Session 13: Multiple approaches to structure in molecular biology

Lectures

L13.1

A personal view of advances in diffraction and imaging methods for structural biology

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After a long period of incremental advances in crystallography and electron microscopy we are in a period of disruptive change. Long wavelengths can all buteliminate the phase problem for biological crystals. XFEL crystal presentationmethods are likely to develop and spread to synchrotrons. XFELs offer subpicosecond time resolution of dynamic events, and this might lead to a renaissance instudies of dynamics at synchrotrons. Meanwhile electrons challenge X-rays for theanalysis of sub-micron crystals and cryo-electron microscopy is increasingly able toproduce high resolution refined atomic models for complex systems. Zooming out tothe cellular level we can see that molecular and even atomic detail will be revealed bycryo-electron tomography. I will discuss technology advances and give some snippets ofresults from the work of my group and more broadly from Diamond.

L13.2

High-resolution single-particle cryo-electron microscopy

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Structural biology for many years relied on high resolution information obtained by macromolecular crystallography. For a long time a limitation of electron microscopy was the fact that it in most of the cases it provided structural information at 6-10 Å resolution which was not sufficient to build a structural model from scratch. Recent developments in single particle cryo-electron microscopy (cryo-EM) allowed to push this resolution limit as high as ~2 Å leading to the so-called "resolution revolution" in cryo-EM. Two main technical challenge of electron microscopy is the low contrast of the images. This is why very many images of particles need to be collected and averaged to increase to signal-to-noise ratio. Moreover, the radiation damage by the electron beam and the movements of the specimen during the data collections were additional factors limiting the attainable resolution. These problems were solved by the development of very sensitive and fast detectors using the CMOS semiconductor technology, termed direct detectors. They allow the recording of movies of the sample during exposure, which in turn allows for the correction of the sample movement and selection of the frames that are not affected by the radiation damage. These developments combined with powerful new software like Freealign and RELION led to a dramatic increase of the resolution of EM maps, which nowadays allow building the structural models of macromolecular complexes without prior knowledge of their structure, which is the essence of the EM revolution. EM is particularly suited for the studies of large and dynamic assemblies which are at the center of the most important questions of modern molecular biology.

In this talk the principles of modern single-particle electron microscopy will be presented along with some examples of our own results obtained using this methodology.

L13.3

Serial crystallography and molecular imaging using X-ray free-electron lasers

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This talk will cover past results and current research in crystallography using X-ray free-electron lasers (XFELs). An XFEL produces single X-ray pulses containing enough photons to create an easily measurable diffraction pattern from a protein crystal only a few microns in size, even though the duration of the pulse is only a few tens of femtoseconds. This allows us to sidestep most of the processes of radiation damage which limit macromolecular crystallography using synchrotron X-ray sources [1], and allows for high time resolution when studying protein dynamics [2].

There are currently two X-ray free-electron lasers operational in the world, the Linac Coherent Light Source (LCLS) in California and the Spring-8 Ångstrom Compact Laser (SACLA) in Japan. Several more facilities will become operational in the next few years. In the few years since XFEL facilities have been available, many different biomolecular systems have been studied using a variety of techniques for delivering protein crystals to the X-ray beam, recording diffraction patterns and processing the data. Great progress has been made in reducing the quantities of sample and measurement time required, while improving the quality of the resulting data [3]. In addition to the purely crystallographic approaches, it has recently been found that diffusely scattered X-rays arising from crystal disorder can be interpreted directly similar to the scattering from a single molecule [4], potentially unlocking a vast new source of information.

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L13.4

Structure determination of protein complexes based on joint NMR and X-ray crystallography data

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BMI1 is a family member of the polycomb group proteins, and emerging data support a crucial role for BMI1 in cancer. *BMI1* is a stem cell gene that determines the proliferative capacity and self-renewal of normal and leukemic stem cells. Overexpression of BMI1 induces transformation and promotes tumor growth *in vivo* in animal models. Furthermore, silencing of *BMI1* impairs cancer cell proliferation and tumor growth in the models of blood cancers and solid tumors, suggesting that BMI1 might represent a valid target for therapeutic intervention.

