Friday, March 20, Session V

L17

STRUCTURAL DYNAMICS OF WrbA PROTEIN PROBED BY MASS SPECTROMETRY

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Tryptophan repressor-binding protein A (WrbA), more correctly called FMN-dependent NAD(P)H:quinone oxidoreductase, is a protein highly interesting for its involvement in oxidative stress defense responses in many organisms. Its biologically functional assembly is a homotetramer held together by non-covalent interactions, whose 1.2 Å high-resolution structure has recently been solved by X-ray crystallography. [1] The present work was aimed at studying the structure-function relationship of WrbA, with main focus on uncovering the dynamics of its behaviour in solution. Specifically, this meant understanding the effects of factors determining its conformational and oligomeric states, where the protein is known to be present in the solution in dynamic equilibrium between monomeric, dimeric and tetrameric species.

Studied protein was produced by overexpression in its natural producer organism *E. coli* and purified to homogeneity by size exclusion and affinity chromatography steps, before being subjected to studies by analytical ultracentrifugation and advanced structural mass spectrometry techniques. Native mass spectrometry coupled with ion mobility enabled us to gently transfer the whole non-covalent WrbA assembly into the gas phase while preserving its structure and showed a dramatic stabilizing effect of FMN cofactor on the protein in its tetrameric form. It also uncovered a positive interdomain cooperative effect which seems to be involved in the binding of FMN molecules to individual WrbA subunits.

Behaviour of WrbA in solution was also probed by hydrogen / deuterium exchange and chemical cross-linking in combination with mass spectrometry. These techniques enabled a more detailed description and structural localization of conformational changes in the WrbA macromo lecule depending on the presence of cofactor and on the varying solution conditions. We specifically studied the effects of solution temperature and the protein concentration, which according to the analytical ultracentrifugation determine the oligomeric state of WrbA by influencing its dimer-tetramer dynamic equilibrium.

In combination with computational modelling and crystallographic data, the combination of complementary structural mass spectrometry techniques provided a novel insight into the behaviour of the WrbA protein in solution and offered an explanation for the mechanism of its oligomerization.

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L18

HYDROGEN/DEUTERIUM EXCHANGE: MOVING FROM EASY PROTEINS TO REAL LIFE STORIES

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H/D exchange coupled to mass spectrometry is already a well-established technique of structural biology. It allows monitoring of protein interactions and structure changes via time resolved kinetics of amide hydrogen exchange. It is often presented as a technique with virtually no limitations in terms of protein size, complexity, or buffer requirements. In this presentation we will critically review these statements by demonstrating methodological developments in our laboratory and their application to several non-trivial protein problems including monitoring of a large number of unusual experimental conditions, big modified proteins, membrane proteins and complex protein mixtures.

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L19

DESCRIPTION OF ALGORITHM FOR ANALYSIS AND 3D RECONSTRUCTION OF LIVING CELL INNER STRUCTURES FROM HIGH-RESOLUTION BRIGHT-FIELD MICROSCOPY IMAGES

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Bright-field microscopy images provide us the best information about the cell and cell interior without any labelling or other changes of the sample.

The first step in our analysis is the calculation of PDG (Point Divergence Gain) [1]. The basic idea of this analysis is that homogeneous objects give the PDG values (information change between two consecutive images in Z-scan) equal zero. Therefore, the second step is selection of all these points. This images are applied as a mask on the original images. Now we are able to do the first 3D reconstructions. The zero value of the PDG does not mean that these objects will have the same intensities in the whole volume.

