Synopsis of PhD dissertation

INVESTIGATION OF THE STRUCTURE, DYNAMICS, THERMAL UNFOLDING AND HIDDEN CONFORMERS OF THE *PENICILLIUM* ANTIFUNGAL PROTEIN

written by

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Debrecen, 2018.

I. Introduction and objectives

Introduction

The combat against fungal infections has higher and higher importance regarding human healthcare as well as in the agriculture. The higher incidence of fungal infections in humans during the last two decades has multiple reasons such as the widespread application of immunosuppressive therapies and diseases with immunodeficiency like AIDS or leukaemia. The development of new antifungal agents is very challenging because the extensive usage of antimycotics – similarly to antibiotics – lead to resistance, and also because – unlike bacteria – fungi are eukaryotic organisms just like mammalians, and therefore the high degree of physiological similarities restrict the number of potential molecular targets for a new drug. These factors together lead to an urgent need of newly developed antifungal drugs which are species-specific, effective and non-toxic. To meet these requirements not only the traditional synthetic molecules are developed, but also biologics which have higher molecular mass and more complex structure as compared to "traditional" drug molecules.

A relatively recently discovered group of antifungal drug candidates are antimicrobial proteins (or also called antimicrobial peptides, abbreviated as AMPs). AMPs are produced by almost all kingdom of organisms including bacteria, fungi and even mammalians. As a result of "biochemical competition" between different microbial fungus species for the habitat, a wide spectrum of potent antimicrobial peptides have been evolved. According to the recent scientific literature[4–13] AMPs secreted by the *Ascomycetes* dividion of filamentous fungi at least ten different AMPs were proved to be a protein with strong antifungal activity. While these molecules differ in their amino acid sequence, specificity and mechanism of action, they share common features too. Antifungal proteins produced by *Ascomycetes* can be characterized with 50 to 60 amino acid residue of length, high net charge and relatively high number of disulfide bridges which results in a very compact and stable molecular structure, while their structural properties such as beta-sheets and the well-folded globular nature reminds them proteins. Probably this beneficial combination of stability and specificity makes them ideal antifungal drug candidates. One of the most extensively investigated member of this group is the *Penicillium* antifungal protein (PAF) which was the subject of our investigations described in my dissertation.

The research of PAF begun more than two decades ago. The gene *paf* and the first isolation of the protein was described by an Austrian research group in 1995.[5] Since then, this group and the other partners who joined to this research publish on the average one publication per year on this topic. The studies cover a wide range of disciplines with the aim of characterization of the biochemical[14,15,5,16], physiological[17–26], or structural[14,27,28] properties of this protein. Our structural biology group at the University of Debrecen joined to this research in 2007. Within two years, this fruitful cooperation resulted in a publication in which the atomic structure of PAF was published for the first time[14]. This publication can be considered as direct precedents of my work. Having the 3D structure in our hands

initiated a bunch of new ideas and opened the window for new research areas such as structure-based protein engineering and structural biology. Moreover, the advantageous attributes of PAF makes it an outstanding protein model for basic spectroscopic research and the development of this two areas often facilitate each other[28,29].

As a BSc student I joined to this exciting research in 2008. After I have completed my BSc and MSc diploma in the same topic, I could to start my post-gradual education holding the biotechnology and structural biology toolkit and with clear aims, that I summarize above in three points.

1. In case of the solution structure of PAF published in 2009[14] the disulfide pattern was not determined because the available structural data was insufficient for unambiguous cysteine-pair assignment. My first aim was to reveal this uncertainty and determine the exact cysteine pairing using a molecular biology approach, by producing modified versions of PAF by cysteine-to-serine point-mutations. I intended to use the unambiguous disulfide bridges for more accurate modelling of the structure.

