276th CSCA seminar (Rozhovory)

Protein Crystallography: Novelties from our laboratories II

Bratislava, January 22, 2008, Institute of Molecular Biology SAS

Programme

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(*Institute of Molecular Biology, SAS, Bratislava*):
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(*Institute of Experimental Oncology, SAS, Bratislava*):
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Nikola Kostlánová
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Patrik Florek
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Tanya Prudnikova
(*Institute of Physical Biology, South-Czech University, České Budějovice*):
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(*Bruker AXS*):
Fast track to a 3D protein Structure using Automated Crystal Imaging, Screening and Data Collection

Organization: Ľubica Urbániková, Institute of Molecular Biology, SAS, Bratislava

RIBONUCLEASE SA2 - MECHANISM OF THE CATALYSIS AND RECOGNITION OF RNA

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Ribonucleases Sa2 and Sa are extracellular guanylspecific ribonucleases isolated from *Streptomyces aureofaciens*, strains BMK and R8/26, respectively. The identity of the primary structures of RNase Sa2 and Sa is 53%. Tertiary structures of these proteins are very similar. Both enzymes specifically hydrolyse the phosphodiester bond of RNA at the 3′-site of guanosine [1]. The amino-acids Glu56 and His86 (RNase Sa2 numbering), which in catalytic reaction act as general acid and general base are conserved in both enzymes. In spite of that, the enzymatic and kinetic parameters of the two enzymes significantly differ [2], for example, the catalytic constant k_{cat} of RNase Sa2 is seven times lower than that of RNase Sa [3]. To better understand the mechanism of RNA cleavage and differences in the catalytic properties of the two riboucleases we have solved structures of RNase Sa2 with free active site and in complex with the catalytic cleavage product (3′-GMP), an analogue of the reaction intermediate (*exo*-2′,3′-GCPT), and an enzyme inhibitor (2′-GMP). Moreover, to better understand the enzyme-substrate recognition we preformed extended molecular docking calculations of mono-, di- and tri- nucleotides into the active site of RNase Sa2.

Structures of complexes were solved with the program *MOLREP* [4] and refined with the program *REFMAC5* [5] against 1.8 and ~ 2.0 Å resolution data. The final crystallographic R-factors were in the range 20-23 % and R_{free} ~ 26 %. Mononucleotides, 3'-GMP, 2'-GMP and 2',3'-GCPT, are bound to the active site of RNase Sa2 in a similar manner as in the complexes with other bacterial ribonucleases, RNase Sa [6, 7], binase [8] and barnase [9]. The guanine base of all three mononucleotides lies essentially in the same position and forms five hydrogen bonds, three with amide groups of Glu40, Asn41, Arg42 and two with carboxy-group of Glu43. It is stabilized by interaction with aromatic rings of Phe39, Tyr87 and Phe90, which form the bottom of the active site. An important role in stabilizing the base has Arg42, the planar ä-guanido group of which lies flat above the guanine base forming so called „closed" conformation of the active site. The orientation of the ribose with respect to the guanine base of mononucleotides differs. In RNase Sa2/3'-GMP complexes the ribose with respect to the guanine is in *anti*-conformation and the ribose adopts C2′-*endo* pucker. In complexes of RNase2/2',3'-GCPT and RNase2/2'-GMP the ribose is in *syn*-conformation and the ribose adopts 3'-*endo* pucker. The phosphate group of 3'-GMP forms hydrogen bonds with Arg67, Arg71, His86 and Tyr87. The phosphate group of 2',3'-GCPT forms hydrogen bonds with Arg67, Arg71, His86, Tyr87, Glu56 and Arg34. The main difference in the phosphate binding site is in the conformation of Arg34 side-chain, which, most likely, depends on the conformation of the nucleotide. When the mononucleotide is in *anti*-conformation, the side-chain of Arg34 is turned outside the active site and does not make any contact with the mononucleotide. When the mononucleotide is in *syn*-conformation the side-chain of Arg34 is turned towards the nucleotide and makes hydrogen bonds with the ribose and phosphate group.

Comparison of active sites of RNases Sa2 and Sa shows that the main differences are in substitution of Arg34 and Arg45 in RNase Sa2 by Gln32 and Val43 in Sa. None of these residues is directly involved in catalytic reaction, however, it is expected that these residues play an important role in the RNA cleavage as shown by site directed mutagenesis in ribonuclease Bi (*Bacillus intermedius*) [10] and barnase (*Bacillus subtillis*) [11]. Molecular docking of different di- and tri-nucleotides suggests the presence of subsites for second and the third nucleotide.

