in the channel pore, leading to nonadditive effects on gating. We also documented a seemingly contradictory mechanism of insecticide *inhibition* via the same cavity, when bound more favorably in the closed state. Our results provide a detailed allosteric model of ion channel gating and modulation, including a critical role for solvation at transmembrane subunit interfaces.

Complex structure studies of GLUT4 and ASPL

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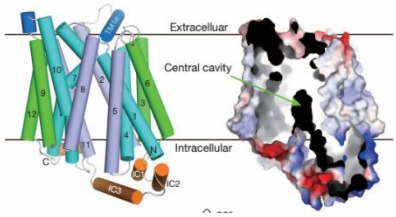
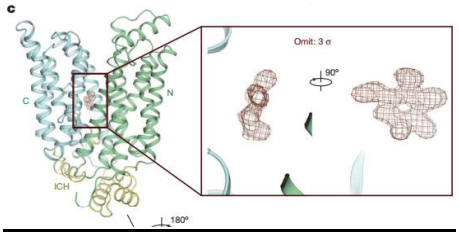
- **Background** Glucose transporters (GLUTs) are a series of proteins responsible for glucose uptake into different cells, in which structures of GLUT1 and GLUT3 have been determined (Figure1 and 2) [1, 2]. Different from GLUT1 and GLUT3 embeded in lipid bilayer of membrane, GLUT4 is expressed in intracellular vesicles of adipocytes and muscle cells, and then transformed to plasma membrane with the stimulation of insulin. And the transfering process of GLUT4 is regulated by TUG, a homogenous protein of ASPL in the cells of mouse (Figure3) [3, 4].





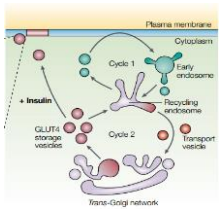


Figure1. Overall structure of huamn GLUT1 [1]. Figure2. Overall structure of GLUT3 [2]. Figure3. Transport of GLUT4 [3].

- **Aim** To characterize the interaction of GLUT4 and ASPL in adipocytes, and determine the structure of GLUT4 and complex structure of GLUT4 and ASPL (homologous protein of TUG in homo sapiens) [4] with Cryo-EM.
- **Methods**

  Protein expression in *Pichia Pastoris*

  Membrane protein purification

  ASPL expression and purification

  Cryo-EM sample preparation

  Data collection, refinement and structure determination

- **References**

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3D DNA origami and what it can help us learn about biology

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Karolinska Institutet

I will talk about DNA origami and its origin, how we translated it to 3D and our new paradigm for polyhedral design of nanostructures. In addition, I will talk about how we think that DNA-nanotechnology can be of great help in learning the tactile alphabet of cells by stimulation using protein decorated DNA-origami 'nano-calipers'. We have been focusing on ephrin-Eph signaling and, more recently Notch signaling. Using a similar technique we have also recently been investigating how the binding kintetics of human antibodies is affected by nanoscale separations between antigens.

Investigating the organisation and function of the membrane proteins of the Type VII secretion system in *Staphylococcus aureus*

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The Type VII/Esx-1 protein secretion system (T7SS), which is found in many Gram positive bacteria, was first described in *Mycobacterium tuberculosis* where it was shown to secrete ESAT6 (early secreted antigen, 6 kDa) and CFP10 (culture filtrate protein, 10 kDa). Both of these proteins are important T-cell targets and essential for the virulence of *M. tuberculosis*. A protein secretion system related to the ESX-1 protein secretion system in *M. tuberculosis* was later identified and characterized in the human and animal pathogen *Staphylococcus aureus*, sharing ESAT6/CFP10-like as well as EssC-like components. EssC proteins are members of a family of the FtsK/SpoIIIE family of ATPases, with three interlocking ATPase domains at the C-terminus. Previous work has shown that the *S. aureus* T7SS machinery comprises six core components of which four, EsaA, EssA, EssB and EssC are integral membrane proteins. While EssC is related to the actinobacterial EccC, EsaA, EssA and EssB, that are essential components of the secretion machinery, are unique to firmicutes suggesting a different mode of action/organisation of the T7SS machinery in *S. aureus*. Indeed, using crosslinking and BN PAGE analysis, we have shown that the EsaA, EssB and EssC proteins individually form homomeric complexes, but do not appear to interact with one another, or EssA[1]. Moreover, it was recently shown that multimerisation of EccC (the EssC homolog in actinobacteria) depends upon the binding of the substrate EsxB to a pocket of the most C-terminal ATPase domain. Surprisingly, we demonstrated through crosslinking that the formation of higher molecular weight multimers of EssC in *S. aureus* is not reliant on the presence of any other Ess component or substrate protein for their assembly[1]. Therefore, it seems that oligomerisation of EssC may be controlled differently to that of EccC.

In addition, the EssC proteins from *S. aureus* strains can be grouped into four variants (EssC1 – EssC4) that have sequence variability in their C-terminal domains. Interestingly, these variants are associated with unique clusters of candidate substrate-encoding genes. In a further study we could show that each EssC variant can interact with the remaining Ess components form strain RN6390 to facilitate secretion of the core component EsxA but not the substrate protein EsxC[2]. Thus EssC appears to be a specificity determinant for T7 substrate secretion in *S. aureus.*

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A nanobody platform for structural characterizations of complement complexes

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The complement system is a tightly regulated proteolytic cascade, central to innate immunity. It consists of more than 50 different proteins and is well-known for being involved in detection, phagocytosis and killing of invading pathogens. The complement system also has important roles in maintenance of host homeostasis, tissue remodelling and neural development. There are three well-characterized pathways for initiation of the complement cascade, known as the classical, the lectin, and the alternative pathway (AP). They all converge on the cleavage of C3 into the 9 kDa fragment C3a and the 176 kDa fragment C3b by complement convertases. C3a is signal molecule known as an anaphylatoxin, which orchestrates the immune response. C3b becomes covalently attached to the surface of the activator and acts as an opsonin, leading to phagocytosis and removal of immune complexes. C3b can also form complexes with the protease factor B, forming an AP convertase which cleaves more C3 into C3b and C3a. When the C3b density on the activator reaches a certain threshold, the substrate specificity of the convertases switches from C3 to C5. This leads to the formation of C5b and the potent anaphylatoxin C5a. C5b initiates the terminal pathway which ends with membrane permabilization and potentially cell death of the activator. Each step of the complement cascade is heavily regulated, and dysregulation is associated with a large number of diseases. Understanding complement regulation has to an increasing extent relied on structural dissection of large complement complexes. This has primarily been through X-ray crystallography, but in recent years small angle X-ray scattering (SAXS) and single particle electron microscopy (EM) has become increasingly more important. Mono-disperse systems are preferred in both methods and larger size and symmetry is advantageous. In several crystal structures of C3b, a head to head dimer with 2-fold rotational symmetry has been observed. This is also true for our recently published structure of C3b in complex with a high affinity nanobody inhibitor of the AP, termed hC3Nb1. Two hC3Nb1 molecules pack against each other and stabilize the C3b dimer. Engineering a solution-stable version of the hC3Nb1 crystal packing dimer could potentially stabilize a structurally homogenous C3b dimer. The C3b dimer could then be used as a platform for structural analysis of the large number of complement receptors and regulators interacting with C3b or other functional C3 fragments such as iC3b, C3(H_2O) or C3c. It could also be useful for studying the AP C3 and C5 convertases. To pursue this, we introduced a free cysteine in hC3Nb1 by site directed mutagenesis, motivated by the hC3Nb1 – C3b crystal structure. We utilized the free cysteine to form either a disulphide linked or mercury bridged nanobody dimer. We formed the C3b-hC3Nb1 dimer and characterized it structurally using single particle negative stain EM and SAXS. We showed that we can also form stable dimers of other functional C3 fragments and that the iC3b-hC3Nb1 dimer can still form a stable complex with complement receptor 3. Our results indicate that the hC3Nb1 induced C3b dimer is a versatile tool for structural characterization of complement complexes involving different functional states of C3.

X-ray structures of three *Proteus mirabilis* fimbrial adhesins:

UcaD, AtfE and MrpH

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The human urinary tract is a common target for bacterial infections. Uropathogenic *Escherichia coli* (UPEC) is the main causative agent for uncomplicated UTI in otherwise healthy individuals. *Proteus mirabilis*, a Gram-negative bacterium famous for its ability to swarm across agar surfaces, is the leading agent causing complicated UTIs in patients with indwelling catheters. *P. mirabilis* utilizes a multitude of virulence factors, including urease, flagella and fimbriae to establish and promote infection.

Fimbriae are filamentous structures assembled from Ig-like protein subunits called 'pilins'. Previous studies indicated critical roles of *P. mirabilis* fimbriae in adhesion and colonization of uroepethilial cells and in the formation of biofilm. Two-domain adhesins (TDAs) are the signature receptor-binding subunits located at the very N-terminal tip of rod shaped fimbrial monoadhesins. TDAs consist of an N-terminal domain (NTD) that is responsible for high affinity binding to receptors (usually carbohydrate), joined through a short linker to a C-terminal pilin domain.

Seventeen fimbrial gene clusters (FGCs) encoding chaperon/usher (CU) fimbrial assembly machineries have been identified in the genome of *P. mirabilis* HI4320. The CU fimbriae of *P. mirabilis* are highly conserved across isolates collected decades apart, from diverse geographical locations. Transcription of the major subunits of all 17 FGCs could be detected in *P. mirabilis* HI4320 log-phase aerated cultures. Proteins from six different fimbriae could be detected by mass spectrometry from sheared bacterial surface of *P. mirabilis* (cultured at 37°C with aeration for 8 h). This is in contrast to UPEC, where the fimbrial genes are quite variable between different isolates, and a single type of fimbrium usually dominates under any given culture condition.

The rich repertoire of fimbriae encoded and expressed by *P. mirabilis* has been suggested to be important for survival in the environment. Most of the *P. mirabilis* fimbriae have not been studied in any detail or not at all, and only little is known about their expression, physical characteristics, and biological functions. Here we present the first three atomic structures of TDA receptor-binding domains from *P. mirabilis* fimbriae:, uroepithelial cell adhesin (UCA), ambient temperature fimbriae (ATF) and mannose resistant *Proteus*-like fimbriae (MR/P). Our work provides an important stepping-stone toward a molecular level understanding of *P. mirabilis* fimbrial adhesins and of their roles in the complex pathogenesis of this important uropathogen.

The Norwegian National graduate school in biocatalysis– BioCat

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BioCat is a national graduate school in biocatalysis granted by The Norwegian Research Council for the period 2016 – 2024. It is established to provide expertise and tools for research and research education within biocatalysis. The school is open to all PhD-students and their research groups in Norway within the field. Biocatalysis is important in many areas including biomedicine, biotechnology, microbiology, and (bio)nanoscience. Additionally, biocatalysis is becoming increasingly important industrial processes and in various diagnostics-gene- and biotechnologies and is expected to be important in the transition of the society to a bio-based economy. The aim of initiating graduate schools in Norway is to unite small scientific communities and increase collaboration between research groups, create student platforms for technical and academic exchange and training, and thereby improve the quality and the skills of the PhD students. BioCat will in particular emphasize aiding career development of early stage researchers, both PhD and Post Doc fellows, in the field.

Six Norwegian universities, four research institutes and the Industrial Biotechnology Network Norway, constitute the BioCat consortium. This collaboration represents a unification of many strong research groups, giving the students great access to both the expertise, facilities and training offered by the Norwegian biocatalysis research community. We wish to get to know the Swedish research-environment and learn more about how the Swedish PhD-schools are organised. So, please feel free to visit BioCat at the Poster-stand.

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Structural and functional characterization of UvrD mediated transcription coupled repair

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Maintaining a steady genome composition is one of the most critical task for the survival of any living organism. Environmental factors, such as that UV irradiation, can lead to deleterious changes of the genetic material, posing a serious threat to cell survival. In order to cope with this threat cells have developed evolutionary conserved DNA–repair pathways, mending DNA lesions and thereby, ensuring genomic stability and integrity. Nucleotide Excision Repair (NER) is one of the major pathways of the DNA–repair in both prokaryotic and eukaryotic cells, which restores diverse types of DNA lesions up to 10–12 nucleotides long [1]. In bacterial cells, NER is mediated by the multistep assembly of the multiprotein UvrABCD complex, which localizes and cleaves damaged base-pairs in a synergized manner [2]. Transcription coupled repair (TCR) is an important sub-pathway of the NER, where concurrent scanning for DNA lesions is performed by the transcribing RNA Polymerase (RNAP), resulting in stalled transcription complexes at damaged sites [3].

