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Structural and functional investigation of non-globular regions in the postsynaptic Drebrin protein

Theses of PhD Dissertation

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I. Introduction

Drebrin (Developmentally Regulated Brain Protein) is an actin-cytoskeleton organizing protein[1], which has key importance in the morphogenesis and organization of the dendritic spine[2]. Earlier studies reported its actin-bundling[3], actin-binding[4], and actin-depolymerizing[5] properties, thereby showcasing its importance in the PSD (Postsynaptic Density).

Drebrin can only interact with filamentous (F) actin and not with the monomeric (G) form[6]. Drebrin binding to F-actin causes structural changes in the F-actin filament, slows down the treadmilling rate and influences the activity of motor proteins such as myosin II on F-actin. Shirao et al have described the importance of Drebrin in the response of dendritic spines to synaptic activity in detail[7]. In this, Drebrin's role is to form a pool of stable actin filaments that help stabilize dendritic spine sizes via a dynamic equilibrium between the Drebrin-bound and unbound filaments. Upon Ca2+-influx caused by synaptic activation, Drebrin and the bound actin filaments exit the synaptic spine, a process termed Drebrin exodus. This, in turn, allows G-actin to fill the spine and polymerize, contributing to spine size enlargement. At the end of the stimulus, Drebrin re-enters the spine and re-establishes the pool of stable actin filaments, stabilizing the enlargement by restoring the equilibrium between different actin forms. Decrease in the availability of Drebrin has been linked to various neurological conditions, primarily Alzheimer's disease (AD), but also to Down syndrome and ageing. The above described model is consistent with the role of Drebrin in maintaining synaptic plasticity and the decline of cognitive functions under certain conditions.

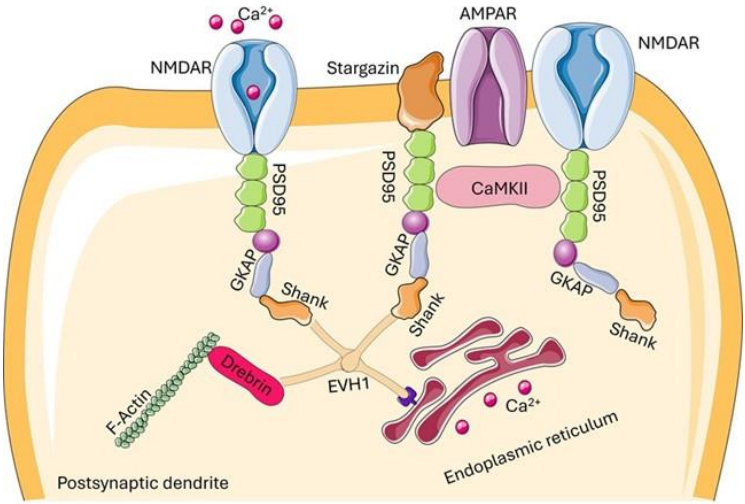

Figure 1: Drebrin interactions in the context of several major postsynaptic proteins. Drebrin is believed to play a key role in anchoring the postsynaptic protein network to the actin cytoskeleton

Drebrin has 2 known isoforms, Drebrin E, which is occurring during embryonal development, and in adults, Drebrin A, which contains an isoform-specific insertion. Drebrin has several non-globular regions, and to date there is no consensus in their structural properties and biological roles.

A primary example is the region between residues 173-238, initially predicted to form a coiled coil[8], then recognized as a likely Single Alpha Helix (SAH)[9], more consistent with the observed monomeric nature of the protein[3]. SAH domains occur mostly in cytoskeletal and RNA-binding domains, but regardless to their importance, very little detailed structural information is available about them.

Although Drebrin contains an ADF-H (actin-depolymerizing factor homology) domain, it does not appear to be its primary actin-binding site[6,10]. The Actin-Binding Domain (ABD) of Drebrin has been first delineated in several in vivo studies, then it has been further investigated via low-resolution analytical methods such as cosedimentation assays. Results from these studies do not concur and

suggest the location of Drebrin ABD in different sequential positions, including the primary binding site between residues 233-300 or 233-317, with KD values in micromolar range. Worth et al. further suggested the possibility of cooperative binding involving N-terminal parts of Drebrin as well. Electron microscopy investigations of Drebrin-decorated actin filaments suggest that the disordered actin-binding region of Drebrin might become folded upon the interaction, although no atomic-level structural model has been proposed[11].