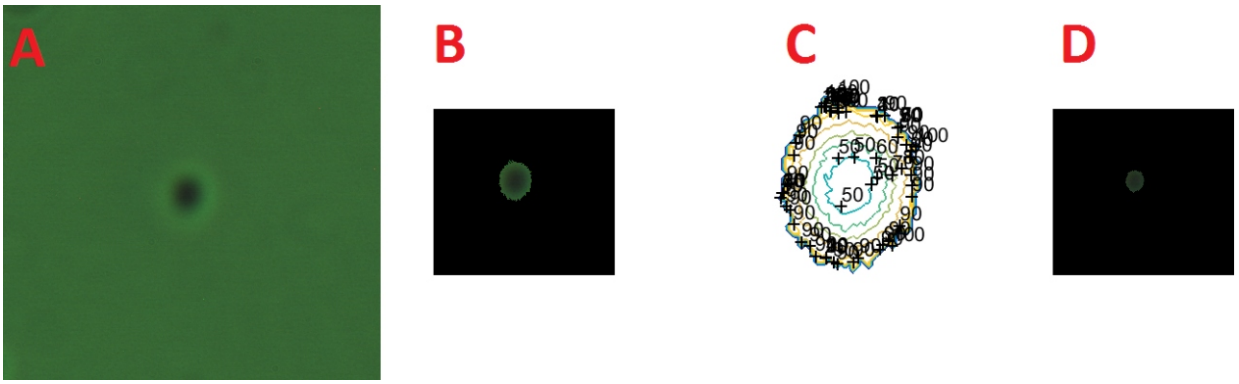

Figure 1. Demonstration of the algorithm on the image of 220nm latex particle. A - Original image, B - Part of the image with zero values of PDG, C – Contour image with different intensities, D – Darkest part of the image.

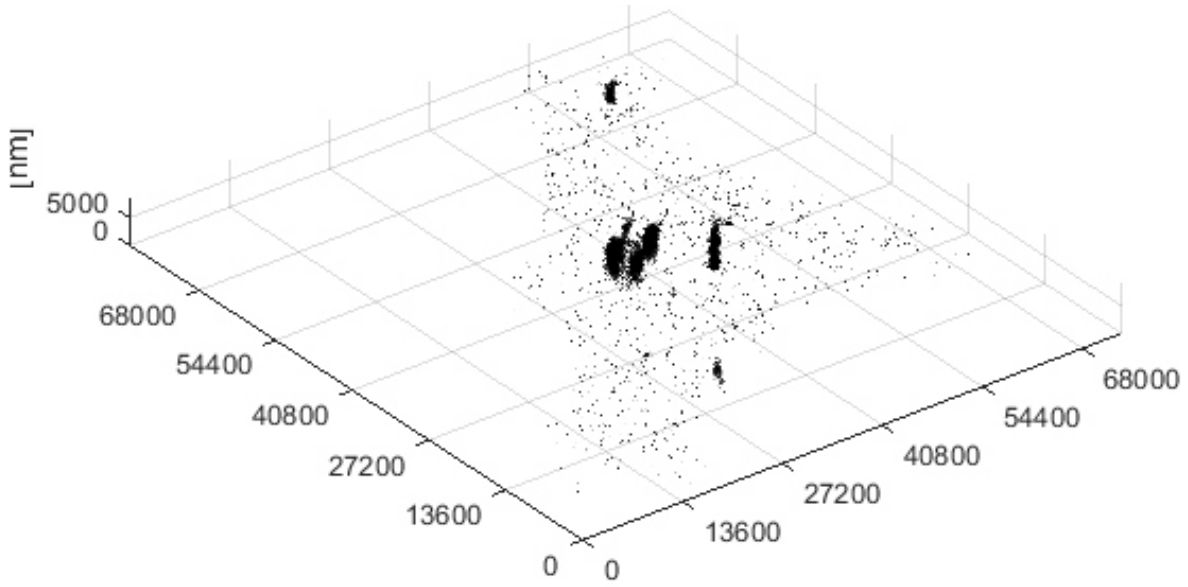

Figure 2. 3D reconstruction of the cell. PDG values are equal to zero – 3D view.