UvrD is a multifunctional DNA Helicase (II) protein which belongs to the superfamily 1 helicases performing 3'–5' helicase function. Recent studies show that UvrD, a member of the UvrABCD NER machinery, also plays a more active role in TCR pathway based on genetic studies corroborating that UvrD mutant bacterial strains show high sensitivity towards UV-induced DNA-damage [4]. UvrD has been proposed to function by back-tracking stalled RNAP in order to recruit NER machinery facilitating DNA-repair and the subsequent restart of transcription.

Our aim is to understand the structure-function relationship of the UvrD helicase mediating TCR by employing an integrated structural biology approach with more focus on solution NMR spectroscopy. We are interested in deciphering domain dynamics of the UvrD protein and transient interactions within different TCR complexes in order to get better understanding of the atomic level details of TCR pathway. Currently, we are optimizing protein expression/purification by testing various UvrD constructs for the structural studies by NMR.

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Snapshots of the water oxidation reaction in photosystem IIusing crystallography and spectroscopy at an XFEL

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Structural characterisation of the catalytic domain of botulinum neurotoxin X – high activity and unique substrate specificity

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Botulinum neurotoxins (BoNTs) are among the most potent toxins known and are also used to treat an increasing number of medical disorders. There are seven well-established serotypes (BoNT/A-G), which all act as zinc-dependent endopeptidases targeting specific members of the SNARE proteins required for synaptic vesicle exocytosis in neurons. A new toxin serotype, BoNT/X, was recently identified. It cleaves not only the canonical targets, vesicle associated membrane proteins (VAMP) 1/2/3 at a unique site, but also has the unique ability to cleave VAMP4/5 and Ykt6 (1). Here we report the 1.35 Å X-ray crystal structure of the light chain of BoNT/X (LC/X) and its apo form at 2.4 Å. LC/X shares the core fold common to all other BoNTs, demonstrating that LC/X is a bona fide member of BoNT-LCs. We found that access to the catalytic pocket of LC/X is more restricted, and the regions lining the catalytic pocket are not conserved compared to other BoNTs. Kinetic studies revealed that LC/X cleaves VAMP1 with ten times higher efficiency than BoNT/B and the tetanus neurotoxin. The structural information provides a molecular basis to understand the convergence/divergence between BoNT/X and other BoNTs, to develop effective LC inhibitors, and to engineer new scientific tools and therapeutic toxins targeting distinct SNARE proteins in cells (2).

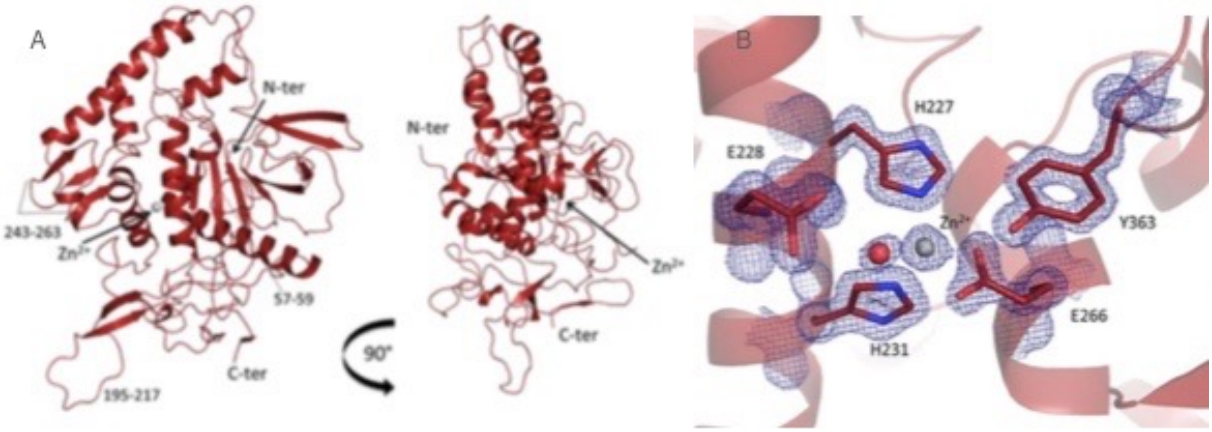

Figure 1: Crystal structure of LC/X. (A) Ribbon representation of LC/X, the zinc ion (grey sphere) and other regions of interest are highlighted. (B) Close up view of the active site of the LC/X.

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Galectin-3: Studying role of fluorines in the protein-ligand interaction to achieve high affinity and selectivity

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Galectin-3 belongs to the galectin family that recognizes carbohydrates. It has a highly conserved carbohydrate recognition domain (CRD) of 130 residues, which is responsible for binding to beta-galactosides. Galectin-3 has been shown to be involved in cancer, angiogenesis and stroke. Its involvement in these important diseases makes it a wonderful drug target. Our previous work[1] showed the mode of binding of lactose and role of structured water molecules in carbohydrate binding site. These results prompted us to explore the molecular recognition and role of water molecules in designing high affinity inhibitors. Natural ligands of galectin-3 almost always have a galactose residue.

Selective small molecule galectin-3 inhibitors are valuable both as research tools to study protein-ligand interactions and as lead compounds in drug discovery. These compounds usually involve galactose-based derivatives, 1- and 3-substitutions of galactose. We have solved numerous protein-ligand crystal structures to study the effect of various substitutions. Fluorines are known to have diverse effects on physicochemical and conformational properties of ligands. Introduction of Fluorines at key positions in ligands has been proven to be promising strategy in lead optimization. Position and amount of fluorination has strong effect on the protein ligand interactions. Fluorines enhance ligand affinity by interacting with both the polar electropositive and hydrophobic groups in protein. Orthogonal multipolar C-F…C=O interactions with both peptide backbone and side chain carbonyls have been found important for Fluorines[2].
Distinct fluorophilic environments in proteins are the ubiquitous peptide bonds, which undergo multipolar C-F ··· H-N, C-F ···C=O, and C-F ··· H-CR interactions. Here we report several structures of galectin-3 CRD with mono-galactose based compounds having fluorines in different positions and numbers.

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Adhesion-Related Novel Giant Proteins of *Lactobacillus kunkeei*

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Lactobacillus spp. are lactic acid producing Gram-positive bacteria that can be found in many foodstuffs such as sour milk, pickled vegetables, fermented fruit juices etc. Many species possess probiotic properties and several have been commercialized.

Lactobacillus kunkeei is an important symbiont of the honeybee and is one of the major bacteria species inhabiting its digestive tract. Genome sequencing revealed a unique family of very large proteins ranging from 320–880 kDa. The family is conserved across all sequenced *L. kunkeei* strains and can be found as an array of 4–5 genes per genome and they make up a substantial part of these diminutive genomes. The gene products are secreted and we hypothesize that they are surface proteins involved in adhesion to host tissues. As such, they may be important probiotic effector molecules as health-promoting properties of probiotic bacteria has been shown to be linked to cell attachment. As part of our investigation to elucidate the function of the giant proteins, we study their structure using electron cryo-microscopy. The current state of the project will be presented.

PrgU represses sex-phermone induced toxicity in *Enterococcus faecalis* pCF10

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Antibiotic resistance and nosocomial infections present major problems in the world, and they are on the rise. One of the most clinically relevant issues is transferable resistance linked to conjugative plasmids, which transfers DNA laterally through Type 4 Secretion Systems (T4SS). Here we focus on the regulation of the T4SS from the conjugative plasmid pCF10 from *Enterococcus faecalis*. All of the genes important for the T4SS in *E. faecalis* pCF10 reside within a single operon under the control of the P_Q promotor. Whether transcription of the P_Q promotor is induced or not is controlled by sex pheromones[1]. In 2016 a new protein involved in the regulation of pCF10 was found: PrgU. PrgU somehow inhibits the transcription from the P_Q promotor, thereby downregulating the expression especially of the T4SS adhesin genes, which if expressed in too high levels are toxic to the cell and leads to cell death[2]. The structure of PrgU has previously been determined by a structural genomics consortium. PrgU contains a PUA domain, which is commonly found to bind to RNA. One hypothesis is that PrgU binds to the intergenic region (IGR) that can be found after the first gene after the P_Q promotor. The IGR contains several predicted transcriptional stop sites, and also interestingly has a predicted rRNA structural fold[3]. Furthermore, PrgU is also predicted to interact with another regulator on the same operon, PrgR. Our specific aims are to 1) Examine the interaction between PrgU, IGR and PrgR. 2) Determine the structure and function of the PrgU-PrgR complex and of PrgU bound to its substrate. Currently we have *in vivo* data that supports the hypothesis that PrgU binding to the RNA of the IGR, based on Northern blots and Pull-down experiments. Using purified PrgU and PrgR we are currently examining their interactions with each other and with RNA using electrophoretic shift assays and isothermal titration calorimetry.

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Structural study of metalloproteins using a drop-on-demand sample delivery at X-ray free-electron laser sources

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Synchrotron X-ray crystallography has proven to be a powerful method for studying the structure of metalloproteins. However, artifacts can be induced using this method, e.g. caused by the photoreduction of metal cofactors by extended exposure to X-rays, or induced by data collection at cryogenic temperatures.

A new method of X-ray crystallography at X-ray free-electron laser (XFEL) sources uses femtosecond pulses to produce diffraction signal at room temperature before radiation-induced changes occur. Consequently, XFELs provide new opportunities to study biologically relevant redox state of metalloproteins. Moreover, when combined with X-ray emission spectroscopy (XES), both global structures and the chemical properties at catalytic sites of metalloenzymes can be studied.

Using a newly developed drop-on-demand sample delivery, coupling an acoustic droplet ejection with a conveyor belt drive optimized for crystallography and spectroscopy measurements [1], we recently obtained X-ray crystal diffraction and XES data of a metalloprotein, simultaneously collected at a XFEL source (SLAC Linac Coherent Light Source, Stanford, USA). In our study, we compare this structure solved from a XFEL source with several structures of the same protein solved from synchrotron X-ray sources, focusing on the metal cofactor and its environment.

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The low-resolution revolution – SAXS in non-structural biology

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Stockholm University

Small Angle X-ray Scattering (SAXS) has in traditional Structural Biology been used to obtain low-resolution structures since the early 1960s. The power of SAXS to obtain structural "blobs" has within the recent years been vastly surpassed by electron microscopy, forcing the method to develop new applications.
SAXS retains its inherent advantage of being in-solution, and increased beamline power and online gel chromatography, has allowed for high-throughput measurement to determine miniscule conformational changes as functions of ligand concentration, pH values, heat or salt concentrations, effectively changing the methodology from structural biology into screening spectroscopy. Additionally, recent software developments have allowed for the SAXS studies of membrane proteins in micelles.
Here we present the effect of magnesium binding on the magnesium transporter CorA and introduce the SAXS derived structural Kd and Hill coefficient parameters.

Fragment Screening at MAX IV: perspectives for high-throughput data collection and processing at BioMAX beamline

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Using small organic compounds to identify and analyse binding clefts in protein has been largely used in the recent years [1]. As both industry and academy grows interest in using such methodology, the ultimate technique that maximises success rate in fragment screening campaigns is still matter of discussion, whereas the previous routines of using biophysical experiments (e.g. Thermal Denaturation Assay) for pre-screening fragment libraries has been criticised as they fail to retain hits, creating a demand for techniques that can identify fragments with some affinity to the biological target and also shed light to its binding mode, such as X-ray macromolecular crystallography [2].

The advent of brighter and more coherent light sources is bringing new challenges to fragment screening projects, and the most notable is the ability to collect a large screening in short period of time. Given the huge amount of data collected during these experiments, it is unrealistic to expect manual processing and analysis on all datasets in order to find the few positive results one is looking for, making automated approaches the key to success [3].

MAX IV macromolecular crystallography beamline, BioMAX, operates with the most advanced light source to date. Recent progress in its instrumentation and software pipelines foresees a few hundred datasets collected in 8-hour shifts, encouraging users to conduct large fragment screening campaigns. The pipeline for data processing will include iterative steps of structure refinement using Phenix and CCP4 suites to achieve levels of structure refinement that will make possible to distinguish possible ligands in the electron density.

This project aims to implement state of art scripts and methodologies at BioMAX to make full use of its high-throughput capabilities, presenting users refined data that dramatically reduce the bottleneck of Fragment-based ligand discovery.

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Human NUDT22 Is a UDP-Glucose/Galactose Hydrolase Exhibiting a Unique Structural Fold

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Human NUDT22 belongs to the diverse NUDIX family of proteins, but has, until now, remained uncharacterized. Here we show that human NUDT22 is a Mg^{2+}-dependent UDP-glucose and UDP-galactose hydrolase, producing UMP and glucose 1-phosphate or galactose 1-phosphate. We present the structure of human NUDT22 alone and in a complex with the substrate UDP-glucose. These structures reveal a partially conserved NUDIX fold domain preceded by a unique N-terminal domain responsible for UDP moiety binding and recognition. The NUDIX domain of NUDT22 contains a modified NUDIX box identified using structural analysis and confirmed through functional analysis of mutants. Human NUDT22's distinct structure and function as a UDP-carbohydrate hydrolase establish a unique NUDIX protein subfamily.