II. Objectives

The aim of my research was to map the intriguing non-globular protein regions of Drebrin on an atomic level which are to date rather understudied as they are experimentally difficult to investigate. My goal was to corroborate the results yielded by earlier functional investigation of Drebrin, and to extend the available knowledge with structural information. To date, there are only 2 publications available, where they describe SAH motifs with structures which have been solved by NMR[12,13], and the understanding of the actin-binding mechanism of Drebrin bears challenges from both sides. F-actin forms a dynamic double helix, unsuitable for X-ray crystallography[14]. The predicted flexible nature of the actin-binding interface in Drebrin, and the high abundance of charged residues there prompted NMR spectroscopy as main method for these studies. In addition to the expression and experimental investigation of protein segments, my goal was to interpret my results from a holistic perspective and apply bioinformatic methods, to build an integrative model that describes the structure and behavior of non-globular protein segments in as much detail as possible.

III. Materials and methods

During my work, I prepared plasmid DNAs containing the genes encrypting the protein segments I wanted to study using a series of molecular cloning procedures. For these, I used a plasmid encompassing the nucleotide sequence encoding the full-length human Drebrin protein (RRID: Addgene_40362) as template DNA. I designed primers for the selected regions, then optimized touch-up PCR techniques to amplify the gene segments, which I cloned into pET-15b (Novagen) plasmids modified in our laboratory. I transformed the resulting constructs into BL21 (DE3) *E. coli* cells and then optimized the protein expression protocols. I developed methods consisting of various chromatographic steps for the purification of the produced recombinant proteins.

I applied CD spectroscopy for the initial, low-resolution structural characterization of the proteins. The bulk of my work was the subsequent part of structural and functional NMR investigations. I resolved the large amount of signal overlap in the low-complexity regions using NMR techniques developed for this purpose: In the case of the D233 construct, the narrow amide-hydrogen chemical shift distribution resulting from its high degree of disorder, and in the case of the single alpha-helix, the spectral crowding resulting from the large number of parallel amide vector dipole-dipole couplings along the transverse axis of the rod-like motif made the resonance assignment particularly difficult. In the first case, I used the 3D (HN)CO(CO)NH experiment based on triple-bond couplings between carbonyl ^{13}C atoms in sequential peptide-backbone neighborhoods[15] and for the latter, I used the 4D (HACA)CON(CA)NH experiment displaying HN(i-1)-N(i-1)-CO(i-1)-N(i) correlations and the 4D (HACA)N(CA)CONH experiment displaying CO(i)-N(i)-(Ni+1)-HN(i+1) correlations [16] (Figure 2).

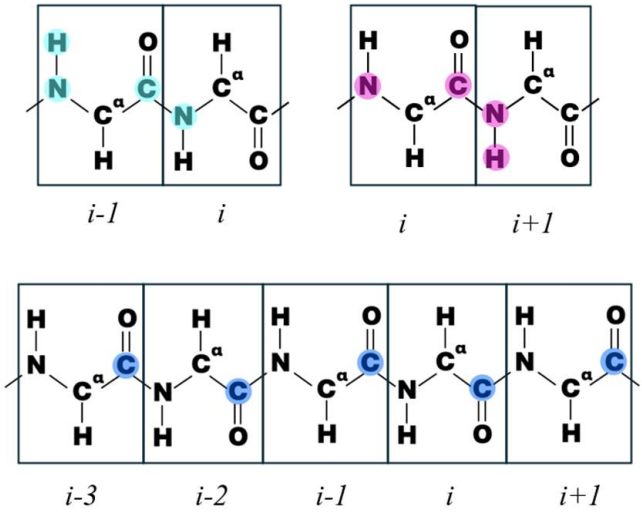

Figure 2: Special NMR techniques for low-complexity proteins used in backbone resonance assignment. The correlated nuclei along the peptide chain highlighted in the 4D (HACA)CON(CA)NH (turqoise), the 4D (HACA)N(CA)CONH (purple) and the 3D (HN)CO(CO)NH (blue) experiments respectively.

The single alpha-helix in Drebrin was investigated by small-angle X-ray scattering on the part of its global structural characterization. Based on the scattering parameters, I first performed an initial, low-resolution structural characterization, and then, supplemented with NMR chemical shift parameters, I developed a complex, ensemble-based methodology for the integrative structural modeling of the protein. Using the DIPEND algorithm developed in our research group[17], I generated an initial ensemble based on our preliminary results, which was sampled using a multiple selection procedure with CoNSEnsX^{+}[18], driven by experimental restraints and supplemented by molecular dynamics simulations. By analyzing the side chain configurations of the structural ensemble and inspecting atom-atom distances of individual conformers, I characterized the diverse occurrence possibilities of transient secondary interactions (salt bridges and cation-pi interactions) that provide the extraordinary stability of the special structural motif.