BMI1 is a central component of the canonical PRC1 complex and has a dual role in PRC1 activity: regulation of H2A ubiquitination activity and mediation of protein-protein interactions. RING domain of BMI1 forms a complex with Ring1A/B proteins, which constitutes the heterodimeric E3 ubiquitin ligase subunit of the PRC1 complex. We have developed potent compounds that bind to Ring1B-Bmi1 and inhibit its E3 ubiquitin ligase activity. In order to determine the structure of Ring1B-Bmi1 inhibitor complex we obtained diffracting crystals. However, structure determination of a complex was not feasible due to insufficient resolution of the diffraction data. To determine the structure of a protein-inhibitor complex, we have assigned chemical shifts and measured intermolecular NOEs between Ring1B-Bmi1 and inhibitor. Hybrid refinement starting from the crystal structure of Ring1B-Bmi1 and employing NOE-based distances yielded high quality structure of a complex.

To understand how Bmi1 participates in protein-protein interactions within PRC1 we have determined the complex with polyhomeotic protein. We have obtained well diffracting crystals; however, the structure could not be determined due to unusual crystal packing. To determine the high resolution structure of a complex, we have again employed joint refinement using the crystal structure and NMR data.

In summary, we will describe how joint refinement using NMR and X-ray crystallography data can be used to provide high resolution structural information for protein-protein and protein-inhibitor complexes.

L13.5

Overview of macromolecular crystallography

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After many years of dynamic development macromolecular crystallography has become a mature technique in the sense that it is available to a wide spectrum of structural biologists rather than a select few specialists. A change of paradigm from a project-driven to a technology-driven research, supported by massive investments in the developed countries, resulted in high-throughput (semi)-automated facilities for protein production, purification and crystallization. Numerous dedicated synchrotron beamlines, distributed worldwide, have revolutionised crystallographic data collection. Some of the beamlines are also set up for high-throughput, remote-controlled, semi-automated crystal screening and data collection. Advanced software allows many structures to be solved in a (semi)-automatic manner. All this has resulted in a rapid increase in depositions in the Protein Data Bank (PDB). Many of the recent structures have not been characterised biologically. It seems as if the rate of production of structural results has overtaken the analytic capacity of the scientific community. Perhaps this is somewhat embarrassing but it is also an opportunity for scientists prepared to do high-level data mining. The PDB itself is also being continually improved, equipped with tools for structure validation, visualisation, searching of the data base and cross-linked with other biological data bases. The high-throughput approach has been compared to 'picking of low-hanging fruits' because the difficult proteins are dropped from the production lines. However, the approach has increased tremendously our knowledge-base of protein structure. Moreover, techniques which have been developed are useful also for scientists following the traditional route: focusing on a chosen project.
Macromolecular crystallography will continue as the major technique of structural biology. It remains unchallenged as a source of high-resolution structures. The high-throughput facilities will probably continue (subject to funding) to deliver large numbers to PDB entries, but besides that there will always be challenging projects that require individual approach and high-level human skills.

L13.6

BioSAXS – a tool for structural studies of complex or disordered biomacromolecules

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Small angle X-ray scattering (SAXS) in recent years has won a status of attractive tool used in structural studies of various biological systems. Growing popularity of this method stems from the availability of synchrotron radiation sources, advances in detector technology (fast hybrid photon counting detectors) and the automation of BioSAXS beamlines. As a result of these technological improvements now remote-controlled experiments are also available [1]. Simultaneously with technological development, many computer packages dedicated to the analysis of SAXS data have been developed, including programs for modelling of low resolution structure or analysis of disordered systems [2-4].
Currently, important applications of BioSAXS technique include structural studies of systems of high conformational disorder and disordered systems. Illustrative examples of these applications are our results of SAXS studies of selected partially structurized proteins (human PrPc protein) and intrinsically disordered proteins. SAXS technique is also applied in the studies of protein complexes or oligomeric structures characterized by low stability. Examples of such structures are, e.g. the complexes of Hsp90, Rar-1 or Sgt-1 proteins [5], which are components of plant defense systems. SAXS studies allow also observation of fast (on a millisecond time-scale) conformational changes of proteins. An example of successful application of SAXS to observe such dynamic processes in solution are the presented conformational changes and oligomerization process of human cystatin C.