2. According to the published ^{15}N-relaxation data[14] PAF can be described as a rigid protein on the ps-ns timescale. Although this corroborate with the extended β-stranded secondary structure and the stabilizing three disulfide bridges, the “rigid molecule theory” was controversial with our initial findings of our thermal unfolding experiments. The decrease of integral values at high and low temperatures without appearing new resonances on the spectra suggested that hidden exchange partner exist in the system. I wanted to investigate this “mysterious” phenomenon in detail and I aimed to explain the unusual spectral behaviour of this system.

3. In the frame of structure-function investigation studies in close cooperation with our partners, we have produced several modified versions of PAF by point-mutations. One of these variants in which aspartic acid was exchanged to serine in the position 19 showed surprisingly low antifungal activity although its overall structure was unchanged according to our preliminary structure investigations. My aim was the comparative investigation of this PAF^{D19S} variant with the unmodified protein, regarding its structure, dynamics and thermal unfolding. We intended to explain the significant functional difference by finding potential differences in protein structure or dynamics.

II. Applied methods, equipments and investigated materials

The investigated materials

The protein PAF is originally produced by the filamentous fungus *Penicillium chrysogenum.* PAF was firstly isolated from its strain ATCC 10002, which is the first expression system that was used for the production of ^{15}N-enriched form of PAF[14].

As our research proceeded, we needed an expression system which produces PAF (and its recombinant variants) with a higher yield. Initially, the yeast *Pichia pastoris* was the system of our choice because it was proved to be an organism that produce well-folded PAF[14] with an acceptable yield (3-7 mg/L) and the fermentation technology allows ^{15}N and/or ^{13}C supplementation of the growth media. The cys/ser

point-mutated variants of PAF were produced in this system, namely PAF^{C7S}, PAF^{C14S}, PAF^{C28S}, $PAF^{C7S\text{-}C36S}$, and $PAF^{C28S\text{-}C54S}$.

Later our partners developed a *Penicillium*-based expression system[32] with even higher yield for PAF (>10mg/L). This strain is the *paf*-knockout *Penicillium chrysogenum* (*Δpaf Penicillium C3)* which has several advantages over the previously applied expression systems. PAF^{D19S} and ^{15}N-^{13}C-labelled PAF were produced in this system according to the protocol we have published[32]. This technology was successfully transferred to the our laboratory in Debrecen.

The chemically produced PAF and its intermediates was synthesized by our partner Györgyi Váradi (University of Szeged, Faculty of Medicine, Department of Medical Chemistry) with native chemical ligation in combination with oxidative folding[27]. The selectively ^{15}N-labelled peptide (Arg-(^{15}N)-Ala-Gln-Ile) that were used as reference compounds for quantitative measurements were also synthesized by her.

The applied methods

During my research I studied the structure of proteins by nuclear magnetic resonance (NMR) spectroscopy. Nowadays, NMR became an indispensable tool of the modern biological research, because it provides abundant and very precious structural information about macromolecules such as nucleic acids and proteins. What makes NMR unique among other techniques is that structural data can be obtained in atomic resolution and at the same time it provides information about molecular motions on a very wide timescale. A general workflow of structure investigation of biologically produced proteins by NMR spectroscopy and computational methods is summarized above on **figure 1**.