Identification and characterization of a novel botulinum neurotoxin

Geoffrey Masuyer*, Sicai Zhang**, Linda Henriksson*, Markel Martínez-Carranza*, Sara Košenina*, Daniel Lundin*, Jie Zhang**, Yi Shen**, Shin-Ichiro Miyashita**, Sulyman Barkho**, Min Dong** and Pål Stenmark*

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Botulinum neurotoxins (BoNTs) are among the most potent bacterial toxins known and are also used to treat an increasing number of medical disorders. There are seven well-established serotypes (BoNT/A-G), which all act as zinc-dependent endopeptidases targeting specific members of the SNARE proteins required for synaptic vesicle exocytosis in neurons. We recently reported a new BoNT serotype named BoNT/X[1], which has the lowest sequence identity with other BoNTs. It cleaves not only the canonical targets, vesicle associated membrane proteins (VAMP) 1/2/3, but also has the unique ability to cleave VAMP4/5 and Ykt6. We confirmed the toxin's biological activity by assembling a small amount of full-length BoNT/X using a transpeptidase (sortase) linking two non-toxic fragments. Assembled BoNT/X is active on cultured neurons and causes flaccid paralysis in mice. We also characterized the catalytic domain of BoNT/X by X-ray crystallography to provide the molecular basis for its high activity and unique substrate specificity[2]. In addition, recent advances in whole genome sequencing have led to the discovery of new BoNT-like toxins such as BoNT/En[3], which is present in a commensal strain of *Enterococcus faecium*.

The discovery of a new BoNT and BoNT-like toxins posts a challenge to develop effective countermeasures, but it also provides novel tools for studying intracellular membrane trafficking, and the potential for new therapeutic toxin that modulate cell secretion.

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Crystal structure and regulation of human β-ureidopropionase

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β-Ureidopropionase (EC 3.5.1.6; βUP) catalyzes the final reaction of the reductive pyrimidine degradation pathway. This pathway degrades uracil and thymine and controls thereby the concentrations of pyrimidine nucleobases and their degradation products in human cells. The chemotherapeutic 5-fluorouracil (5-FU) is degraded by the same sequence of reactions, and thereby rendered ineffective. Today, 5-FU is still one of the most prescribed chemotherapeutic in colorectal, gastric and breast cancer therapy. Due to the narrow therapeutic window and rapid metabolic inactivation, cancer patients suffering from β-ureidopropionase deficiency are potentially at risk of developing severe toxicities following intake of 5-FU.

In this study we could confirm that the activity of human βUP is dependent on the oligomeric state of the enzyme, which changes in response to ligand binding. Furthermore, we revealed that shifts in pH also affect the quaternary structure of the enzyme. In order to further investigate the oligomerization-dependent allosteric regulation, key residues at potential subunit interfaces were mutated via site-directed mutagenesis. These residues were selected based on the crystal structure of *Drosophila melanogaster* βUP (DmβUP). The crystal structure of one of the created enzyme variants, HsβUP_T299C, could be determined at a resolution of 2.1 Å. The gained knowledge about structure and function of human βUP will facilitate prediction of which gene mutations are likely to cause β-ureidopropionase deficiency and increased risk of developing severe 5-FU toxicity for cancer patients.

Exploring [FeFe] hydrogenase using synthetic chemistry

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[FeFe] hydrogenases are metalloenzymes that catalyse the interconversion between H_2 and protons with remarkable efficiency. The reaction occurs at the H-cluster featuring an organometallic dinuclear [2Fe] subsite. Synthetic chemistry has long been a powerful tool in studies of this cluster via the preparation of biomimetic model compounds, and in 2013 we could how such synthetic complexes can be introduced into the enzyme itself under *in vitro* conditions.[1, 2] This provides a direct link between biomimetic chemistry and biology, and allows us to manipulate the enzyme using synthetic chemistry. Here I will present how the concept of artificial maturation can be extended to *in vivo* conditions and the apo-enzyme activated using synthetic cofactors inside living cells (Fig. 1),[3, 4] providing us with a novel tool for *in vivo* studies of [FeFe] hydrogenase.

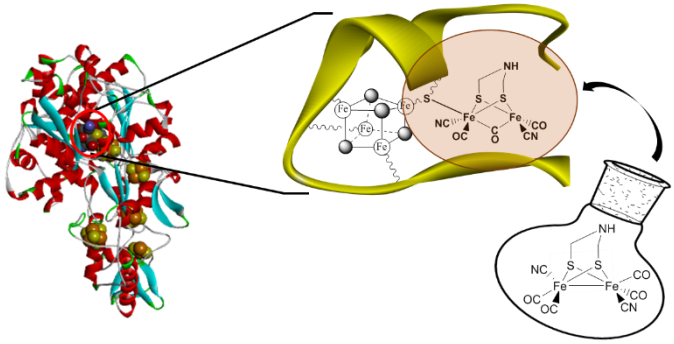

Figure 1. Schematic representation of the artificial maturation technique.

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New structural insights into the regulation of aquaporins

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Obesity is a major threat to global health and metabolically associated with glycerol homeostasis. Glycerol flux across plasma membranes, a key component for maintenance of body glycerol levels, is facilitated by aquaglyceroporins. Here we present the first crystal structure of a mammalian aquaglyceroporin determined at 2.3 Å resolution. The structure reveals an unusually wide selectivity (ar/R) filter, and a unique cytoplasmic gate formed by pore-lining residues and a loop. *In vitro* and *in vivo* functional data disclose a glycerol-specific pH-dependence and pinpointed a pH sensor of the glycerol facilitator, corroborating with the structural findings. Molecular dynamics simulations indicate how gate opening is achieved at low pH inducing local structural rearrangements widening the channel. These findings display a novel type of aquaporin regulation important for controlling adipocyte volume and body fat mass. Thus, targeting the novel gate to simulate constitutive glycerol release may open up a novel avenue to treat obesity and related metabolic diseases.

A pilot macromolecular 3D structure determination project

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In 2015, the Swedish light source MAXIV decided to fund a HPC-MX software installation called PReSTO (https://www.nsc.liu.se/support/presto/). All Swedish structural biologists can access the PReSTO installation by request membership in the pilot SNIC project entitled as this abstract. SNIC stands for Swedish Network of Infrastructure Computing and is a collaboration of Swedish HPC centres funded by the Swedish research council (www.snic.vr.se).

For MX computation, the pilot project allocation is sufficient for the Swedish research community. A structural biologist may want to run molecular dynamics or use cryo-EM calculations and should therefore apply for their own compute time allocation with SNIC using their MX pilot membership simply to have MX software access.

Thinlinc enables remote graphics applications such as coot/chimera/ccp4mg/pymol for model building, visualization, displaying and analysing surface properties and making presentation movies.

The PReSTO project started out at NSC Triolith and we decided to use easybuild (https://easybuild.readthedocs.io/en/latest/) for simplified sharing of the HPC-MX installation with LUNARC Aurora and the MAXIV cluster. Since HPC system architecture differ between sites, minor adjustments to the MX installation environment are applied locally, however the MX installation itself is identical between the three sites (Triolith/Aurora/MAXIV)

MX software may contain a few parallel subroutines and the total execution time is always limited by the serial parts of the software (Almdahl´s law). If the software is 50% parallel the maximum speedup factor is 2 because the serial part will use half the time even if the parallel part runs infinitely fast. If the software is 95% parallel the maximum speedup factor is 20 since 5% of the job will be serial.

Together we benefit of using HPC as a research community because

A. long jobs such as phenix.mr_rosetta can be run on a compute node while attempting other software at other compute nodes or manual model building at the login node
B. the software stack being more complete and contain fewer BUGs since a community will try more options (i.e. find more BUGs) than any single experimentalist will do
C. The software stack is professionally maintained (support/updates/system administration) by trained personnel from the leading Swedish compute centres.

Besides several compute centres encourage and support installation of software at the interest of the user. We expect more HPC adapted MX software from the developers in the future with respect to parallelism and queue system integration and are ready to take initiatives inviting developers to (Sweden/MAXIV) to discuss these matters.

Towards the characterization of the ribosomal intersubunit B3 bridge dynamics using NMR spectroscopy

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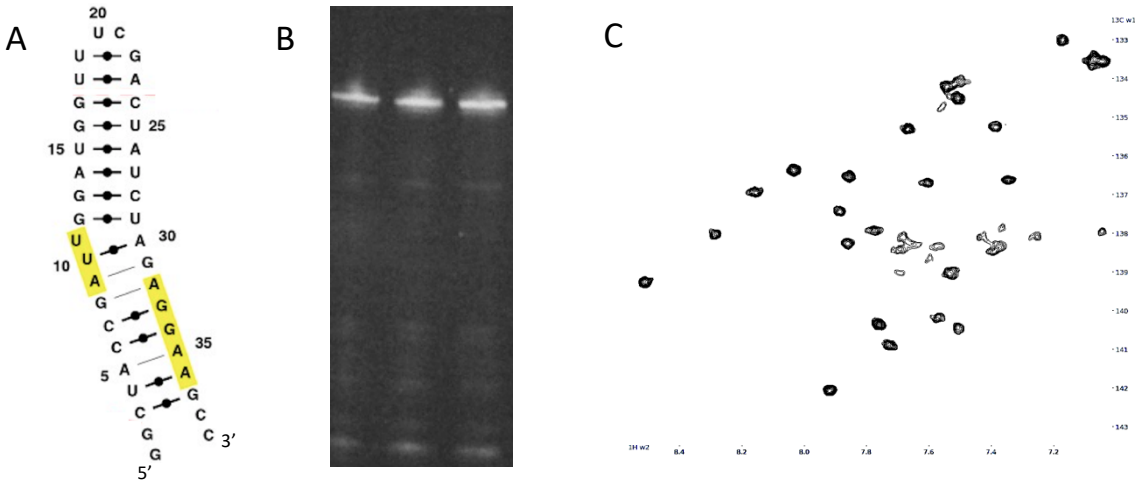
Multiple snapshots of the ribosome in different structural states have been obtained using X-ray crystallography and Cryo-EM. However, translation and subunit association structural changes are still poorly understood at the atomic level. In contrast to other techniques, NMR spectroscopy and specifically relaxation dispersion experiments allow the study of the dynamics involved in these processes.

The subject of this study is the highly conserved B3 bridge, which forms between helix 44 of the small subunit of the ribosome and helix 71 of the large subunit. Along with other ribosomal bridges, B3 bridge is located in the core of the subunit interface and is considered to be the pivot point for the ratchet-like intersubunit movement that occurs during the translocation of the mRNA-tRNA complex along the ribosomal sites A, P to E.

To allow the study of the B3 bridge, short RNA constructs (Fig. A) that mimic the B3 native conformation are produced via *in-vitro* transcription (Fig. B) and further purified with ion-exchange HPLC. Then, once the resonances have been assigned in the construct (in progress Fig. C), excited states can be can be characterized with relaxation dispersion experiments.

The goal of the project is the complete characterization of excited states on the B3 bridge. Excited states are short lived and minutely populated conformations that can play major roles in the biological function of a given biomolecule. Such conformers can also be targeted and stabilized by drugs. Thus, both the *E. coli* –as a prokaryotic model– and the human intersubunit B3 bridge are being studied in order to be able to selectively target different excited states and potentially develop new antibiotics.



A: Secondary structure prediction of the 39nt construct used as model for h. sapiens B3 bridge in helix 44. Nucleotides labelled in yellow are involved in intersubunit ribosomal interactions. B: Crude transcription reaction product previous to purification in a 15% denaturing PAGE stained with EtBr containing the main transcript of interest and other non-desired shorter and longer products. C: ^{1}H, ^{13}C HSQC for the protons connected to aromatic carbons of B3 construct shown in A measured with ^{13}C natural abundance.

Structural and dynamic properties of c-Myc $_{1-158}$ as revealed by NMR and small-angle X-ray scattering (SAXS).

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c-Myc proteins play prominent roles in cell cycle progression, apoptosis and cellular transformation processes. These proteins act as a universal upregulator of gene expression, except early genes in cells. Regulation of c-Myc expression is crucial for obtaining normal cell functions and since it regulates the transcription of a wide range of genes; even small changes may influence the cell growth, proliferation, apoptosis, differentiation and transformation (Meyer and Penn 2008; Levens 2010). The Myc protein comprises a large intrinsically disordered region, comprising conserved so-called Myc Box (MB) regions. We have previously characterized the MBI region, which is highly mutated in cancer, and identified a new MB0 region, which is essential for cell growth (Helander et al., Structure 2015). In this work, we will present our recent progress in characterizing an extended Myc fragment, Myc-1-200, by NMR, small-angle-X-ray-scattering (SAXS), and biophysical analysis. Myc-1-200 includes three essential Myc boxes: MB0, MBI and MBII. Our analysis sheds light on the transient interactions of these MBs interact and how these interactions are regulated by post-translational modifications.