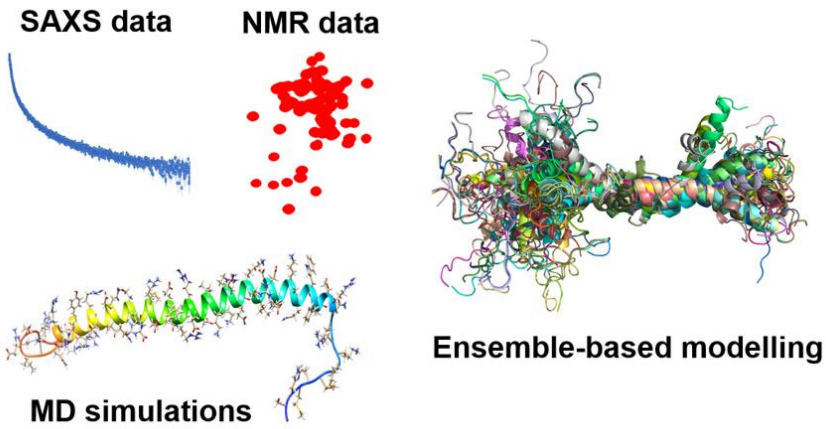

Figure 3: Simplified representation of the integrative modeling methodology. A model consisting of 60 conformers was created using a selection procedure driven by SAXS and NMR experimental data and aided by MD simulations.

I predicted the secondary structural features of the D233 construct in advance based on the amino acid sequence and then characterized them by secondary chemical shifts of the aliphatic carbon atoms. For the rapid, fingerprint-like examination of the secondary structural elements, I utilized a method based on the overlaid representation of $^{1}H^{15}N$ bTROSY and $^{1}H^{15}N$ HSQC spectra. To investigate the Drebrin-Actin interaction, I performed NMR titrations and t2 relaxation measurements.

IV. Results and thesis points

1./a: *I prepared plasmid DNA containing the sequence encoding the previously predicted single alpha-helix region in the postsynaptic Drebrin protein. I transformed this nucleic acid construct into E. coli cells, then optimized the bacterial expression and recombinant protein purification protocols, thus preparing a protein sample suitable for NMR structural analysis.*

1./b: *To characterize the Drebrin-actin interaction, which is yet to be described on an atomic level with consensus, I designed the D233 construct, prepared the necessary plasmid DNA, and then further developed the methods for the expression and purification of the single alpha-helix to prepare a protein sample suitable for NMR functional studies.*

To produce the regions, I intended to investigate, I designed primers that are compatible with the plasmid vectors most commonly used in our laboratory. After successfully amplifying and purifying the DNA regions, I managed to prepare expression plasmids encoding each protein by optimizing restriction digestions and ligation reactions with several attempts. Then, I validated by Sanger sequencing whether they contained the desired gene regions in the correct locations. After that, I transformed them into BL21 (DE3) cells, and then I concluded that the non-globular regions were expressed to a much higher extent at 37°C. The addition of protease inhibitor additive proved to be crucial during the ultrasonic cell extraction, since in the absence of tertiary structure, proteins are much more accessible to protease enzymes, thereby causing significant protein loss. I separated the target proteins from the bacterial cell extract by Ni-NTA affinity chromatography. The expression tag was removed by TEV protease. In the case of Drebrin SAH, an S-column ion-exchange chromatography performed at pH=6.0 proved to be effective, as I managed to bring the numerous charged amino acids to the same protonation state. Subsequently, I ensured by size exclusion chromatography that I had separated the target protein from the added TEV protease and the cleaved protein segment. Since the D233 construct does not contain amino acids detectable at 280 nm, it was advisable to minimize the chromatographic steps after TEV cleavage. For this, I developed a Q-column purification that bound the

enzyme and the cleaved protein segment, but allowed the target protein to flow through, thereby isolating it.

2./a: *I confirmed the monomer structure of the single alpha-helix in aqueous solution by CD spectroscopy and mass spectrometry. I performed the complete* ^{1}H-^{15}N-^{13}C *resonance assignment of the protein backbone, and the partial resonance assignment of the protein side chains by 4-dimensional NMR experiments. I demonstrated the local structural preference of the protein through Cα and Cβ secondary chemical shifts.*

The shape of the CD curve recorded in the wavelength range of 190-260 nm clearly showed the characteristic properties of alpha-helices (local maximum at 215 nm, local minimum at 222 nm). By examining the temperature dependence of the protein through the values measured at 222 nm between 285 K and 350 K, I demonstrated that the unfolding of the protein is non-cooperative, thus ruling out the coiled coil and confirming the monomeric occurrence of the helical motif.