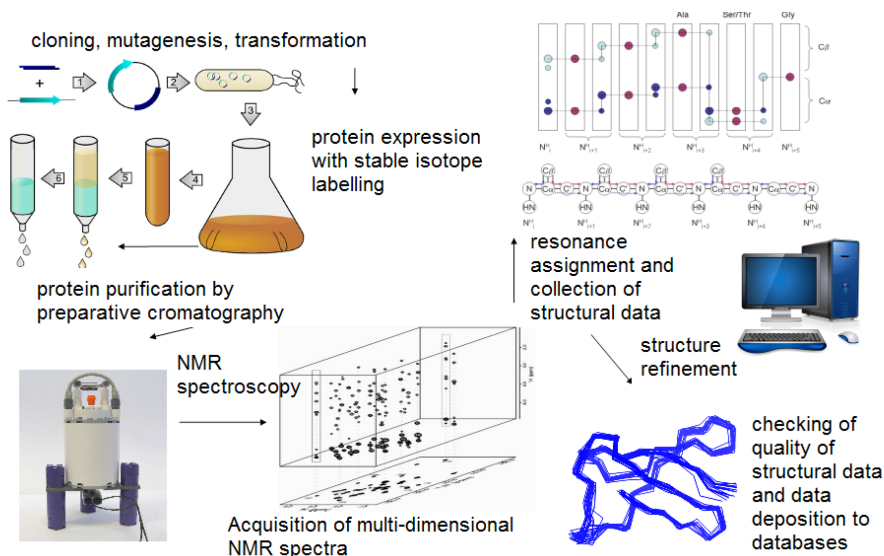

Figure 1.*: Main steps of structure investigation of biologically produced proteins by NMR spectroscopy.*

De novo protein structure determination

In order to acquire multi-dimensional NMR spectra on PAF and its variants, we labelled PAF by two type of isotope-labelling schemes: uniform ^{15}N- or uniform ^{15}N-^{13}C-labelling. According to this two approaches, two type resonance assignment strategies were applied as showed on **figure 2.**

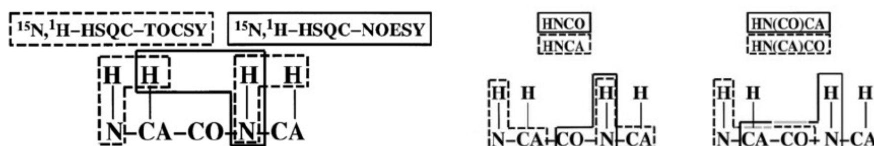

***Figure 2.**: The applied strategies for protein backbone resonance assignment. (Left) typical 3D NMR experiments of the ^{15}N- labelled and (rigth) ^{15}N-^{13}C. Dashed lines indicate inter-residual-, solid lines show intra-residual correlations.*

After backbone and sidechain resonance assignment, the crosspeaks of (^{15}N- and/or ^{13}C-resolved) 3D NOESY spectra were assigned to the corresponding hydrogen pairs automatically by means of the ATNOS-CANDID protocol. This process was simultaneously applied with the simulated annealing molecular dynamics package Cyana 2.1. The robustness of the resulting structure models was tested by systematic exclusion of H-H atom pairs during calculations. The final structure was refined based on torsion angle restrains which were calculated from ^{13}C backbone chemical shifts with the TALOS+[35] algorithm.

Modelling of thermal unfolding

Thermal unfolding of PAF was investigated with a series of quantitative ^{1}H-^{15}N-HSQC experiments recorded at different temperatures in a wide temperature range (-20 - +80°C). The temperature-dependence of the crosspeak volumes were fitted based on the Gibbs-Helmholtz equations (and its extended version).

Investigation of protein dynamics and exchange processes

Protein dynamics at the ps-ns timescale was investigated by the measurement of T_1, T_2 ^{15}N relaxation times and heteronuclear NOEs. Relaxation data was evaluated according to the second model of Lipari-Szabó formalism with the aid of the Bruker Dynamics Center.

The directly not-observable low populated conformers were detected by CEST-NMR (Chemical Exchange Saturation Transfer)[37] method and CEST data was evaluated with the Chemex method[37].

The sidechain solvent accessability was determined by the CLEANEX[38] method.

Equipment, experiment conditions and applied software

The majority of NMR spectra were recorded on a 500 MHz Bruker Avance II (Bruker BioSpin GmbH, Rheinstetten, Germany) spectrometer equipped with a 5 mm three channel probehead with z-gradient, at the University of Debrecen. For the temperature-dependent experiments the same spectrometer was

equipped with a broadband inverted probehead with proton detection. NOESY experiments were repeated on a Bruker Avance II 700 MHz spectrometer with the kind help of Dr. Andrea Bodor at the ELTE University in Budapest.