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Status of the beamlines for Macromolecular Crystallography at MAX IV

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MAX IV is the first operational ultimate storage ring, which is offering synchrotron radiation at various energies for many scientific communities.

Among the first beamlines in user operation, BioMAX, the first macromolecular crystallography (MX) beamline, can be used to create high resolution 3D data from crystalline macromolecules to better understand the structure functional relationship of this complex matter. BioMAX has been designed as a work horse beamline to support all kinds of established crystallography methodologies and runs at a high automation level. The experimental station is equipped with a MD3 micro-diffractometer, an Eiger 16M hybrid pixel detector and an ISARA sample changer, which allows for complete data collections in seconds.

Recently the seconds MX beamline has been funded by the Danish Novo Nordisk Fonden. MicroMAX will become a micro-focussing beamline, which will allow for investigating micrometer sized protein crystals at room temperature using serial crystallography. Here the complete diffraction dataset is composed from several thousand's single frame, partial datasets of a large number of crystals. Using a pink-beam option, MicroMAX can be exploited also for time resolved crystallography in the microsecond time resolution. After a 4 years construction period, the beamline will be ready for first experiments.

Within this presentation the current status and future directions are discussed.

ION TOF MEASUREMENTS ON AEROSOLISED BIOPARTICLES TO STUDY HYDRATION LEVELS FOR EXPERIMENTS WITH X-RAY LASERS

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An open question in delivering a beam of intact bioparticles into the pulse train of an X-ray free-electron laser (X-ray FEL) concerns the level of hydration of these particles at the interaction point inside the vacuum chamber. Anything in the path of the X-ray beam is "sample", including gas molecules, and particles need to be injected into the X-ray pulse train at reduced pressure, without any container to reduce this background [Seibert et al. 2011; Hantke et al. 2014]. Injected particles spend microsecond-milliseconds inside the vacuum chamber before intersecting the X-ray beam. Simulations of the injection and evaporation process have been performed [Patriksson et al., 2007; Iavarone et al. 2007, Friemann, et al. 2009; Marklund et al., 2009; Wang et al., 2009]. These studies show that structural water molecules prevent unfolding of biosamples over a broad temperature range, and hydrated macromolecules retain their conformational integrity in the gas phase. Structural changes become significant if proteins are let to dry out completely. On the other hand, if the solvent layer is too thick, it will reduce contrast in X-ray studies and introduce various degrees of heterogeneity, making the assembly of the diffraction patterns impossible. It is therefore essential to the success of these experiments that the amount of hydration is well controlled. Our goal is to study this problem and develop new strategies for optimizing aerosol injection towards high-resolution imaging.

In a simulated FEL experiment, we use a high-power femtosecond laser at 800 nm wavelength to mimic the FEL beam. This beam is powerful enough to trigger the ionisation and hydrodynamic explosion of the sample particles. Ion time-of-flight measurements are then used to report on the elementary composition of the particle.

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Human mitochondrial pyruvate carrier 2 as an autonomous membrane transporter

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The active transport of glycolytic pyruvate across the inner mitochondrial membrane is thought to involve two mitochondrial pyruvate carrier subunits, MPC1 and MPC2, assembled as a 150 kDa heterotypic oligomer. Here, the recombinant production of human MPC through a co-expression strategy is first described; however, substantial complex formation was not observed, and predominantly individual subunits were purified. In contrast to MPC1, which co-purifies with a host chaperone, we demonstrated that MPC2 homo-oligomers promote efficient pyruvate transport when reconstituted into proteoliposomes. The derived functional requirements and kinetic features of MPC2 resemble those previously demonstrated for MPC in the literature. Distinctly, chemical inhibition of transport is observed only for a thiazolidinedione derivative. The autonomous transport role for MPC2 is validated in cells when the ectopic expression of human MPC2 in yeast lacking endogenous MPC stimulated growth and increased oxygen consumption. Multiple oligomeric species of MPC2 across mitochondrial isolates, purified protein and artificial lipid bilayers suggest functional high-order complexes. Significant changes in the secondary structure content of MPC2, as probed by synchrotron radiation circular dichroism, further supports the interaction between the protein and ligands. These results that provide the initial framework for the independent role of MPC2 in homeostasis and diseases related to dysregulated pyruvate metabolism will be presented and discussed.

Structural and functional studies of heavy metal ion transporters

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The performance of biological systems is highly affected by the concentrations of transition metal ions such as copper, which is controlled by ion channels and transporters. Thus, comprehensive structural and functional understanding of such regulating proteins is warranted, as a paving step also towards therapeutic interventions.

Herein, we summarize our structural characterization efforts on selected heavy metal transporters from bacteria and yeasts. The targets were produced to high yields and extracted in detergent and lipid mixtures. Purification strategies, including affinity and size-exclusion chromatography, were then devised and the purified protein of one target yielded initial crystals. A positive outcome of this work will shed fundamentally new light on structural/functional properties of the approached copper transporters.

Cryo-EM structure of Mycobacterial respiratory supercomplex III-IV

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Respiratory chain complexes (complex I-IV) transfer electrons to terminal substrates (oxygen in the case of aerobic respiration), and establish a proton gradient across the respiratory membrane. It is well established for many organisms that the classical respiratory complexes I-IV can also organize into higher order structures; so called supercomplexes or respirasomes. In general, Mycobacteria harbor homologues of the mitochondrial BC1 complex and the aa_3-type cytochrome c oxidase, but lack a soluble cytochrome c. Instead, the Mycobacterial cytochrome c is of a di-heme type, contains a transmembrane helix and is an integral part of the supercomplex. Here, the *Mycobacterium smegmatis* respiratory supercomplex III_2-IV_2 (SC) is biochemically and structurally characterized. Direct electron transfer activity of SC is demonstrated by the full reaction sequence resulting in O_2 reduction by electrons provided by quinol (DMNQH2) or TMPD/ascorbate. The supercomplex also displays ligand (CO) binding activity (to the bi-nuclear centre of cyt aa_3-Cu_B). Interestingly, mass spectrometry analysis revealed 4 previously unidentified protein components of the SC, including the Cu-Zn superoxide dismutase (SodC). The presence of SodC was further confirmed by an unexpected additional density in the Cryo electron microscopy (Cryo-EM) 3D structure, and by detection of SodC activity within the supercomplex. The Cryo-EM structure also supports the expected IV-III_2-IV arrangement of the supercomplex.

Structural Basis and Dynamics of the Autoinhibition of NusA

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To ensure efficient DNA transcription RNA polymerase (RNAP) is regulated by an array of transcription factors at different phases of transcription: initiation, elongation and termination. One of these factors N-utilization substance A (NusA), an elongation factor that also is involved in termination, could be identified in bacteria and archaea [1]. Furthermore, it acts as a so-called antitermination factor during transcription by its recruitment to stalled RNAP encountering pause-sites or DNA-damage in form of lesions. The latter marks it as an important player in DNA-surveillance in a process called transcription-coupled repair (TCR). Structurally, NusA is a 55 kDa protein, composed of six domains; amino-terminal domain (NTD), S1, KH1, KH2, acidic repeat 1 (AR1) and acidic repeat 2 (AR2). The S1, KH1 and KH2 domains make up the so called SKK domain, while AR1 and AR2 are part of the carboxyl-terminal domain (CTD). Each of the NTD, SKK, AR1 and AR2 domains were revealed to have distinct function [2, 3] as they interact with different molecules. The central SKK domain interacts with nascent RNA, however this interaction seems to be hindered by AR2 through formation of intramolecular interdomain complex resulting to autoinhibition [4].

Previous studies on *Escherichia coli* NusA has been based on sub-construct. Therefore, we intend to, by solution nuclear magnetic resonance (NMR) spectroscopy, study initially the structural basis and dynamic of full-length *E. coli* NusA autoinhibition. This part of the study marks an important first step in our goal to discern the role of NusA within the different transcription associated complexes. I will present our preliminary data of studying the basis of NusA-autoinhibition by sophisticated NMR-methods.

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A time-resolved study of the light-gated ion pump ChannelRhodopsin-2.

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Cryo-crystallography is a key technique that allows a high resolution static representation of a given protein structure. Unfortunately this method is time-consuming and difficult to realize. However, time-resolved serial crystallography allows us to probe the structural changes happening inside the protein, but is not used to study the overall tertiary structural changes as a result of the crystal lattice constraints. On the other hand, time resolved wide angle scattering (TR-WAXS) is a promising method for observing structural changes in proteins, but limited by its low resolution due to the random orientation in solution. Therefore, combining these techniques, we can obtain information about the protein conformation changes modeled with a high resolution crystallographic structure.

Here, we study ChannelRhodopsin-2 (ChR-2), a light-activated proton pump belonging to the Rhodopsin family where the depolarisation of the H^+ selectively allows the passive movement of cations through the cell membrane. It is an integral membrane protein composed by seven transmembrane helical domains with Retinal covalently attached to a Lysine residue through the Schiff base. Upon light stimuli, the retinal isomerizes and the protein enters in a photocycle.

The crystal structure of ChR-2 at cryogenic temperatures is available, while only electrophysiology and spectroscopy techniques have been used to make time-resolved studies. Through the study of its photocycle with the help of TR-WAXS and time-resolved serial crystallography, we aim to have a more complete overview of its different conformational changes, to better understand the function of this medically relevant protein. The project is still in progress.

Structure of the chloroplast ribosome with chl-RRF and hibernation-promoting factor

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Photosynthetic organisms convert light energy into chemical energy, producing oxygen and building a variety of organic compounds. The photochemical reactions underpinning this process in plants and algae are hosted by the chloroplast. The chloroplast has its own genome that encodes for approximately 100 genes and it also has its own dedicated machinery for translation: the chlororibosome. Protein synthesis in the chloroplast is highly regulated; it fluctuates in response to light levels and it is believed to be coordinated with the synthesis, recycling and insertion of chlorophylls, a process, which is essential for the correct folding of parts of the photosynthetic proteins. To provide new insights into the structural specialities of the chlororibosome we purified chlororibosomes from spinach and used them for cryo-EM. The resulting map of 3 Å enabled the modelling of 53 proteins and approximately 4400 nucleotides. The model of the chlororibosome shows that uL23c is missing a loop of 15 amino acids, which in bacteria constricts de tunnel and plays a role in co-translational processes. The absence of the loop in uL23c not only widens the exit tunnel but also gives rise to a 30 Å long channel that runs perpendicular to the exit tunnel towards the periphery of the chlororibosome (Figure 1). The accessibility of this channel is further enhanced by rearrangement of the chl-rRNA. In addition, the recycling factor chl-RRF and hibernation-promoting factor HPF are resolved in complex with the chlororibosome, providing a portrayal of an organelle protein synthesis machinery with regulatory factors.