The extremely high abundance of charged amino acids, their repetitive occurrence (Fig. 4), and the parallel alignment of the amide vectors of the rod-like motif along the transverse axis posed a problem that was impossible to overcome with conventional resonance assignment techniques. With the help of the additional indirect ^{15}N dimension introduced in the 4D experiments, I was able to observe multiple correlations that made the spin systems clearly identifiable after considerable iterations. With the Cα and Cβ chemical shifts thereby became accessible, I was able to confirm the strong preference of the protein towards the locally helical structure.

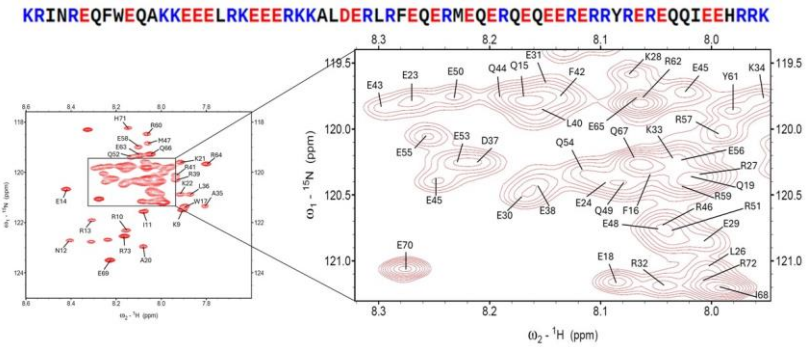

Figure 4: Sequential assignment of the Drebrin SAH domain. Low complexity and high abundance of charged residues are highlighted in blue (positive charge) and red (negative charge). The panel shows the ^{1}H-^{15}N HSQC spectrum of a single alpha helix in 50 mM NaCl, 17 mM NaH_2PO_4, 3 mM Na_2HPO_4, pH 6.0, and 25°C. The inlet highlights the extreme NH signal overlaps in the central region of the spectrum. Assigned chemical shifts are indicated by single-letter amino acid codes.

2./b: *Using NMR experiments specialized for disordered proteins, I performed the full ^{1}H-^{15}N-^{13}C resonance assignment of the D233 protein backbone and the partial resonance assignment of the protein side chains. The obtained chemical shifts indicated a high degree of disorder in the protein, with the exception of the helical region observed at the N-terminal section.*

I confirmed the high degree of disorder of the D233 construct containing the actin-binding region of the Drebrin protein by preliminary CD spectroscopy experiments, therefore I applied a specific NMR methodology for the resonance assignment of such proteins. In the (HN)CO(CO)NH experiment, I bypassed the degeneracy of chemical shifts of the amide protons by relying on long-range carbonyl-carbonyl correlations (Fig. 5). In the experiment, I exploited the particularly favorable relaxation properties of ^{13}C carbonyl nuclei in disordered proteins, thereby using an especially long acquisition time with a semi-constant time t1 evolution in the ^{13}C indirect dimension. In the MOCCA-XY16 pulse sequence, I was able

to correlate up to 5 sequentially adjacent carbonyl signals using an extremely long (500 ms) isotropic mixing time, thereby significantly accelerating the otherwise complex resonance assignment procedure. Partial side chain resonance assignment was performed using the (H)CC(CO)NH experiment, and the chemical shifts of the aliphatic carbon atoms were used to characterize the secondary structure propensities of the protein using the CheSPI program. In addition to the high degree of disorder, I somewhat surprisingly confirmed the presence of a helical motif of approximately 15 amino acids in length near the N-terminus of the protein.