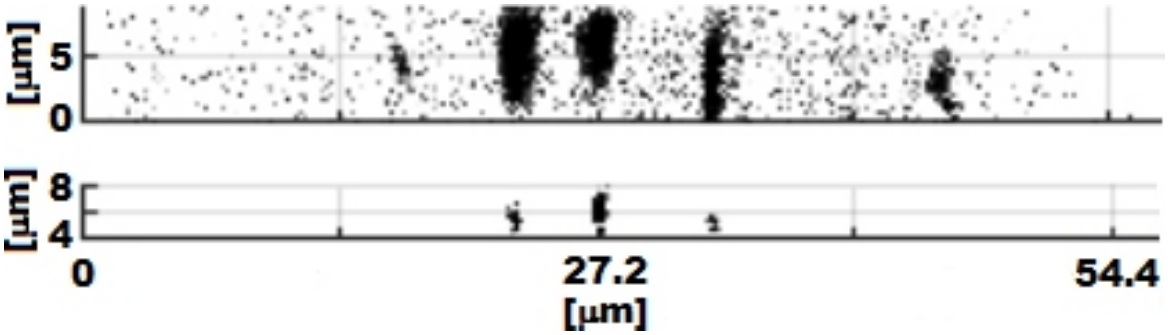

Figure 3. 3D reconstruction of the cell. PDG values equal to zero (top) and their darkest parts (bottom) – side view.

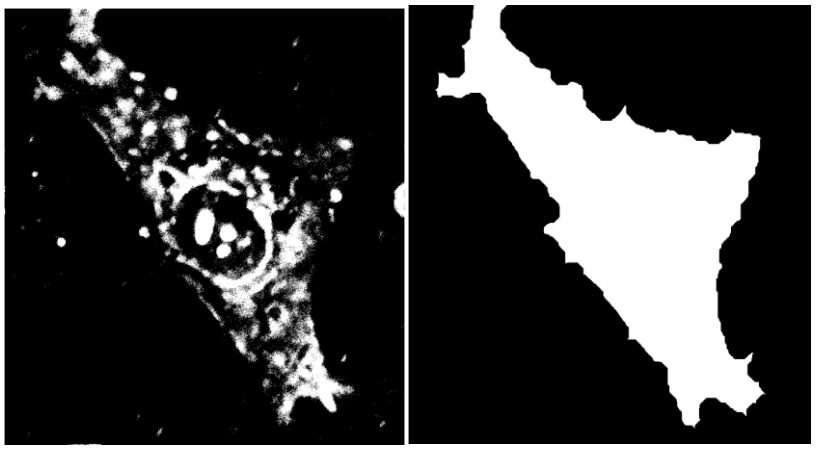

Figure 4. Left image – Sum of images with lower intensities, Right image – final mask of the cell.

In the bright-field microscopy (diffraction images) the focused objects are the darkest ones. To obtain the image of the real object, the next logical step is to find layers with the same intensity. This is performed by finding contours in z-stack images (Fig. 1).

This PDG analysis is used for estimation of the cell size and will be followed by analysis of differential images. Differential images gives us more precise and more localized information about individual organelles and primitive diffracting objects (Figs. 2, 3).

Differential images can be also used for detection of the cell (Fig. 4).

R. Rychtáriková, T. Náhlík, R. Smaha, J. Urban, D. Štys Jr., P. Císař, D. Štys, in *ISCS 2014:Interdisciplinary Symposium on Complex Systems (Emergence, Complexity and Computation 14)*, edited by A. Sanayei, O.E. Rössler, I. Zelinka (Switzerland: Springer), 2014, pp. 261-267.

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L20

SPECTROSCOPIC STUDIES OF PHOSPHOENOLPYRUVATE CARBOXYKINASE FROM *MYCOBACTERIUM TUBERCULOSIS*

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Tuberculosis, the second leading infectious disease killer after HIV, remains a top public health priority. The causative agent of tuberculosis, *Mycobacterium tuberculosis* (Mtb), which can cause both acute and clinically latent infections, reprograms metabolism in response to the host niche. Phosphoenolpyruvate carboxykinase (Pck) is the enzyme at the center of the phosphoenolpyruvate-pyruvate-oxaloacetate node, which is involved in regulating the carbon flow distribution to catabolism, anabolism, or respiration in different states of Mtb infection. Under standard growth conditions, Mtb Pck is associated with gluconeogenesis and catalyzes the metal-dependent formation of phosphoenolpyruvate. MTb Pck contains nine cysteine residues that might co-determine the redox state and conformation of Pck under different conditions. Structural alignment showed that Cys-273, located within the putative P loop of MTb Pck, is probably the hyperreactive cysteine residue, which is typical for the GTP-dependent Pck family and coordinates binding of Mn^{2+} in the active site [1]. The minor structural changes of Pck in the absence or in the presence of reducing agents were reported and the presence of one disulfide bridge was suggested [2].