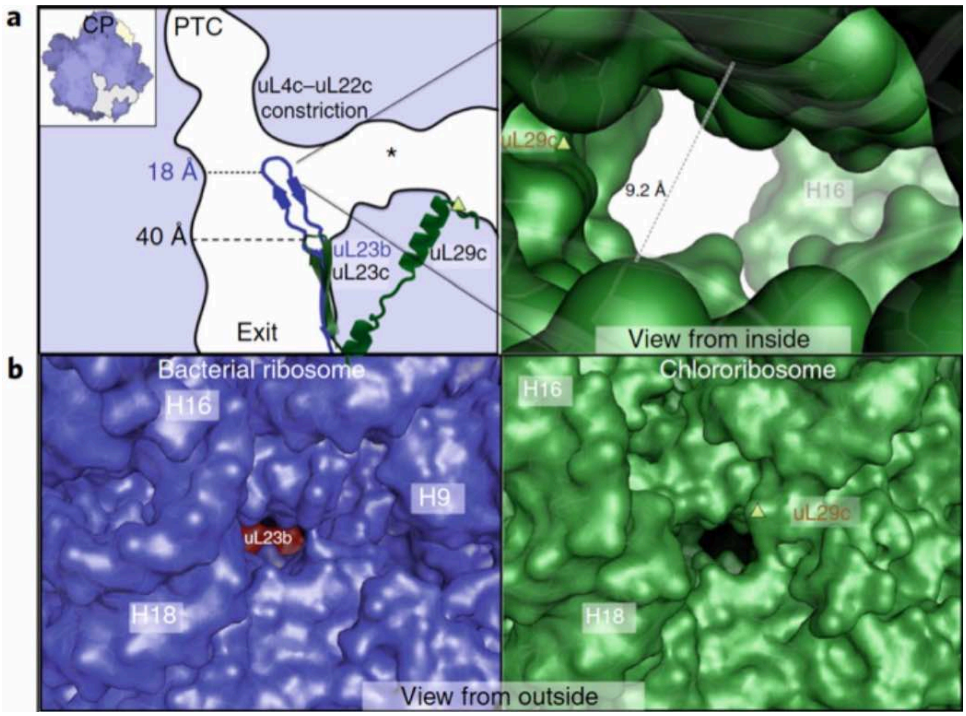

Figure 1: The exit tunnel. (A) Shortening of the loop of uL23c (green) as compared to uL23b (blue), results in opening of a new channel. (B) The view from outside of the channel shows that the channel is not present in the bacterial ribosome

Allostery in ubiquitination – an integrated structural biology study of UBE2E1

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Protein function relies on conformational dynamics on a wide range of time scales. In particular, grasping the molecular origins of allostery and conformational ensemble shifts in critical systems holds important keys to understanding protein interactions in health and disease. However, the width of time scales involved means that we run a severe risk of being too limited in our perspective if we restrict our use to the one or two experimental methods within our "safe zone". We will present recent work in our group that shows how we jointly use NMR, crystallography, small angle scattering and molecular simulations to analyse proteins involved in transcriptional regulation and ubiquitination and their interactions. In particular, we will show how mutational analysis has been used as a functional probe to analyse through-domain allosteric networks in the E2 conjugating enzyme UBE2E1, as identified by NMR and molecular dynamics simulations.

NUDT15 Hydrolyzes 6-Thio-DeoxyGTP to Mediate the Anticancer Efficacy of 6-Thioguanine

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Thiopurines are a standard treatment for childhood leukemia, but like all chemotherapeutics, their use is limited by inherent or acquired resistance in patients. Recently, the nucleoside diphosphate hydrolase NUDT15 has received attention on the basis of its ability to hydrolyze the thiopurine effector metabolites 6-thio-deoxyGTP (6-thio-dGTP) and 6-thio-GTP, thereby limiting the efficacy of thiopurines. In particular, increasing evidence suggests an association between the NUDT15 missense variant, R139C, and thiopurine sensitivity. In this study, we elucidated the role of NUDT15 and NUDT15 R139C in thiopurine metabolism. In vitro and cellular results argued that 6-thio-dGTP and 6-thio-GTP are favored substrates for NUDT15, a finding supported by a crystallographic determination of NUDT15 in complex with 6-thio-GMP. We found that NUDT15 R139C mutation did not affect enzymatic activity but instead negatively influenced protein stability, likely due to a loss of supportive intramolecular bonds that caused rapid proteasomal degradation in cells. Mechanistic investigations in cells indicated that NUDT15 ablation potentiated induction of the DNA damage checkpoint and cancer cell death by 6-thioguanine. Taken together, our results defined how NUDT15 limits thiopurine efficacy and how genetic ablation via the R139C missense mutation confers sensitivity to thiopurine treatment in patients. *Cancer Res 2016; 76(18); 5501–11.*

Enteroccous faecalis PcfF: A Ribbon-Helix-Helix protein responsible for recruitment of DNA and Relaxase to initiate conjugation

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Bacterial conjugation requires type IV secretion systems (T4SS) for transferring conjugative plasmid from donor to the recipient cell. This is an important process for bacteria to acquire multi-drug resistance. In our model conjugative plasmid pCF10 from the gram-positive bacteria *Enterococcus faecalis*, the substrate processing at the beginning of the conjugation is possible with the help of the accessory factor, PcfF. This protein is essential to facilitate the processing of the DNA substrate. pCF10 contains an origin of transfer (*oriT*) having an inverted repeat (IR) region and a nick site (*nic*). PcfF has been shown to bind to the IR of ds-*oriT*. There it is thought to recruit PcfG, the Relaxase, which binds and subsequently nicks the DNA and catalyses the formation of ssDNA. PcfF and PcfG together with the substrate DNA is called the relaxosome. PcfG remains covalently bound to one of the copies of the processed ss-DNA. This complex is the substrate being transferred into recipient cells via the T4SS. The components of G- T4SS have been characterised structurally in detail, however structural and functional information from G+ T4SSs is sorely lacking.

Here we report the crystal structure of PcfF to 1.8Å resolution, the first such structure of an accessory protein from G+ T4SSs. The structure was solved using SAD experiments of Se-incorporated protein crystals. The structural analysis reveals a dimeric functional assembly of PcfF, we verified that this dimeric form also exists in solution by GEMMA analysis. The whole architecture comprises a DNA-binding domain and a stalk domain. These two domains are connected via two parallel β-strand from each monomer (R51-D54). Presumably, these might act as a hinge region, possibly for conformational flexibility during formation of a relaxosome. The potential DNA-binding domain is contributed by the first 50 residues from each monomeric unit. These residues fold in to dimeric ribbon-helix-helix (RHH) domains. The RHH domain is a signature fold of the counterpart accessory proteins from T4SSs involved in conjugation and also found in transcriptional repressor. The RHH domain enables PcfF to bind DNA in a sequence-specific manner. Two anti-parallel β-strand contributed from each monomer possess conserved residues. Structural analysis propose that these conserved positive residues bind into the major groove of duplex-*oriT* DNA. Electrophoretic mobility shift assays (EMSA) confirmed the binding of the wild-type PcfF to the ds-*OriT* with a sub-micromolar affinity. Site-directed mutagenesis of these conserved residues resulted in abolished DNA binding, confirming that they indeed are responsible for binding to the DNA.

The C-terminal stalk-domain is the hypothetical binding site for a cognate relaxase. The structure of this domain is exclusively helical with four parallel α-helices, containing an indentation with conserved polar residues. This indentation seems to be the hypothetical sight for accommodating pcfG during processing of DNA to initiate conjugation. Mutagenesis of residues in this binding interface is ongoing along with EMSAs and further structural studies to get the detailed information on the complete assembly of a relaxosome.

Redox processes in complex I assembly

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Structure and function of human K2P ion channels at the SGC

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Two-pore-domain potassium channels (K2Ps) comprise a major and structurally distinct subset of the mammalian K+ channel superfamily, and underlie the background, or leak, currents that control the membrane potential. They are divided into six subfamilies, TWIK, TREK, TASK, TALK, THIK, and TRESK, which are activated by various stimuli such as pH, membrane stretch, lipids and G proteins. They assemble as dimers, forming a pseudotetrameric pore with a large extracellular cap domain. Their importance is demonstrated by their linkage to both physical and mental diseases such as pain, diabetes, pulmonary hypertension, Alzheimer's disease and depression. Currently, there are no pharmaceuticals available that directly target this family of proteins. Structures of TREK-1 and TREK-2 have been determined at the SGC, illustrating the mechanism of regulation by mechanosensitivity. We aim to determine additional structures of novel K2P channels involved in disease.

Positive and Negative Contributions to Enzymatic Selectivity Supported by Coinciding Amino acid Residues

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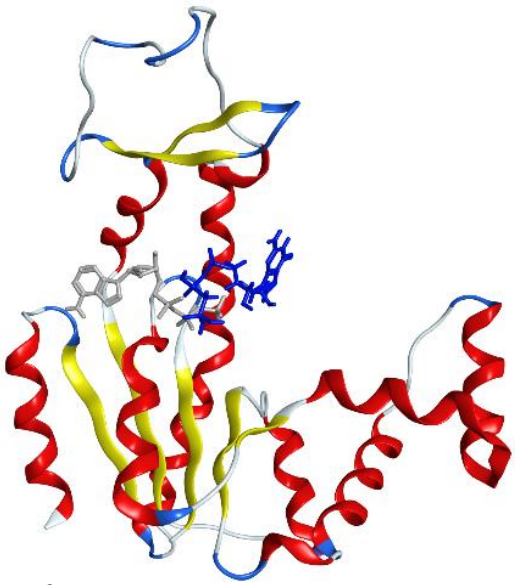
The enzyme adenylate kinase (Adk) has a two order of magnitude higher activity when using ATP to phosphorylate AMP compared to using, the chemically related, GTP. We have shown that the molecular basis for this selectivity is that Adk binds GTP in a catalytically inhibited conformation instead of inducing a catalytically competent structure the way ATP does. Coinciding residues mediate, in part, the interaction interfaces supporting ATP and GTP recognition.[1]

The importance of being able to select ATP over GTP as a phosphorous donor is crucial in order to protect the GTP pool. GTP has specific roles in the cell, mainly in signalling pathways.

By combining NMR spectroscopy and synthesis of novel ATP-analogs, we have further shown that one specific hydrogen bond between the adenine moiety of ATP and the backbone of the enzyme is pivotal for this selectivity. This specific interaction is conserved over a large part of all eukaryotic protein kinases suggesting that the selectivity mechanism we present is general throughout the kinase family.



1.7Å-X-ray structure of GTP bound adenylate kinase (PdB: 6F7U). The blue ligand is GTP. The correct binding of ATP is indicated by the grey ligand.

[1]Rogne P, et al. (2018) Molecular mechanism of ATP versus GTP selectivity of adenylate kinase. Proceedings of the National Academy of Sciences.

The SciLifeLab Drug Discovery and Development Platform

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Biophysical Screening and Characterization facility, DDD Platform, Dept. of Cell and Molecular Biology and Dept. of Chemistry-BMC, Uppsala University*DDD Platform, Dept. of Medicinal Chemistry, Uppsala University, **DDD Platform, Dept. of Medical Biochemistry and Biophysics, Karolinska Institutet

The Drug Discovery and Development (DDD) Platform is a national infrastructure within SciLifeLab aimed at helping Swedish academic groups progress therapeutic ideas towards a preclinical proof-of-concept. We take on projects either with a small molecule focus or with an idea for an antibody therapeutic. The current portfolio consists of 17 full projects where 11 are prioritized and 6 are supported when resources are available. All projects are evaluated bianually by the platform steering board. The platform consists of 8 facilities with different areas of expertise, including assay development and high throughput compound screening, antibody generation, protein expression, biophysical characterization, medicinal chemistry, pharmacological profiling, analysis of drug metabolism and pharmacokinetics data, and finally target safety analysis in collaboration with Swetox.

Projects that enter the platform will be supported with strategic advice as well as hands on lab work in the techniques needed to move the drug idea forward. This is done in collaboration with the academic group who provides their expert knowledge of the system and continues to work with their field of expertise. The platform provides know-how and instrumentation and the academic group pays for consumables and instrument maintenance. Importantly, the researcher retains all rights and ownership during this process.

Towards cryo-electron microscopy of heterogenous states of a model ligand-gated ion channel

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Pentameric ligand-gated ion channels are important mediators of electrochemical signal transduction in the nervous system and other pathways. Recent progress in protein biochemistry and structural biology has shed light on topology and possible gating mechanisms of these channels, yet the functionally relevant endpoints of gating and modulation in this system remain unclear. In order to obtain alternative structural data in this system, we optimized protein purification, reconstitution, and grid preparation conditions for structure determination of a model ion channel using cryo-electron microscopy (EM). We prepared samples at equilibrium between active and inactive states, with the goal of quantifying distinct classes within individual cryo-EM grids. Modification of carbon support, glow-discharging vapor, and blotting time improved particle distribution and orientation, allowing us to identify interpretable 2D classes, and initiate 3D classification. This work promises to inform methods development in cryo-EM of a biophysically relevant membrane protein, with insight into structure populations in varying agonist conditions.

Novel ATP-cone-driven allosteric regulation of ribonucleotide reductase via the radical-generating subunit

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Ribonucleotide reductases (RNRs) are key enzymes in DNA metabolism, providing the only known de novo pathway for the biosynthesis of deoxyribonucleotides (dNTPs), the immediate precursors for DNA synthesis and repair (Hofer *et al*, 2012). Class I RNRs consist of a large, catalytic subunit and a smaller, radical-generating subunit, which together form the active complex. Allosteric mechanisms control substrate specificity and overall activity of RNRs. Acting as a regulatory master switch, the allosteric activity site of ~100 amino acid residues, called the ATP-cone (Aravind *et al*, 2000), senses intracellular nucleotide concentrations by competitive binding of ATP and dATP. When ATP is bound, the enzyme is active, and when concentrations of deoxyribonucleotides rise, binding of dATP switches the enzyme off. This mechanism ensures sufficient but not excessive amounts of nucleotides.