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WrbA BRIDGES BACTERIAL FLAVODOXINS AND EUKARYOTIC NAD(P)H:QUINONE OXIDOREDUCTASES

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The solved crystal structure of the flavodoxin-like protein WrbA with oxidized FMN bound reveals a close relationship to mammalian NAD(P)H:quinone oxidoreductase, Nqo1. Structural comparison of WrbA, flavodoxin, and Nqo1 indicates how the twisted open-sheet fold of flavodoxins is elaborated to form tetramer that extend catalytic function from one-electron transfer between protein partners using FMN to two-electron reduction of xenobiotics using FAD. The structure suggests a possible novel physiological role for WrbA and Nqo1. The 21 kDa protein WrbA from *E. coli* is the founding member of a family of flavodoxin-like proteins conserved from bacteria to higher plants that are implicated in cellular responses to altered redox conditions and other kinds of stress *via* their two-electron NAD(P)H: quinone oxidoreductase (Nqo) activity). The crystal structure of oxidized tetrameric *E. coli* WrbA rationalizes functional distinctions with dimeric Nqos and monomeric flavodoxins, and suggests a novel function for WrbAs and Nqos.

WrbA monomer (Figure 1A) adopt the canonical sandwich fold of the flavodoxins with FMN bound at the C-terminal end of the parallel 5-stranded sheet but with characteristic flanking insertions and the ability to form tetramers (Figure 1B) without a dedicated multimerization domain Helix ' is part of a conserved sequence interrupting 5 that is typical of long-chain but not short-chain flavodoxins (Figure1C). Helix 6 is part of a highly variable subdomain inserted after 2 in Nqos (Figure 1D) and some flavodoxins.

Overlay of 1QRD and WrbA monomers (Figure 1D) indicates that cofactor selectivity is influenced by residues in a short insertion after 5b that is characteristically conserved in WrbAs. The adenine riboside portion of FAD is substituted by an irregular segment that lies in a groove on the WrbA surface along the N-terminus of 1 at the same depth as the cofactor, with the sequence $_{171}$RQP$_{173}$ occupying the position of the adenine and ribose rings. The WrbA binds FMN in the same relative location as that of flavodoxins (Figure 1C), but the disposition and redox properties of FMN are entirely different. Two protruding loops enclose the flavodoxin isoalloxazine in a narrow crevice lined by aromatic residues, leaving the dimethylbenzene ring edge exposed as an electron conduit.

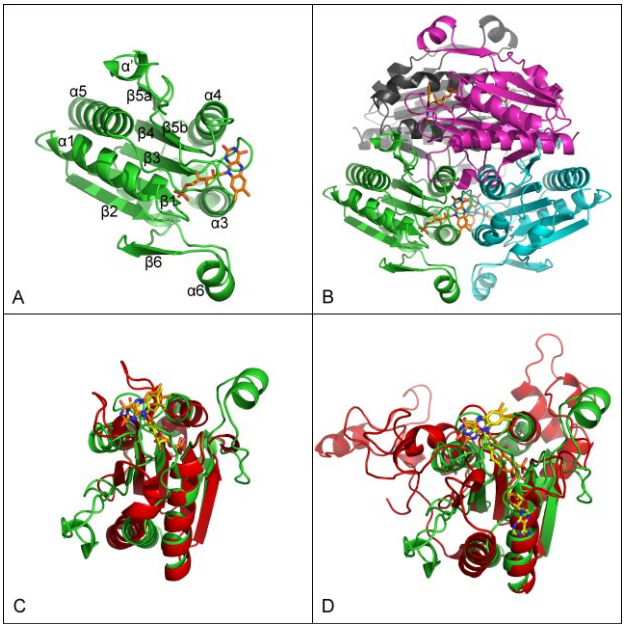

Figure 1.

STRUCTURAL STUDIES OF SACCHARIDES BINDING PROTEINS

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Lectins are a class of proteins of non-immune and non-enzymatic origin that bind carbohydrates specifically and reversibly. They express numerous biological activities, which are based on their acting as recognition determinants in diverse physiological and pathological processes including fertilization, pathogen-cell adhesion and recognition, inflammatory response and others. A number of pathogen microorganisms utilize lectin-carbohydrate interaction to recognized and infect host organism. The comprehension of the molecular mechanisms which gives a pathogenic bacterium the ability to invade, colonize and reorient the physiopathology of its host is a goal of primary importance and such studies may direct the conception of new strategies to fight these pathogenic agents 1 .