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L13.7

MS in structural studies of difficult protein targets

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The application of mass spectrometry for protein studies extends far beyond classic proteomic analysis in which proteins and their posttranslational modifications are identified and/or quantified. Recent developments of new approaches, like ion mobility separation – IM or technical improvements in the methods already known (like measurements of the hydrogen-deuterium exchange kinetics - HDex) or a combination of both (solution/gas phase HDex combined with IM and/or ETD) enable efficient application of MS for protein structure studies. These methods allow structural insight into a large group of targets difficult in structure analysis. It is estimated that a stunning 25–30% of eukaryotic proteins are mostly disordered, while more than half (!!) of eukaryotic proteins and more than 70% (!!) of signaling proteins have long regions of disorder – IDRs [1]. Due to a variety of reasons access by classic methods (crystallography or NMR) to this vast part of structural proteome, including IDRs and other difficult protein targets, like strongly oligomerising systems, is severely limited. A major breakthrough in the methodologies available to study the structures of this class of proteins is thus a must. In this respect recent developments of MS-based approaches to protein structure analysis are the most promising path. Application of such methods in characterization of several protein systems will be presented. Among them are oligomerising peptides, including Aβ peptide, main neurotoxic agent in Alzheimer' s disease, responsible for synaptic dysfunction and neuronal injury, kinetochore complex, intermediate filaments, histone pre-mRNA cleavage complex, etc.

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L13.8

Computational modeling of macromolecular complexes

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Macromolecular complexes play fundamental roles in many biological processes, such as the regulation of gene expression, RNA splicing and protein synthesis. Structures of some of these complexes have been experimentally determined, providing insight into mechanisms of their biological activities. However, for a great majority of large biological machines, high-resolution structures are only available for some isolated components, often accompanied with low-resolution information about the overall shape (e.g. from cryo-EM or SAXS) or about the proximities and interactions of these components (e.g. from chemical cross-linking experiments). Given the scarcity of experimentally determined structures, computational techniques can be used to integrate heterogeneous pieces of information, guide structure elucidation and subsequently determine the mechanisms of action and interactions between the components.

I will present methods for computational modeling of protein-protein and protein-nucleic acid complexes developed in our laboratory, in particular PyRy3D, a program that predicts structures based on low-resolution experimental data and enables the construction of models even for very large complexes with components of unknown or disordered 3D structure. Our software is available at http://genesilico.pl.

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L13.9

Structural databases and data mining

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X-ray crystallography is one of the most detailed "microscopes" available today for examining macromolecular structures. However, structures are only simplified models of target proteins and/or nucleic acids, and should be seen as a framework for generating hypotheses to be explored. Numerous biochemical and biophysical experiments, including new diffraction experiments, can and should be performed to verify or falsify these hypotheses. Processing of structural information, particularly when combined with functional, experimental, and sequential data in the context of pathways or interaction networks with other bio-macromolecules and/or bioactive chemical compounds, increasingly requires the use of Big Data paradigms for effective data management, as well as for checking data integrity and accuracy. This is easy to say but extremely difficult to implement. A combination of advancements in high-quality data harvesting, validation, mining, and data management tools would make it possible to convert high-throughput pipelines into high-output pipelines in target-based drug discovery and academic biomedical research.

Posters

P13.1

Structural studies of *Helix aspersa* agglutinin – a diagnostic tool

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Helix aspersa agglutinin (HAA) is a protein found in the albumen gland of the garden snail. Agglutinins, also known as lectins, belong to a differentiated group of proteins exhibiting sugar-binding properties. Due to this fact, they are interesting research targets for medical diagnostics. HAA is a lectin that recognizes the epitopes containing α-d-N-acetylgalactosamine (α-d-GalNAc). Generally, agglutinins cause coagulation of cells possessing specific glycosylation, which can be used to diagnose and determine progress of some serious diseases and therefore glycoproteins are good biomarker targets, *e.g.* in diagnostics of different type of cancer, kidney disease, immunoglobulin A (IgA) nephropathy or type-2 diabetes. The changes of IgA and IgD glycosylation patterns were also identified by HAA binding in patients with hyperimmunoglobulinaemia D and periodic fever syndrome.