We present the spectroscopic study of the Pck and its mutants C119S and C198S to characterize this disulfide bridge. The mutation sites were chosen with respect to detail crystal structure analysis of Pck and represent the cysteines, which might form disulfide bond. Circular dichroism and Raman spectroscopy supported by mass spectroscopy was used for this purpose.

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L21

MEASUREMENT IN BIOLOGICAL SYSTEMS

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The system theory of Zampa [1] gives the framework for the analysis of information provided by the measurement. It explains the crucial importance of the system model for understanding of measurement in dynamic systems. Crucial term in this respect is the complete immediate cause $C_{k,l}$ of the consequence $D_{k,l}$

$$C_{k,l} \quad \bigcup_{k,l\ i,j} D_{i,j}$$

where k and i demarcate time instants at which the measurement is performed and l and j are time instants which make provision for the causality between measurements.

In many technical systems, i.e. electrical or mechanical, we have rather good models which enable us both to determine the extent of time needed for determination of the $C_{k,l}$ and to analyze the causality within intervals between measurements. Non-linear dynamical system may also reach recurrent behaviour which may be in ergodic state [2,3] or, in other words, be Lyapunov stable [4].

In the physico-chemical equilibrium systems we assume no system memory, the state of the system does not depend on the path by which it was achieved, i.e.

$$C_{k,l} \quad D_{k,l}$$

Biological systems are not in chemical equilibrium and we also do not have good models for their time evolution. They are dynamical self-organized systems, structured outside equilibrium, and for their time evolution we may refer to qualitative simplified models of time evolution of cellular automata [5]. We consider travel through the zone of attraction along which a few well defined, common and structured states are visited and observed. Biological systems such as living cells are re-started before achievement of the recurrent / ergodic state, higher organisms evolve more freely and are “alive” only through their offsprings. Consequences of these findings for measurement in self-organised systems and adequate models will be shown and solutions for adequate reporting of biological systems

will be shown [6]. Our findings also explain sources of inconsistences and irreproducibilities in contemporary biology [7,8].

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Friday, March 20, Session VI

L22

QUANTITATIVE ANALYSIS OF BIOMOLECULAR INTERACTIONS WITH MICROSCALE THERMOPHORESIS (MST)

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Microscale Thermophoresis (MST) allows for quantitative analysis of protein interactions in free solutions and with low sample consumption. The technique is based on thermophoresis, the directed motion of molecules in temperature gradients. Thermophoresis is highly sensitive to all types of binding-induced changes of molecular properties, be it in size, charge, hydration shell or conformation. In an all optical approach, thermophoresis is induced using an infrared laser for local heating, and molecule mobility in the temperature gradient is analyzed via fluorescence. In addition to fluorescence by labels or fusion proteins attached to one of the binding partners, intrinsic protein fluorescence can be utilized for MST thus allowing for label-free MST analysis. Its flexibility in assay design qualifies MST for biomolecular interactionanalysis in complex experimental settings, which we herein demonstrate by addressing typically challenging types of binding events from various fields of life science. The interaction of small molecules and peptides with proteins is, despite the high molecular weight ratio, readily accessible via MST. Furthermore, MST assays are highly adaptable to fit to the diverse requirements of different biomolecules, e.g. membrane proteins to be stabilized in solution. The type of buffer and additives can be chosen freely. Measuring is even possible in complex bioliquids like celllysate and thus under close to in vivo conditions and without sample purification. Binding modes that are quantifiable via MST include dimerization, cooperativity and competition.