In RNRs, the ATP-cone has been found exclusively in the catalytic subunit. In two class I RNR subclasses whose catalytic subunit lacks the ATP-cone, we discovered ATP-cones in the radical-generating subunit. We demonstrated that the ATP-cone in the *Leeuwenhoekiella blandensis* radical-generating subunit regulates activity via quaternary structure induced by binding of nucleotides. ATP induces enzymatically competent dimers, whereas dATP induces non-productive tetramers, resulting in different holoenzymes. The tetramer forms by interactions between ATP-cones, shown by a 2.45 Å crystal structure.

In summary, lack of an ATP-cone domain in the catalytic subunit was compensated by transfer of the domain to the radical-generating subunit. To our knowledge, this represents the first observation of transfer of an allosteric domain between components of the same enzyme complex.

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Time-resolved structural studies of ba_3-type Cytochrome *c* Oxidase

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Cellular respiration is the process where electrons that are extracted from the breakdown of nutrients are used to produce storable chemical energy in the form of ATP. The generation of ATP demands a series of electron transfers through the respiratory chain; this chain is a multi-complex consisted of different red-ox active enzymes located in the membrane matrix of cells. The last electron accepting complex in this chain is Cytochrome *c* oxidase (CcO) which reduces di-oxygen into two water molecules at the same time as it works as a proton pump. The free energy from the red-ox reactions is stored as an electrochemical proton gradient, and there is yet no clear picture of how the proton pumping actually works in the C*c*Os.

The methods we use to study the structural properties of ba_3-type C*c*O are Serial Femtosecond Crystallography (SFX) and Wide-angle X-ray scattering. We have so far developed a convenient method for batch-crystallization in lipidic cubic phase (LCP), which has generated the first room-temperature structure of ba_3-type C*c*O, free from radiation damage (Andersson et al., 2017). We also performed time-resolved experiments of ba_3 C*c*O for both SFX and WAXS, where the aim has been to see if we can elucidate the proton transfer reactions and localize the proton-loading site (PLS). The PLS is a site within the C*c*O suggested to be the place where protons are gated before they are pumped over the membrane matrix (von Ballmoos et al., 2012). The time-resolved approach has been performed in a pump-probe mode with carbon monoxide-binding studies. CO is considered to mimic the binding chemistry of O_2 (Einarsdóttir et al., 2015). By studying the more straightforward ba_3 oxidase which only has a single proton-conducting channel, the long-term goal is to elucidate the PLS and the fundamental pumping mechanism among all cytochrome *c* oxidases.

ANDERSSON, R., SAFARI, C., DODS, R., NANGO, E., TANAKA, R., YAMASHITA, A., NAKANE, T., TONO, K., JOTI, Y., BATH, P., DUNEVALL, E., BOSMAN, R., NUREKI, O., IWATA, S., NEUTZE, R. & BRANDEN, G. 2017. Serial femtosecond crystallography structure of cytochrome c oxidase at room temperature. *Sci Rep,* 7**,** 4518.

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Structural basis for selective targeting of proteasome deubiquitinases by enone-containing compounds

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The ubiquitin proteasome system (UPS) is a promising area for drug development where targets such as E1/E2 ubiquitin ligases and deubiquitinases (DUBs) contain functional cysteines. Cysteines can be targeted by compounds containing □□□□unsaturated ketones (enones) by hetero-Michael addition but the extent of selectivity that can be achieved is controversial. In collaboration with our pharmacology collaborators, we addressed this question by screening a library of ~ 5000□enone-containing compounds for inhibition of proteasome processing using a cell-based assay. Connectivity map (CMap) analyses showed induction of patterns of gene expression characteristic of proteasome inhibitors. By a range of biophysical, biochemical and cellular methods, we characterized 10 different active drugs and found evidence of selective inhibition of USP14 and proteasome DUB activity. The results suggested that all compounds can bind to a USP14 crevice close to the active site and some compounds also bind covalently, presumably to the active cysteine. We finally demonstrate limited developmental toxicity and significant antineoplastic activity of enone DUB inhibitors in zebrafish embryos. These findings challenge the view of general reactivity and toxicity of enones and show that drug candidates showing selectivity to cysteine-containing proteasome DUBs can be identified. In this presentation, we describe our most recent advances in interpreting the structural and functional properties of USP14 by a combination of crystallography, NMR and small-angle X-ray scattering.

Computational Development and Analysis of Time Resolved Protein Dynamics

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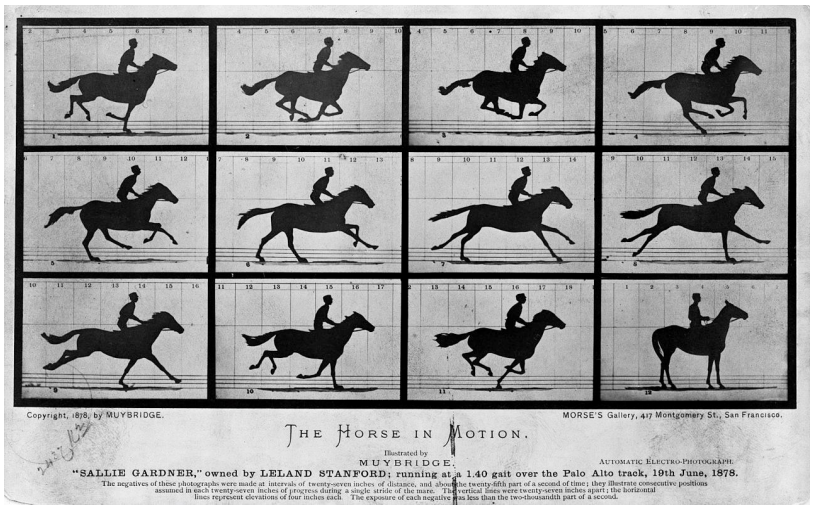
The horse in motion (1878) is considered the first motion photography ever produced, in order to answer the scientific question of whether all four hooves of a horse is ever off the ground at the same time while it's galloping. Similarly, in the molecular world, the single static representation of macromolecules, such as proteins, was the first way of viewing such structures. However, a static picture is inadequate when describing the dynamics of a protein, which is essential to the performance of its biological function.

Recent developments of X-ray scattering techniques allow us to measure protein dynamics and intermediates in solution. By combining Molecular Dynamics simulations with experimental WAXS difference curves, we can fit the most likely tertiary structures that represent this data.

This requires developing a software suite for TR-WAXS data analysis. Experiments are performed at various x-ray sources and can differ in methodology. The project aims to develop a standardized method for the data analysis of experimental data.

Finally, for membrane proteins, the presence of the lipid bilayer adds to the difference scattering effects which are difficult to model. This project will look at developing theoretical models that can correctly describe the lipid bilayer.



Visualizing the inhibitory synapse: Structural studies of the Glycine receptor and gephyrin using electron microscopy

Andreea Scacioc*, Tales Rocha de Moura*, Carsten Mim
*shared authorship

1. *Background*

Our understanding how signals are transmitted and integrated in the nervous system, depends on our knowledge about the regulation of inhibition and excitation. The glycinergic synapse is a great model system to gain insights into inhibition because of its simple anatomy. Moreover, studying the glycinergic synapse is synonymous to gaining knowledge about the mechanism of pain to control it.

Glycine receptors (GlyR) are the main components of the glycinergic synapse. They are ligand gated ion channels conducting chloride as soon as glycine binds to their extracellular domain. On their cytosolic side, GlyRs bind gephyrin – a scaffold protein. Gephyrin is important for anchoring and trafficking GlyRs, but also to recruit other components of the glycinergic synapse.

Recently, two structures of the GlyR were determined. However, these structures miss functionally important parts of the GlyR, i.e., the gephyrin binding loop. Moreover, these structures are determined in detergent rather than in a lipid membrane. Similarly, no full-length gephyrin structures are available neither of the gephyrin in solution nor its scaffolding lipidic membranes.

2. *Aims, approaches and preliminary results*

Aim 1 – <u>Visualizing the full-length Glycine receptor in a lipidic membrane (or stabilized by amphipols):</u>
We hypothesize that the GlyR interactions with lipids are important for its conformation. This is why, we aim to visualize, using electron microscopy and electron crystallography, the GlyR in 2D crystals, nanodiscs and amphipols. The GlyR purification was modified in towards reconstitution into a lipidic environment.

Aim 2 – <u>Atomistic studies of full-length gephyrin in solution and as a membrane scaffold:</u>
Once the heteropentameric GlyR is purified and reconstituted in a lipid membrane, the next building block towards the synthetic inhibitory synapse is the addition of its scaffolding protein, gephyrin. We aim at understanding the scaffolding mechanism of gephyrin and, in particular, the transition from the solution conformation of gephyrin to its scaffold conformation. Towards this end, the full-length gephyrin purification was optimized for cryo-EM purposes. We tested two cross-linking reagents to stabilize the protein and reduce the degree of freedom. The protein was further separated with a glycerol gradient. Finally, we collected data for gephyrin in solution using cryo-EM. We obtained a preliminary low resolution model for its full-length conformation. However after evaluating the cross linking reagent and applying a restrictive selection of particle, based on noise artifacts and quality of particles, we obtained 2D classes with higher resolution features. These classes resemble projections of X-ray structure of a gephyrin domain. A strong orientational bias prevented us from reconstructing these classes reliably. Next, we will focus on collecting a larger data set including images of the sample at high tilt.

The 8-oxo-dGTP hydrolase NUDT1 from *Arabidopsis thaliana* also utilizes non-nucleotide monoterpene substrates

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Arabidopsis thaliana NUDT1 (AtNUDT1) belongs to the Nudix family of proteins, which have a diverse range of substrates, including oxidized nucleotides (1). The hydrolysis of oxidized dNTPs is highly important as it prevents their incorporation into DNA, thus preventing mutations (2). AtNUDT1 is the sole Nudix enzyme from *A. thaliana* shown to have activity against 8-oxo-dGTP, a common oxidized nucleotide (3). The structure of AtNUDT1 in complex with 8-oxo-dGTP was solved *via* X-ray crystallography. Structural comparison with homologues from bacteria and humans revealed a conserved overall fold. Analysis of the 8-oxo-dGTP binding mode indicated that the residues Asn76 and Ser89 interact with the O8 group of the substrate, a feature not observed in related structures solved to date. Kinetic analysis of wild-type and mutant AtNUDT1 confirmed these active site residues to have an influence on 8-oxo-dGTP hydrolysis. In addition to nucleotide substrates, a recent study showed AtNUDT1 is also able to hydrolyse monoterpene compounds. In this study, the kinetic parameters for the monoterpene GPP and several precursors were determined, which indicated that AtNUDT1 actually hydrolyses monoterpene substrates much more efficiently than 8-oxo-dGTP. The diversity of reactions catalysed by AtNUDT1 suggests that this Nudix enzyme from higher plants has evolved in a manner distinct to its homologues from both humans and bacteria.

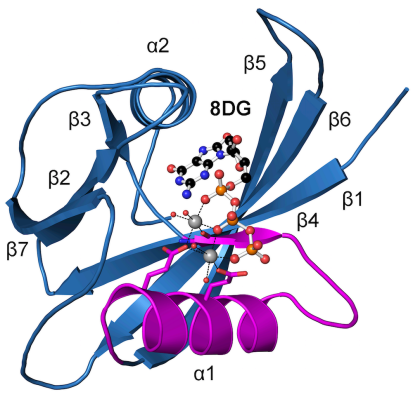

Figure 1. Crystal structure of the AtNUDT1 8-oxo-dGTP complex. Ribbon representation of the AtNUDT1 monomer. The secondary structure elements, α-helices (1-2) and β-strands (1-7) are labelled. The highly conserved Nudix motif is coloured magenta. The 8-oxo-dGTP (8DG) ligand is depicted as a ball-and-stick model. Magnesium ions involved in ligand coordination are shown as grey spheres.

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PrgB promotes Aggregation, Biofilm Formation and Conjugation through DNA binding and Compaction

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Gram-positive bacteria deploy type IV secretion systems to facilitate horizontal gene transfer. The T4SSs of Gram-positive bacteria rely on surface adhesins as opposed to conjugative pili to facilitate mating. *Enterococcus faecalis* PrgB is a surface adhesin that promotes mating pair formation and robust biofilm development in an extracellular DNA (eDNA) dependent manner. Here we report the structure of the adhesin domain of PrgB. The adhesin domain binds and strikingly also compacts DNA in vitro. In vivo, PrgB deleted of its adhesin domain does not support cellular aggregation, biofilm development and conjugative DNA transfer. PrgB also binds lipoteichoic acid (LTA), which competes with DNA. Our findings support a hypothetical mechanism whereby: i) PrgB forms long-range intercellular contacts through binding of eDNA filaments and through DNA condensation establishes shorter range contacts; ii) PrgB then alternatively binds LTA exposed on target cell surface to stabilize mating junctions enabling highly efficient T4SS-mediated gene transfer.