Although several reports have already described the use of HAA as a diagnostic tool, this protein was not characterized on the molecular level. Here, we present the structural information about lectin, isolated from mucus of *Helix aspersa* (garden snail), and its isoforms varying by few amino acids residues confirmed by crystal structures. The amino acid sequence of the protein was established based on high resolution (1.38 Å) electron density maps and verified by Edman degradation. The biological assembly of HAA is a hexamer formed by three dimers, with monomers of each dimer connected by a single disulfide bridge. The crystal structure and MALDI-TOF mass spectrometry analysis provide the detailed information about a large part of the HAA natural glycan chain. The topology of the α-d-Gal*p*NAc binding cleft and interaction with lectin are very well defined in the structure and fully confirmed by STD HSQC NMR spectroscopy. Results from all of these experiments provide structural clues regarding HAA specificity and opens possibilities to rational modifications of this important diagnostic tool.

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P13.2

Circular Dichroism analysis of the intrinsically disordered C-terminal fragment of Methoprene tolerant protein (Met)

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Drosophila melanogaster Methoprene tolerant protein (Met) has been recently identified as the long-sought juvenile hormone (JH) receptor. Met plays a significant role in control of insect development and maturation and participates in the multiprotein complex that is involved in the cross-talk of the 20-hydroxyecdysone (20E) and JH signaling pathways. Bioinformatics analysis indicated that Met belongs to the bHLH-PAS transcription factors family and besides defined domains has disordered fragments, mainly in the C-terminal region (MetC). The bHLH-PAS family C-terminal regions are usually responsible for the regulation of protein composite activity.

In this study, we show the results of the Circular Dichroism (CD) spectroscopy analysis of MetC. The measurements were examined in standard buffer (20 mM Tris-HCl and 150 mM NaCl) and after a 1-h incubation in buffer supplemented with guanidine hydrochloride (GdmCl) or 2,2,2-trifluoroethanol (TFE). The level of thermal denaturation was analyzed as the temperature increased from 20°C to 90°C.

The CD spectrum of MetC exhibits properties typical for intrinsically disordered protein (IDP): it shows a minimum near 200 nm. A small negative peak is present near 222 nm, suggesting the existence of some residual ordered structure. The CDPro spectra deconvolution software revealed that MetC is mainly unordered (64.0 ± 5.3%). The addition of a denaturing agent resulted in a less negative ellipticity value at approximately 222 nm, indicating the loss of residual structure. In the presence of TFE, negative peaks near 222 nm and 206 nm appear, suggesting that the amount of secondary structure increased and MetC acquired α-helical structures. The increase of temperature caused decrease in the ellipticity value at 222 nm, indicating the formation of a secondary structure. However, the change was not enough obvious to assume that MetC exhibits propensity for folding.

Altogether, the above results demonstrate the disordered character of the MetC with residual secondary structure that disappears under denaturing conditions. Short ordering can be explained by MetC interactions with orphan nuclear receptor FTZ-F1. We suggest that the multiplicity of conformations adopted by the MetC is crucial for its activity as a biological switch modulating the cross-talk of different signaling pathways in insects.

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P13.3

Chemical knockout of the anti-papain activity in the chicken egg white cystatin

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Mammalian legumain (EC 3.4.22.34) is a cysteine endopeptidase predominantly active in lysosomes. Legumain is overexpressed in the majority of human solid tumours and its overexpression is associated with enhanced tissue invasion and metastasis. While the correlation of legumain with the tumorigenesis is well established, there have been efforts to inhibit legumain itself as a causal target in tumour progression. Cystatins are naturally occurring potent inhibitors of papain and papain-like enzymes but selected members of the family 2 cystatins have been shown to possess a reactive centre loop different to the papain inhibitory site. This is an independent active centre for legumain-type proteases in the molecule. The modification of cystatin resulting in selective elimination of anti-papain activity could provide a useful molecular tool for studying the physiological and pathological processes specifically associated with legumain activity. The inhibition of the papain-like cysteine peptidase by the cystatin is due to the tripartite enzyme-binding domain, with C-terminal segment containing a Pro-Trp pair. The hydrophobicity of this tryptophan was thought to be critical for the papain-like substrate binding and supporting catalytic action.Here we report a chemical modification of chicken cystatin that impairs the anti-papain activity of the inhibitor but does not affect its anti-legumain activity. We chose ovocystatin because it is a model protein for investigation on cystatins and its biological activity was confirmed in many studies. The chemical knockout was obtained by reaction with 2-Hydroxy-5-nitrobenzyl bromide (HNB, Koshland reagent) that covalently modifies the Trp104 residue in the molecule. The reaction was monitored by UV-VIS and fluorescence spectroscopy. The anti-papain activity of the inhibitor was measured colorimetrically against BANA as a substrate. The anti-legumain activity was assessed fluorometrically using Z-Ala-Ala-Asn-AMC. The modified inhibitor revealed an additional band at 410 nm in UV-VIS scan, characteristic for HNB. Accordingly, a quenching of Trp fluorescence was also observed. The molecule fully retained the anti-legumain activity, while only residual anti-papain activity (10%) was detected. The data confirmed the selective knockout of the anti-papain activity of chicken cystatin by the modification of Trp104 in the molecule.