The contribution is focused on structural studies on *Ralstonia solanacearum* lectin RSL. A 9.9 kDa fucose-binding lectin has been found in bacterial extract and consequently prepared as recombinant protein. Ultra-high resolution diffraction data to 0.94 Å were collected from crystals of the recombinant RSL: -methyl-fucose complex at ESRF, Grenoble. Excellent phasing was obtained using the RSL/Se-methyl fucoside complex, showing the crystals to contain three monomers, each of two 4-stranded -sheets, with two sugar sites per monomer. The three monomers associate to form a 6-bladed -propeller; the first time such an arrangement has been observed. The structural studies of RSL double mutant (N77G/G84S), in which one of the binding site is altered, soaked in XXFG (the fucosylated nonasaccharide present in plant cell wall) clearly showed the nonreducing trisaccharide Fuc1-2 Gal1-2Xyl in non-altered binding site. The structural data has been supplemented by ITC microcalorimetry and surface plasmon resonance studies [2].

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STRUCTURAL STUDIES OF ATP-DEPENDENT PROTEASE

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ATP-dependent proteases represent the essential degradation system in the cells. Four ATP-dependent proteases: cytosolic - Lon, Clp, HslUV and membrane FstH proteases exist in prokaryotes. In eukaryotic cells, 26S proteasome constitutes the most abundant and elaborate ATP-dependent degradation machinery. In organelles (mitochondria, chloroplasts and peroxisomes), this function is ensured by analogues of ATP-dependent proteases present in prokaryotic cells.

ATP-dependent proteases are important for the biogenesis and homeostasis of living organisms. They are responsible for the degradation of damaged or misassembled proteins and of non-assembled protein subunits that might be harmful to the cells. ATP-dependent proteases also help control the concentration of several regulatory proteins. In prokaryotes, they are involved in stress response, pathogenicity, cell signaling and development (1). In eukaryotes, they are important for the proper functioning of organelles and for the immune and stress responses. Deficiencies in ATP-dependent proteases are connected with such severe disorders as dystrophies, neurodegenerative diseases and cancer and are thought to affect aging as well.

All ATP-dependent proteases operate as powerful multimeric machines. They differ widely in their complexity but nevertheless, they share common architectural features. Energy-dependent proteases usually form barrel-shaped oligomers with narrow central pore. They contain the essential and characteristic regions, proteolytic active sites and ATPase(s), which both are necessary for their activity. The proteolytic active sites that catalyze peptide bond cleavage of specific intracellular proteins are sequestered in a hollow interior chamber, typically constructed from rings of six or seven subunits or domains. The conserved ATPase(s) transduce the energy of nucleo-

tide-triphosphate binding and hydrolysis into cycles of conformational changes (2).

Lon protease is the simplest ATP-dependent protease but the three-dimensional structure of the intact protease has not yet been resolved. Lon protease creates homo-oligomeric ring complex in which each identical subunit contains two key-domains: an ATPase domain and a proteolytic domain. Both of these domains are covalently linked and mutual close cooperation between them is required for the full functionality of Lon. The proteolytic domain possesses a serine-lysine catalytic dyad in the active site (3). Our studies of degradation mechanism of Lon protease showed that the degradation initially occurs on the surface of folded proteins within the hydrophobic amino acids surrounded by charged amino acids (4). Lon protease seems therefore to employ a unique mechanism of degradation of endogenous substrates. In case of the other ATP-dependent proteases, the degradation supposes the binding of the protein substrate to the ATPase, unfolding and translocation to the proteolytic cavity before the degradation. Structural domains of Lon are essential not only for the ATPase and protease activity but also for stabilization of its oligomeric structure. Lon protease represents the most flexible ATP-dependent protease and we succeeded in preparation of the crystals of protease domain of the human Lon that are prepared for the diffraction data collection.

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BIOCHEMICAL STUDIES OF SPOIISA-SPOIISB TOXIN ANTITOXIN SYSTEM FROM *B. subtilis*

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SpoIISA-SpoIISB protein toxin-antitoxin system resembles many of the two component bacterial programmed cell death modules [1]. However, in spite of the similarities, precise biological role of SpoIIS system during *Bacillus subtilis* life cycle is still not clear.