Structural characterization of an ancient aquaporin

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Aquaporins are crucial membrane proteins, responsible for balancing the osmotic balance of every cell by acting as a transmembrane channel for water and small solutes. We are interested in getting more insight into the evolution of aquaporins with respect to their specificity and regulation and we therefore focus on an old ancestor, Barnacle Aquaporin 1 (bAQP1) from the species *Balanus improvisus.* Understanding the principals of transport mechanisms within the barnacle aquaporins will not only shed light on the molecular process, but also how the osmoregulation developed in the eukaryotic tree of life. We have produced functional barnacle homologues to high yields in the yeast *Pichia pastoris*. Previous crystallization setups with the wild type protein crystals were not diffracting better than 10 Å, also optimization trials failed to give better results. As part of the optimization procedure, molecular truncations have been evaluated aiming to support protein contact for crystallization. Using the optimized version of bAQP1 in conjunction with a favourable detergent (OGNPG), lead to initial crystals, diffracting to 7 Å already from the initial plate setup. We now focus on optimizing the different lead conditions for crystallization in order to obtain a high-resolution structure from this ancient protein, which will also be a support for our strategy for crystallization optimization.

Development of serial millisecond crystallography at BioMAX beamline

Anastasiia Shilova

MAXIV laboratory, BioMAX beamline, Lund University

Synchrotron radiation is one of the key factors for the tremendous success of macromolecular crystallography during the past decades. More than 90% of all protein structures in the PDB database were solved by crystallography using synchrotron radiation sources and around 95 % of them were determined from cryocooled crystals[1,2]. In this way, a whole data set can be collected from one flash- cooled crystal. Data collection at cryogenic temperatures drastically reduces radiation damage effects. However, structures determined using cryo-freezing techniques are limited by static nature of frozen crystals.

With the advent of X-ray free electron laser sources (XFELs), serial crystallography - a new method of data collection where thousands of crystals are delivered to the beam and exposed in random orientation at room temperature - was developed. Recently, serial crystallography approaches have started to appear on the more flexible and widely available microfocus beamlines at synchrotron sources.

This work describes the implementation of different sample delivery approaches to perform room temperature serial crystallography at BioMAX beamline and future perspectives for the MicroMax beamline at MAXIV synchrotron, which will provide a very small and intense X-ray beam. One of them is high viscosity injector-based serial millisecond crystallography, similar to the experiments with injectors originally developed at XFELs. A continuous stream containing micro-crystals is injected into the microbeam. An alternative solid support approach is based on scanning micro-crystals deposited on silicon nitride membranes, that has the lowest background scattering among all other presented methods. Successful application of these methods to study room-temperature molecular dynamics will be discussed.

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The structure and function of a membrane superoxide oxidase

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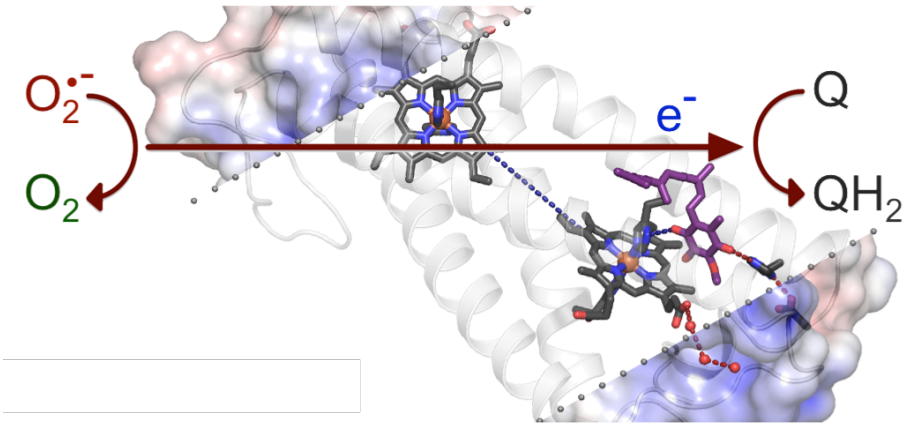
Superoxide is a reactive oxygen species (ROS) that causes damage to proteins, lipids and DNA. Despite its implications for many diseases, up until now the only known universal mechansim for protecting the cell from superoxide is a class of enzymes called *superoxide dismutases (SOD)* that disproportionate superoxide to water and hydrogen peroxide.

We have established the function and solved the 2.0 Å crystal structure of an integral membrane superoxide *oxidase* from *E.coli:* Cytochrome b561 (CybB), a di-heme protein previously suggested to play a role in the respiratory chain. The structure reveals a potential quinone-binding pocket at the cytoplasmic heme and a water channel connecting the latter with the cytoplasm. There is also a putative superoxide-funneling, positively charged patch on the periplasmic side of the protein, surrounding the partially exposed periplasmic heme.

We show that CybB is only 50% reduced by Ubiquinol but can be fully reduced when NADH dehydrogenase II is added to the mixture, a reaction that is mediated by superoxide. The superoxide oxidation rate of CybB is very fast (in the diffusion-limited range), and CybB effectively quenches superoxide generated at the membrane in artificial membranes and respiring *E.coli* membrane vesicles. Based on this, we suggest that this enzyme is named Superoxide Oxidase (SOO).

Our current work is focused on the connections between the various biochemical and structural properties of CybB/SOO, and on how the enzyme performs its function *in vivo*.



Scavenging of superoxide by a membrane bound superoxide oxidase
Lundgren C†, Sjöstrand D†, Biner O†, Bennett M, Rudling A, Johansson A-L, Brzezinski P, Carlsson J, von Ballmoos C*, Högbom M**
Nat Chem Biol. 2018 (Accepted)
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Protein dynamics governing the function of the DegP serine protease-chaperone

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Protein quality control is one of the essential functions found in cells from all kingdoms of life and it is achieved by the interplay of dedicated proteases and chaperones. The dysregulation of the protein quality control machinery can lead to cell death and often it is found at the foundation of diseases such as cancer, age-related disorders or neurodegenerative diseases like Parkinson's or Alzheimer's [1]. The HtrA protein family (high temperature requirement A) is a group of heat-shock induced serine proteases, which are widely spread across different species [2]. One of the bacterial HtrA protein family members is the homo-oligomeric DegP-protease, playing a crucial role in the biogenesis and degradation of β-barrel outer-membrane proteins within the periplasmic space of gram-negative bacteria [3]. Although it is known that DegP is an essential protein in *E.coli* during elevated temperature conditions [4], its functional and structural details are so far only partially understood.

The aim of our study is to track the molecular changes of different DegP oligomeric states at the atomic level underlying its function by using advanced NMR techniques and consequently decipher DegPs role in the assembly and maintenance of bacterial outer membrane proteins. Since the size of DegP can vary from 50 kDa to 1 MDa, sophisticated isotope labeling schemes are used to obtain high-resolution NMR spectra.

Our preliminary results from 2D [^{15}N,^{1}H]-TROSY-HSQC as well as 2D [^{13}C,^{1}H]-HMQC spectra indicate an unexpected degree of dynamics of structural elements of DegP within its hexameric state. Furthermore, we initially observed differences in chemical shift within the obtained NMR-spectra of the different oligomeric states studied, possibly indicating structural changes upon transition between the oligomeric states. The observation of possible structural adaptions seems to be modulated by temperature and might prove to be a crucial aspect for regulating DegP activity.

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microRNA function through structural perturbation

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microRNAs (miRNAs) are small, non-coding RNA molecules that manipulate messenger RNA (mRNA) translation. The interaction of miRNAs with their target mRNAs is guided by a critical and complementary seed region (~6 nucleotides at the miRNA 5' end) and an imperfect base-paring pattern outside the seed region, which is often predicted to be highly dynamic[1]. The human miR-34a is a mediator of tumour suppression[2], and interacts with more than 100 known, validated mRNA targets[3]. How a single miRNA can both select its targets specifically, and also modulate a diverse network of mRNA targets, is poorly understood.

Preliminary results suggest that there may be structural differences between the interaction of miR-34a with three selected targets; the mRNA transcripts for SIRT1 (member of the p53 pathway)[4], CD44 (cell adhesion molecule)[5], and PNUTS (involved in apoptosis and DNA repair)[6,7] (Figure 1). The current project aims to elucidate the structural determinants of miR-34a function at high resolution via a combination of biophysical methods, including Electrophoresis Mobility Shift Assay (EMSA), thermal melting assays, and solution state NMR spectroscopy. This work could aid the understanding of whether differential binding modes mediate the miRNA-mRNA selection process.

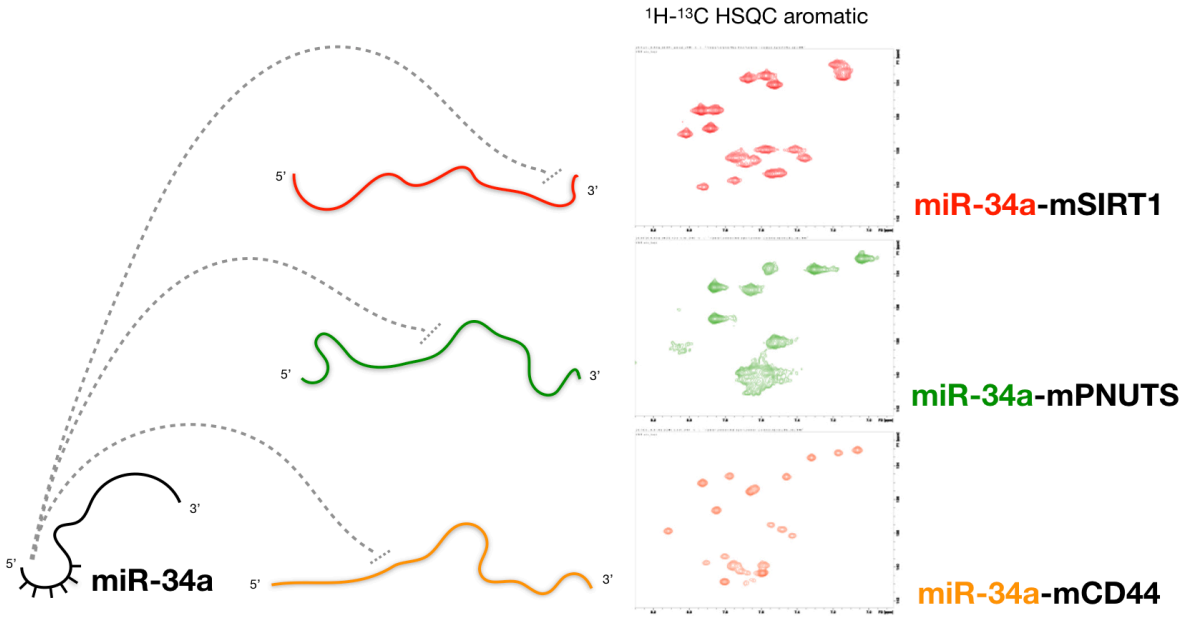

Figure 1. Comparison of the aromatic regions of respective ^{1}H-^{13}C HSQC spectra for complexes of miR-34a with mRNA targets mSIRT1, mPNUTS, and mCD44 indicate possible differential modes of binding. The NMR data were collected by Lorenzo Baronti, Karen Schriever, and Luís Silva respectively.

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Functional and structural characterization of phage SAM hydrolase enzymes

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By screening for bacteriophage DNA that could rescue an auxotrophic *E.coli* strain, a set of three S-adenosylmethionine hydrolases from environmental samples of phage DNA were recently identified (1). The phage-encoded SAM hydrolases could in an un-predicted manner complement an *Escherichia coli* knock-out strain of an enzyme involved in threonine biosynthesis. The mechanism behind this rescue was found to be an up-regulation of the genes involved in methionine biosynthesis, regulated by the transcriptional repressor MetJ in complex with SAM, when SAM was degraded by the SAM hydrolase. SAM hydrolases have been proposed to play a role in combating the Type I RM defense system of bacteria. The only previously known enzyme from this family comes from the T3 phage and was discovered in the 1960's. Until now, there has been no structural and little functional or mechanistic knowledge of this type of enzyme, which is the only described enzyme with trialkylsulfonium hydrolase activity. We have started to characterize the newly discovered SAM hydrolase enzymes as well as the T3 SAM hydrolase. We have determined the first structures of a SAM hydrolase by X-ray crystallography. Furthermore, we have developed an enzymatic discontinuous assay to study the kinetics of the enzymes and shown that the T3 SAM hydrolase has higher activity compared to the newly discovered SAM hydrolases.