P13.4

RNA ligand recognition by TPR proteins

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The tetratricopeptide repeat (TPR) motif typically comprises 34 amino acids and adopts a helix-turn-helix fold. It is found in a wide variety of functionally unrelated proteins present in prokaryotic and eukaryotic organisms. TPR motifs are usually present in multiple copies as tandem arrays that generate solenoid-type scaffolds well-suited for mediating protein-protein interactions. Typically, TPR domains bind specific peptide ligands and undergo little or no structural rearrangement upon ligand binding. Recently it was shown that IFIT (interferon-induced proteins with tetratricopeptide repeats) proteins directly bind RNA. Crystal structure of human IFIT5 in complex with PPP-RNA reveals a novel arrangement of TPR domains that binds PPP-RNA in a 5' end-dependent, but non-sequence specific manner (Abbas *et al*., 2013). It represents the first example of the TPR protein bound to nucleic acid ligand. In our studies of IFIT proteins we aim to investigate how TPR proteins adapted to bind RNA, and the role of homo- and heterooligomerization. We will use other vertebrate IFIT homologs to get insights into the molecular basis of a TPR-nucleic acid recognition. We plan to compare the RNA-binding interfaces of TPR proteins and other helical repeat proteins known to bind RNA.

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P13.5

Biochemical markers of drug induced liver injury (DILI) in HIV-infected patient treatment by HAART

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To date, hepatotoxicity has been associated with all currently used antiretroviral drug (ARV) regimens. Although the mechanisms of drug-induced liver injury are poorly defined, it is likely that many factors contribute to the hepatic effects of a specific drug.

The study included 180 HIV-infected patients among the observed 39.3% of patients not receiving HAART, 60.7% – were on ART, according to clinical treatment protocols. In 14.4% of patients had a change of ART regimen due to ineffectiveness or intolerance. Co-infection of HCV/HBV virus was diagnosed in 7% of patients, about which antiviral causal treatment was not carried out. The observed decrease in our study mitochondrial antibody (AMA M2) with 1.12±0,136 to 0.74±0.044 IU/ml in the serum of HIV-infected patients receiving antiretroviral therapy, compared with HIV-infected, not receiving ART, indicating non-cholestatic effect HAART, causing drug induced liver injury (DILI).

To analyze the importance of the study parameters held by ROC-analysis, which showed a high predictive value in the diagnosis of DILI. The greatest diagnostic value is the ratio of GGT/AMA-M2:

With the separation by cut-off point of the curve, evaluated the sensitivity and specificity of this parameter is found using the split point. Revealed the prognostic value of the ratio GGT/AMA-M2 to predict BOB arising during therapy HIV by ARV. So at the cut-off point of ≥28.99 DILI can be predicted (sensitivity 0.81, specificity of 0.67; p=0.025).

P13.6

New β-lactoglobulin variants with increased affinity to tricyclic antidepressants

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Tricyclic antidepressants (TCA) are a class of drugs used to treat depression and other mood disorders. An overdose of TCA is one of the most common drug poisoning accidents [1]. TCA are tightly bound to plasma proteins and there is no specific antidote for them, however, extracorporeal life support therapy with the use of albumin dialysis recently has become an object of intensive studies [2]. While serum albumin has low binding selectivity, a new class of proteins can be designed to replace albumin and significantly improve treatment efficiency.