In this work we present new findings concerning of biochemical properties of SpoIIS proteins and their possible implications for mechanisms of SpoIISA toxicity and an ability of SpoIISB to cure the lethal effect of SpoIISA.

We focused on interactions between molecules of predicted cytosolic domain of SpoIISA (C-SpoIISA) as well as on its binding with SpoIISB protein. According to our gel filtration experiments, it appears that switch between dimeric and multimeric forms of C-SpoIISA can be reversibly preformed by addition/removal of divalent cations. On the other hand, as shown by gel filtration and non-denaturating PAGE, SpoIISB seems to affects C-SpoIISA dimers in the opposite manner and its binding leads to disintegration of the dimer and formation of a heteropentameric complex.

The complex formed between cytosolic domain of SpoIISA and SpoIISB proteins were shown to resist treatment with high molar chaotropes. This strong interaction between both SpoIIS proteins appears to be carried out by opposite charges presented on their surfaces, since only extreme pH leads to breakage of the binding.

We attempted to prepare crystals of C-SpoIISA, SpoIISB, and their complex, respectively. However, as indicated by NMR experiments, SpoIISB does not possess any rigid fold and thus probably its molecular structure could be achieved only from complex with SpoIISA. We succeeded with crystallization of C-SpoIISA and C-SpoIISA-SpoIISB complex, which both were prepared from the same sample of co-expressed C-SpoIISA and SpoIISB. During crystallization we observed cleaving off the His_6 tag fused on N-terminus of SpoIISA. Similar cleavage was observed with full-length SpoIISA during its expression in *Escherichia coli* [2] and this can be also achieved by incubation of the crystallization samples in presence of divalent cations. Prepared crystals were positively tested for their ability diffract X-rays.

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FAST TRACK TO PROTEIN 3D STRUCTURE USING AUTOMATED CRYSTAL IMAGING, SCREENING AND DATA COLLECTION

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There are a number of hurdles in getting the three dimensional structure of a protein once it has been purified and characterized: Getting X-ray grade crystals, collecting data on these crystals, and solving the structure once the data have been collected.

We present new system solutions that will lower these hurdles by:

- Simplifying the process of getting good crystals
- Speeding up the characterization of grown crystals for X-ray diffraction
- Collecting more accurate data *in house*

The Crystal Farm is an integrated crystal imaging system which stores up to 400 crystallization plates (more than 35,000 experiments) and automatically images crystallization experiments at regular intervals, scheduled by the user. It allows remote access via a web browser and optional scoring software.

The X8 PROSPECTOR system, consisting of a highly reliable, low maintenance micro-focus sealed-tube x-ray source, Montel multilayer optics and sensitive Platinum^{135} CCD detector is ideal for rapid screening and characterization of the smallest crystals.

Once large enough crystals have been obtained they can be characterized crystallographically using the X8 PROTEUM diffraction system. The Proteum^{plus} software suite incorporates screening routines that will automatically parameterize crystal quality, giving feed-back on the unit cell, space group, diffraction power, mosaicity etc. With the increased brilliance of a micro-focus rotating anode generator X-ray source it is now possible to collect redundant and high quality data in house, faster and on smaller samples.

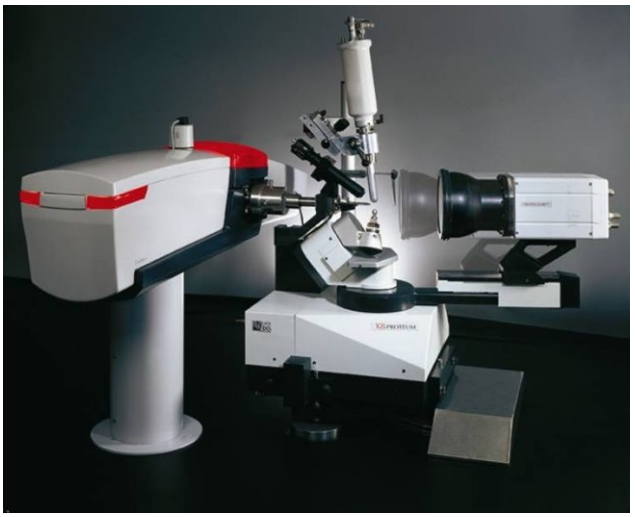

Figure 1. The X8 PROTEUM, in combination with a MICROSTAR-H can be used for rapid screening of protein samples, as well as complete data collection.