1. Jon Jerlström Hultqvist, Omar Warsi, Annika Söderholm, Michael Knopp, Ulrich Eckhard, Egor Vorontsov, Maria Selmer, Dan I. Andersson "A bacteriophage enzyme induces bacterial metabolic perturbation that confers a novel promiscuous function" (under revision)

Chemical design of functional and dynamic protein assemblies

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Protein-enriched bacterial outer membrane vesicles - A native scaffold for outer membrane protein studies

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Most studies characterizing the folding, structure, and function of membrane proteins rely on solubilized or reconstituted samples. Whereas solubilized membrane proteins lack the functionally important lipid membrane, reconstitution embeds them into artificial lipid bilayers, which lack characteristic features of cellular membranes including lipid diversity, composition and asymmetry. Here, we present a new and unique method utilizing outer membrane vesicles (OMVs) released from *Escherichia coli* to study outer membrane proteins (Omps) in the native membrane environment. Enriched in the native membrane of the OMV we characterize the assembly, folding, and structure of OmpG, FhuA, Tsx, and BamA. Comparing Omps in OMVs to those reconstituted into artificial lipid membranes, we observe different unfolding pathways for some Omps. Our observations highlight the importance of the native membrane environment to maintain the native structure and function relationship of Omps. Our fast and easy approach paves the way for functional and structural studies of Omps in the native membrane.

NMR, a molecular microscope to characterize the peroxiredoxin-Hsp70 interaction slowing down yeast aging

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Peroxiredoxins (PRDXs) are thiol active peroxidases and signaling enzymes involved in many biological processes in the cell such as in the defense against oxidative stress, in the regulation of peroxide levels or in light signalling[1]. Their numerous functions derive from their different structural states and diverse localization in many organisms (mammals, plants, yeasts). They have also been shown to be involved in many disorders ranging from different cancer-types to ischemia. Their activity is based on the presence of a peroxidatic cysteine within their active site and involve thioredoxin, thioredoxin reductase, and sulfiredoxin (Srx) in their catalytic pathway. All PRDXs share a Rothman fold and possess quaternary structure ranging from monomer to 20-mer in dependence of their redox states[2].

Recently, PRDXs were found to play a role as modulators of the rate of aging in yeasts and act in lifespan extension through the anti-aging intervention caloric restriction[3]. In a follow-up study it was shown that the anti-aging effect of Tsa1, the major cytosolic PRDX in *Saccharomyces cerevisiae*, was linked to the molecular chaperone Hsp70 and that Tsa1 recruits Hsp70 to aggregated proteins through a H_2O_2-specific redox switch. More specifically, the sulfinylation of Tsa1-C48 supported Hsp70 binding to damaged proteins accumulating in the aging process. In addition, subsequent Tsa1 reduction by Srx was required for the disaggregation of misfolded and aggregated proteins[4]. Besides the fact that Tsa1 and Hsp70 physically interact, the details of this interaction at the molecular level as well as the functional consequences for both enzymes so far remain elusive.

Therefore, by employing the powerful tool solution NMR-spectroscopy we will study the mechanism of Tsa1-Hsp70 interaction, in order to characterize this complex in detail and to decipher the functional interaction of these enzymes. As classical solution state NMR-approaches are limited regarding to the size of the system, we will initially construct stable smaller Tsa1-variants using specific mutations[5]. Furthermore, we will apply methyl-TROSY NMR experiments in combination with methyl-specific isotopic labeling for the study of the larger wild-type protein complex. Indeed, [^{1}H-^{13}C] labelled methyl probes within deuterated proteins leads to intense and well-resolved peaks for supramolecular complexes[6, 7 8]. Initial experiments on specific-methyl-labeled Tsa1 showing the feasibility of the approach will be presented.

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NMR studies of the underlying dynamics of the protein export chaperone SecB in complex with its clients

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The general secretion (Sec) pathway provides an essential mechanism to guarantee a path for proteins requiring to be translocated in a folding competent state across or directly integrated into the inner bacterial membrane [1]. Within this pathway SecB acts as a holdase chaperone forwarding these pre-proteins as an ensemble to the SecA-motor protein associating them to the transmembrane translocase complex SecYEG [2,3]. However, the structural and dynamical details of this holdase–client ensemble and subsequent pre-protein relay remain poorly understood.

In its functions SecB utilises a classical rigid assembly with short-lived individual hydrophobic contacts while keeping the rest of the pre-protein in a restricted conformational ensemble. Previous studies have shown that the transmembrane domain of Outer-membrane protein A binds SecB in a fluid-globule state, an interaction based on avidity with a characteristic re-orientation of the client on the chaperone binding interface [4]. Such a combination of characteristics creates a dynamic playground for advanced NMR techniques to characterize the structural and dynamical properties of this chaperone–client interplay. I will present preliminary data studying the holdase properties of SecB and the influence of the signal-sequences targeting the client protein to the SecYEG-translocon.

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Apolipoprotein N-acyltransferase (Lnt) in a lipoprotein bound state

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In bacteria, lipoproteins are important components of the cell envelope and are responsible for many essential cellular functions including nutrient uptake, secretion, cell wall integrity, and antibiotic production. They are produced by the post-translational modifications of proteins by the covalent attachment of lipids that occurs via a sequential 3-step process controlled by three essential membrane bound enzymes. The first step, carried out by Prolipoprotein diacylglyceryl transferase (Lgt), involves the transfer of an N-acyl diglyceride group to what will become the N-terminal cysteine. This is followed by the cleavage of the Lipoprotein signal peptide by LspA to form apolipoproteins. The last step, unique to Gram negative bacteria, is the N-acylation of the terminal cysteine by Apolopoprotein N-acyltransferase (Lnt) to form the final mature lipoprotein. Here we report the crystal structure of the last enzyme in the pathway, Lnt from *Escherichia coli*. The structure consists of a transmembrane domain containing 8 helices and a globular perplasmic domain with a nitrolase fold. The enzyme crystalized with 2 molecules arranged within the asymmetric unit in such a way that could mimic one possible mode of apolipoprotein docking to Lnt. The active site sits just above the membrane interface and appears to be partially shielded by are large flexible loop. The crystal packing implies that the incoming peptide interacts with this large flexible loop suggesting a possible mechanism by which it could enter and exit the active site. These insights into apolipoprotein docking to Lnt could aid in the development of new antibiotics targeting this essential bacterial enzyme.

Structural basis of mammalian RNA polymerase II pausing, pause release, and elongation activation

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Metazoan gene regulation often involves pausing of RNA polymerase II (Pol II) in the promoter-proximal region. Paused Pol II is stabilized by the protein complexes DRB sensitivity-inducing factor (DSIF) and negative elongation factor (NELF). Here we report the cryo-electron microscopy (cryo-EM) structure of the paused Pol II-DSIF-NELF elongation complex (PEC) at 3.2 Å resolution. The structure reveals a tilted DNA-RNA hybrid that impairs binding of the nucleoside triphosphate substrate. NELF binds the polymerase funnel to restrain Pol II mobility, contacts the trigger loop, and prevents association of the anti-pausing factor TFIIS. NELF possesses two flexible tentacles that contact DSIF and the exiting RNA. One tentacle contains the RNA binding domain of subunit NELF-E and may contribute to NELF recruitment when nascent RNA emerges onto the Pol II surface. These results define the paused Pol II state and provide the basis for understanding NELF function in promoter-proximal gene regulation.

We then show that formation of a pause-released Pol II elongation complex (EC) in vitro requires the kinase activity of the positive transcription elongation factor b (P-TEFb) and the PAF1 complex (PAF1c). The pause-released EC is activated by SPT6. The cryo-EM structure of the pause-released and activated Pol II-DSIF-PAF1c-SPT6 EC (EC*) was determined at 3.1 Å resolution. PAF1c competes NELF from the Pol II funnel for pause release. P-TEFb phosphorylates the Pol II linker to the C-terminal domain (CTD). SPT6 binds to the phosphorylated CTD linker and opens the RNA clamp formed by DSIF. EC* adopts the post-translocated state and can accept nucleoside triphosphate substrates for productive RNA elongation. Together, these results provide the basis for Pol II pausing, pause release, and elongation activation.

Electron crystallography for studying protein structures from micron- and nano-sized 3D crystals

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Recent developments of novel electron diffraction techniques have shown to be powerful for determination of atomic resolution structures from micron- and nano-sized protein crystals, too small to be studied by single-crystal X-ray diffraction.[1–4] At Stockholm University, we are dedicated to further develop and improve methods so that protein structures can be routinely solved from MicroED data collected on micron- and nano-sized crystals (Figure 1). Currently, preparing cryo-EM samples of protein crystals is one of the major bottlenecks in the process. Depending on crystallization conditions, protein crystals can behave very differently. The same also applies to single protein molecules (i.e. single particle cryo-EM sample). Double-sided blotting before the vitrification process takes away majority of the precious specimens (single molecules or protein crystals) from the TEM grid. Meanwhile, single-sided blotting is often not efficient in removing the excess liquid making the sample too thick for TEM experiments. On the other hand, in order to obtain suitable crystal sizes for electron diffraction data collection, additional fragmentation processes are often required.[5] Here, we present some of our preliminary results on improving the cryo-EM sample preparation of protein crystals (1) of sizes between 3-5 µm (too small for most X-ray sources, but too large for TEM) and (2) grown in highly viscous mother liquid (i.e. ~45% PEG 400). More importantly, electron diffraction data collected on these crystals and crystal fragments could be used to solve their structures. We believe this development is an important step forward towards the routine 3D electron diffraction data collection on protein crystals.

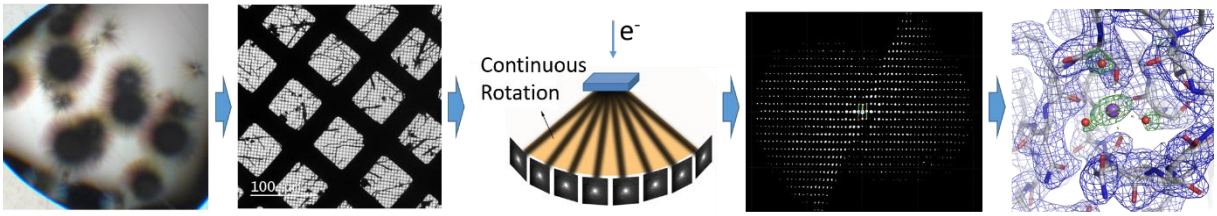

Figure 1 – Electron crystallography for studying protein structures from micron- and nano-sized crystals

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Structural determination of the Bri2 BRICHOS domain: relevance to Alzheimer's disease

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Background: The Bri2 BRICHOS domain contains about 100 amino acid residues and is associated with neurodegenerative and amyloid diseases. Of potential relevance to Alzheimer's disease, Bri2 BRICHOS inhibits amyloid-β peptide (Aβ) fibrillation and reduction of of neurotoxicity in vitro and in animal models. We have shown that different quaternary structures of recombinant human (rh) Bri2 BRICHOS have different molecular chaperone functions. The Bri2 BRICHOS dimer could assemble into larger polydisperse oligomers, which are partly linked by inter-chain disulfide bridges and built up of an even number of subunits. These oligomers efficiently inhibit non-fibrillar aggregation of thermo-denatured citrate synthase (CS), thereby showing general chaperone activity. Conversely, the small size species are more efficient than the large oligomers in suppressing Aβ42 fibril formation, by blocking the secondary nucleation and the elongation process of fibril formation[1].

The aim is to elucidate the molecular structure of the Bri2 BRICHOS oligomeric chaperone. Here, we present a novel 3D map in negative stain obtained from this complex[1]. Recently collected cryo data will add more details to this structure.

Methods: rh Bri2 BRICHOS was expressed in Shuffle *E. coli* cells. Oligomers were isolated and the molecular size (~370 kDa) was evaluated by Size Exclusion Chromatography (SEC)**.** Single particles were imaged using a Jeol JEM2100F transmission electron microscope (TEM) (Jeol, Japan). Micrographs for data collection were recorded on a DE-20 direct electron detector[1]. Cryo data were collected on a Titan Krios TEM, equipped with a K2 Summit image filter/ direct electron detector, at SciLifeLab, Stockholm Sweden.

Results: Bri2 dimers assembled into large oligomers. The 3D map in negative stain, available at the EM Data Bank (EMD-3918), showed an apparent two-fold symmetry at a resolution of 16.5Å. A volume comparison indicated that around 24 Bri2 BRICHOS subunits could be accommodated in this oligomer, which is in good agreement with masses estimated by SEC[1]. Recently obtained cryo data showed similarities to the negative stain data, where a non-symmetrized 3D map revealed an approximate twofold symmetry.

Significance: High resolution data will provide insight into the mechanism behind how the Bri2 BRICHOS domain can affect qualitatively different aspects of protein misfolding by varying the assembly state. It will further provide deeper insight into the molecular basis of amyloid diseases and potential new treatment strategies.

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