Every experiment for structure determination was measured in 10 mM sodium-phosphate solution containing 40 mM NaCl at pH=6.0 and 298K in Shigemi tube. The temperature-dependent spectra were recorded with the same solvent conditions in normal 5 mm glass tube except sub-zero experiments, for which 1 mm glass capillaries were used.

NMR data was collected with the aid of Topspin 2.1 software. Sequence specific resonance assignment was completed with the aid of CARA 2.8.1. In case of titration-type of experiments assignments were transferred with the CCPN Analysis 2.2 program. For NOE peak-picking and assignment the *ATNOS-CANDID*[39] protocol was used in combination with Cyana 2.1. Dihedral angles were determined with *TALOS+*[35]. For molecular graphics, *PyMOL™ Molecular Graphics System*, 1.7.0.0. was used. Modelling of thermal unfolding was performed with in-house written scripts in the *Matlab 7.7* environment.

III. New scientific results

The most important achievements of this work is summarized above in six propositions.

1. By the analysis of 3D triple resonance NMR spectra complete ^{1}H-^{15}N-^{13}C signal assignment was achieved.

During the signal assignment of ^{15}N-^{13}C labelled PAF the previous resonance date was not considered. The comparison of the completeness of ^{1}H and ^{13}C resonance assignments is shown on **Figure 3.** Chemical shift data was validated and deposited to the „Biological Magnetic Resonance Data Bank" (http://www.bmrb.wisc.edu/) (BMRB ID:19657). Due to the double labelling strategy, completeness of both ^{1}H, and ^{13}C increased significantly, resulting more structural restrains for structure determination.

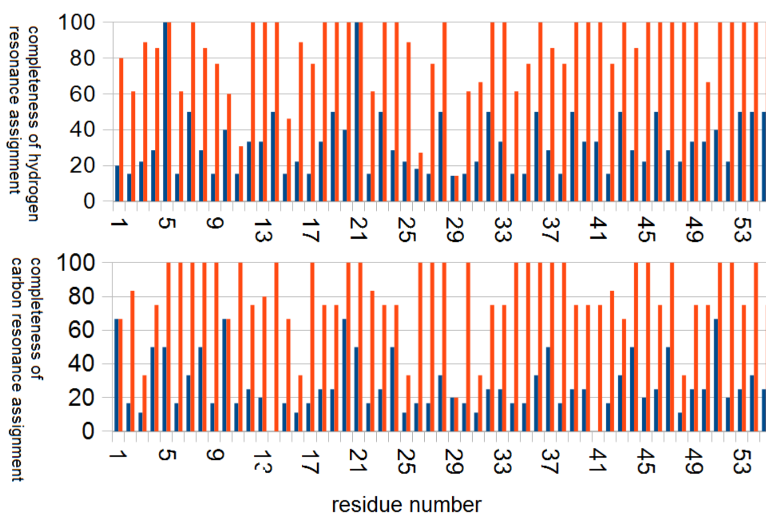

Figure 3. *Completeness of ^{1}H and ^{13}C resonance assignment of PAF. The previously published dataset is shown with blue, the completeness of the new assignment is shown with red.*

2. It was proved that all the six cystein residues are indispensable for proper folding of PAF.

In order to investigate the disulfide pattern of PAF, cysteine deficient versions were designed and expressed in recombinant *Pichia pastoris* expression system. The structure investigation of single- and double- mutated protein forms showed that the lack of any cysteine residue in the amino acid sequence leads to the loss of tertiary structure and therefore all the six cysteine residues are necessary for the maturation process of PAF.