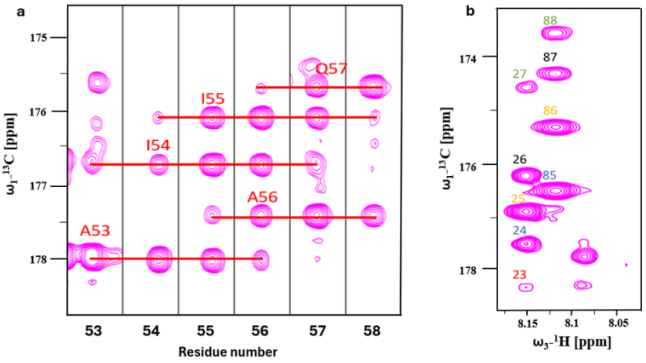

Figure 5: a, Strip plots for adjacent residues of D233 from 3D (HN)CO(CO)NH experiment. (b) A zoomed F1-F3 region of the 3D (HN)CO(CO)NH spectrum showing the correlation between multiple carbonyl signals (green:i+1, black:i, orange:i-1, blue:i-2, red:i-3). In highly resolved areas it is possible to recognize up to 5 carbonyl correlations following each other in the sequence.

3: *Driven by NMR and SAXS data, further aided by molecular dynamics simulations, I constructed an ensemble-based model of the single alpha-helix, which reflects both the local and global structural parameters revealed by experiments and highlights the formation of a transient network of salt bridges and cation-pi interactions that provide the extraordinary stability of the helical motif.*

I confirmed by small-angle X-ray scattering experiments that Drebrin-SAH occurs as a monomer even over a wide concentration range. I

confirmed the hypothesis of a structurally elongated molecule with dimensionless Kratky diagrams, and I refined the preliminary idea of a rod-like shape, since the scattering surface can rather be described by the shape of a twisted banana.

Driven by the Cα and Cβ chemical shifts from NMR experiments, and by SAXS data, I developed a complex ensemble-based methodology for the structural characterization of the single alpha-helix motif. From the initial 5000-member conformational ensemble generated by the DIPEND algorithm, I generated a sub ensemble by parallel selection under two experimental restraints, which I enriched with molecular dynamics simulations with a fixed peptide backbone to sample possible side chain configurations. I repeated the selection procedure based on NMR and SAXS data on the resulting sub ensemble, thus obtaining a final ensemble of 60 conformers (Fig. 6).

I performed atom-atom distance analysis on conformers that well reflect both local and global structural preferences to characterize the transient network of secondary bondings responsible for the extraordinary stability of the helical motif. With my results, I revealed the possibility of numerous salt bridges and cation-pi interactions that can be understood as the net result of the dynamic change of the electrostatic force that provides the stability of the elongated single alpha helix monomer in aqueous solutions.

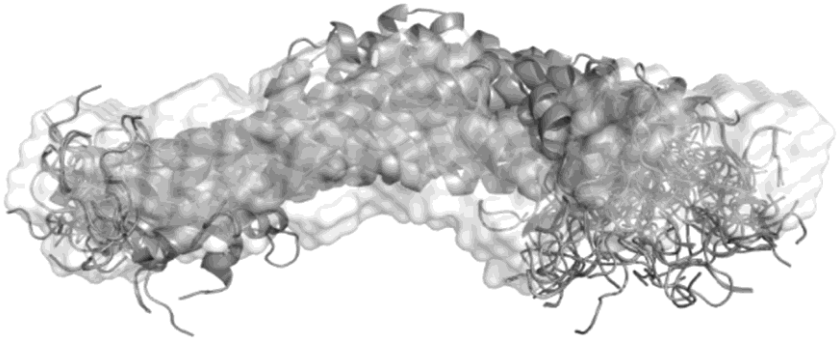

Figure 6: Spatial alignment and comparison of the ensemble (grey ribbons) derived from NMR and the overall shape of a model (white surface) reconstructed *ab initio* from SAXS data.

4: *To characterize the Drebrin-actin interaction at the atomic level, I optimized NMR titrations and t2 relaxation measurements. Based on the changes observed in the D233 signals upon addition of F-actin, I was able to set up a method to confirm the binding, which can serve as the basis for more detailed studies in the future.*

After the successful resonance assignment, I revealed the "fingerprint-like" information of the D233 construct, i.e. I clearly identified the NH signal belonging to every amino acid in the $^{1}H^{15}N$ HSQC spectrum of the protein. This paved the way for determining the region of the protein involved in the interaction, for which I first tried the most conventional NMR titrations. By adding unlabeled F-actin solution to the ^{15}N-labeled D233, I observed changes in most of the D233 signals. As a control experiment, I also titrated the D233 protein with an ATP solution, since this was also present in the buffer ensuring the polymerization of F-actin, while earlier the D233 resonance assignment was performed on an ATP-free sample. At that time, I observed that increasing the ATP concentration also clearly causes observable perturbations in a significant proportion of the D233 signals. Since several of these peaks also changed upon addition of the F-actin solution containing ATP, the approach based solely on NMR titrations to determine the interaction interface became significantly more difficult.