β-Lactoglobulin (BlgB) is a protein belonging to lipocalin family. As other lipocalins [3], BlgB can be modified to gain affinity to low-molecular targets. New BlgB variants, with potential application in extracorporeal removal of protein-bound toxins been designed and produced. Several stable lactoglobulin mutants possessing multiple substitutions in the binding pocket and having an affinity to TCA have been obtained.

Mutations have been introduced to BlgB gene by PCR *QuikChange* protocol. Proteins were expressed in *Origami B(DE3)* and purified by ion-exchange and size-exclusion chromatography. BlgB and their complexes with TCA have been crystallized by hanging drop technique. X-ray diffraction data (1.54 Å, 120 K) were collected on *SuperNova* and *Rigaku* diffractometer. Structures were solved by molecular replacement. Structural stability was monitored by CD while binding constant for BlgB-TCA interactions were determined by ITC.

Determined crystal structures (1.9-2.3 Å) revealed that individual BlgB mutants with a different combination of substitutions in positions: 39, 56, 58, 92, 105 and 107 are able to accommodate in β-barrel such TCAs as e.g. amitriptyline, chlorpromazine and desipramine. Depending on the BlgB variant, TCA structure and its orientation in the binding pocket the aliphatic chain of the drug is directed toward CD or EF loop. The three-ring system of each TCA is located inside modified binding pocket which has been shaped to gain unique geometry that is complementary to TCA structure and dimensions. TCA interact with modified proteins mainly by π-π stacking and hydrophobic interactions.

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P13.7

Hydrophobic interactions – the main driving force of folding nucleation site formation in the engrailed homeodomain protein (EnHD)

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The engrailed homeodomain (EnHD) is a small three-helix bundle DNA binding protein with a sequence length of about 60 amino acids. Because of its small size and simple native state topology EnHD is very often used in the study of protein folding mechanism. It was shown that the EnHD protein's folding mechanism is complicated and at least one stable folding intermediate was well experimentally characterized (Religa *et al.*, 2007). Religa and coworkers (Religa *et al.*, 2007) have shown that HTH (helix II and helix III) motif from EnHD is monomeric and folded in solution, preserving essentially the same structure as in full-length protein and that the N-terminal part of the protein (helix I) is entirely unfolded. Moreover, it was shown that the truncated version of the EnHD protein composed of helix II and III that form HTH motif, is fully active and binds with a high specificity to DNA sequence (Graunt *et al.*, 1993).

In this study we chemically synthesized a series of peptides corresponding to secondary structure elements (appropriate helices) of the EnHD protein. Peptides' structures and their conformational dynamics were investigated by using NMR spectroscopy in water solution. The goal of our research was to search for possible nucleation sites on the folding pathway of the EnHD protein and determine the main driving forces responsible for folding and native structure stabilization. Results of our research show that protein region corresponding to the C-terminal alpha-helix (helix III) is the best candidate to be a nucleation site of the EnHD protein. Peptides which sequences are based on helix III sequence do form stable three-dimensional structures in solution, however they do not resemble alpha-helices. Three-dimensional structures are stabilized mostly by hydrophobic interactions.

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CRASS – software for identification of conformational rearrangements in alternative RNA secondary structures

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Most of RNA molecules can fold into multiple secondary structures. This ability is widely used by regulatory RNAs, e.g. riboswitches, where acquisition of alternative structures play crucial role in regulatory mechanism. Computational approaches for RNA secondary structure prediction usually results with set of suboptimal structures. Recently, high-throughput methods of RNA chemical probing have been developed, which allow for transcriptome – wide estimation of RNA secondary structures in different conditions (ex. *in vivo, in vitro*) (Spitale *et al*., 2015; Talkish *et al*., 2014). The ability to identify the condition-dependent structural rearrangements of the RNA molecules could lead to identification of novel RNA regulatory mechanisms. Here, we present the software for identification of conformational changes between alternative secondary structures of RNA (CRASS). The software employs the abstract shapes notation of RNA structure (Giegerich *et al*., 2004), which enables identification of domain-wise structural rearrangements. The result is comprehensive list of domains and motifs within the RNA structures which reveal structure switching potential. The provided output files enable straightforward visualization of structural changes within the RNAs of interest. CRASS is applicable to any set of alternative RNA foldings, derived either from probing experiments, crystal structures or computational predictions.

References:

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