After that, I determined the r2 relaxation rates of the NH signals of the protein in the free and bound states formed upon addition of F-actin, with the same D233 concentration and buffer content. Interestingly, interpreting the process as a "one-step NMR titration", when superimposing the spectra of the free and bound forms, I experienced only minor differences (chemical shift perturbation, line broadening for a small fraction of the NH signals), however, the r2 relaxation rates increased to a similar extent for practically all amino acids. Based on the results, I concluded that the kinetics of protein-protein interaction occurs on a time scale where the r2 relaxation rates average out in such a way that they lose their sensitivity to the chemical shifts existing in the free and bound forms. For this reason, the effect is not localized to certain regions of the sequence, raising the possibility of a higher order motion governing the interaction.

When superimposing the $^{1}H^{15}N$ bTROSY and $^{1}H^{15}N$ HSQC spectra recorded from the D233 protein, I made an interesting observation: If we take a scalar coupling with an average value of 93 Hz regardless of the amino acid type and then shift one of the spectra by half of that value, the vast majority of the NH signals overlap as expected, but I still experienced a well-defined remaining shift in the case of about 15 peaks. Based on the CheSPI results, these 15 amino acids are exactly the helical segment near the N-terminal that can be interpreted as practically the only structural element of the disordered protein. This observation raises the possibility of developing a fast, fingerprint-like method aimed at determining the local, possibly transient structural elements of mostly disordered proteins.

V. List of publications

Journal publications of the theses

Varga, S., Péterfia, BF., Dudola, D., Farkas, V., Jeffries, CM., Permi, P., Gáspári, Z. (**2025**). Dynamic Interchange of Local Residue-Residue Interactions in the Largely Extended Single Alpha-Helix in Drebrin. Biochemical Journal, vol. 482, no. 08, pp. 383–399, https://doi.org/10.1042/BCJ20253036

Varga, S., Kaasen, JM., Gáspári, Z., Péterfia, BF., Mulder, FAA. (**2025**). Resonance assignment of the intrinsically disordered actin-binding region of Drebrin. Biomolecular NMR Assignments https://doi.org/10.1007/s12104-025-10239-0

Further journal publications

Szabó, AL., Nagy-Kanta, E., Varga, S., Jáger, EA., Pongor, CI., Laki, M., Laki, AJ., Gáspári, Z. (**2025**). Diffusion-based size determination of solute particles: a method adapted for PSD proteins, FEBS Open Bio, submitted

Karjalainen, M., Kontunen, A., Anttalainen, A., Mäkelä, M., Varga,S., Lepomäki, M., Anttalainen, O., Kumpulainen, P., Oksala, N., Roine,

A., Vehkaoja, A. (**2022**). Characterization of signal kinetics in real time surgical tissue classification system. Sensors and Actuators B: Chemical, Volume 365, 2022, 131902, https://doi.org/10.1016/j.snb.2022.131902.

Conference talks

-Conference presentation at the "3rd ML4NGP MEETING on Machine Learning and Non-globular proteins, Vilnius, May 20-23, 2025" conference
-Conference presentation at the "Hungarian NMR Working Committee Meeting, Balatonszemes, May 8-9, 2025" conference
-Conference presentation at the "Hungarian Molecular Life Science Conference 2025, Eger, March 28-30, 2025" conference
-Presentation at the "Zechmeister László Lecture Competition, Hungarian Academy of Sciences, 2024.11.15." lecture competition, 2nd place award
-Conference presentation at the "Hungarian NMR Working Committee Meeting, Balatonszemes, May 23-24, 2024" conference

Conference posters

-Poster at the "46th Danish NMR meeting, Rebild January 30-31, 2025" conference
-Poster at the "Small Angle Neutron and X-ray Scattering from biomacromolecules in solution, 16 – 20 September 2024 | Grenoble, France, organized by European Molecular Biology Organization" course
-Poster at the "The 48th FEBS Congress, 29 June – 3 July 2024, Milano, Italy" conference
-Poster at the "45th Danish NMR meeting, Roskilde January 29-30, 2024" conference
-Poster at the "NMR for combatting diseases: from cancer to SARS-CoV-2 - Sesto Fiorentino, Italy, 27/03/2023 - 31/03/2023, organized by ICGEB" course
-Poster at the "iNEXT-Discovery 2023 Experimental and computational aspects of Structural Chemistry and Biology 22-24 June 2023 Budapest, Hungary" conference

VI: References

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