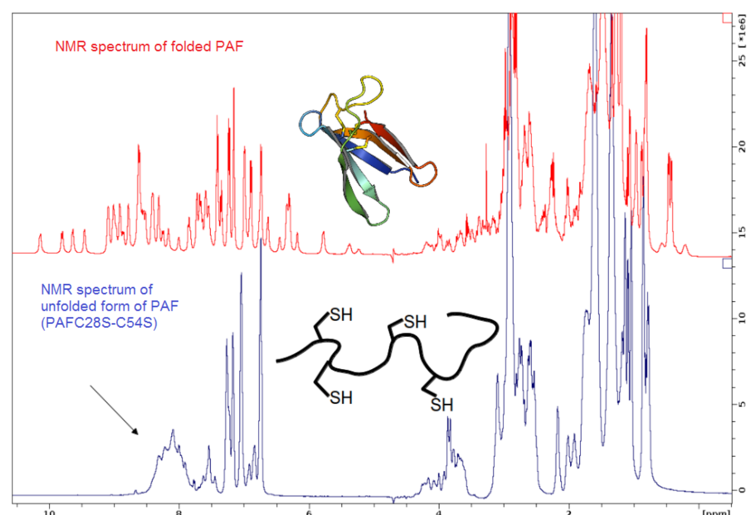

Figure 4. *Red: ^{1}H NMR spectrum and ribbon representation of the folded native PAF. Blue: ^{1}H NMR spectrum and theoretical "structure" of the unfolded $PAF^{C28S-C54S}$ cysteine/serine doubly-exchange PAF.*

3. It was confirmed by NMR spectroscopy that the structure of the synthetic PAF is identical with the native one.

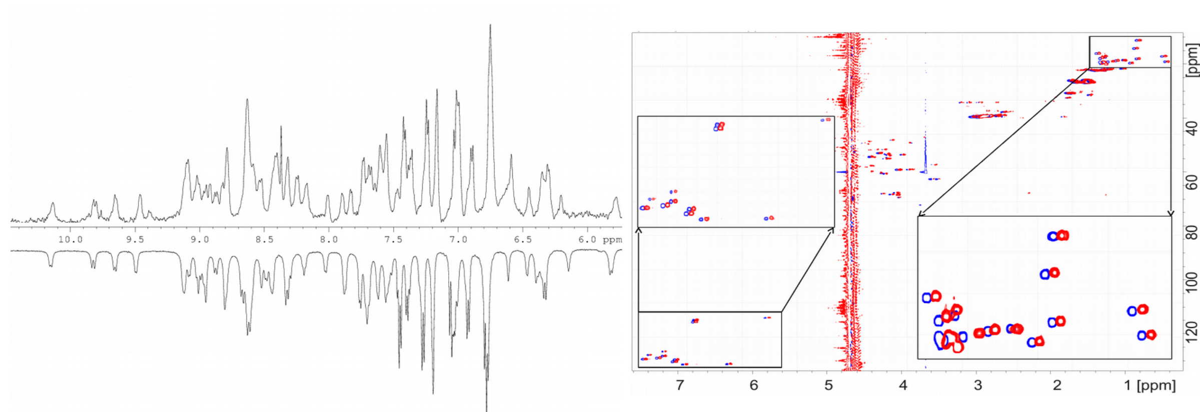

Figure 5. *Left: Amide and aromatic region of the ^{1}H NMR spectra of the synthetic (upper) and biologically produced (lower) PAF. Right: ^{13}C-^{1}H correlation spectra (blue: Penicillium-produced, red: synthetic PAF).*

Based on 1D ^{1}H and natural isotopic abundance 2D ^{1}H-^{13}C HSQC spectra (as shown on **figure 5.**) the tertiary structure of PAF synthesized by the combination of native chemical ligation and oxidative folding[27] is identical with the structure of the isolated one (which is produced by *Penicillium chrysogenum*). The fact of this structural identity was the final proof for the previously ambiguous disulfide pattern. The order of cysteine connections was determined independently by mass spectrometry in case of the synthetic PAF.

As a confirmation of our partner's observation we were able to reproduce the oxidative folding of the reduced linear form of PAF as it was observed by a series of ^{1}H NMR experiments **(figure 6.)**.

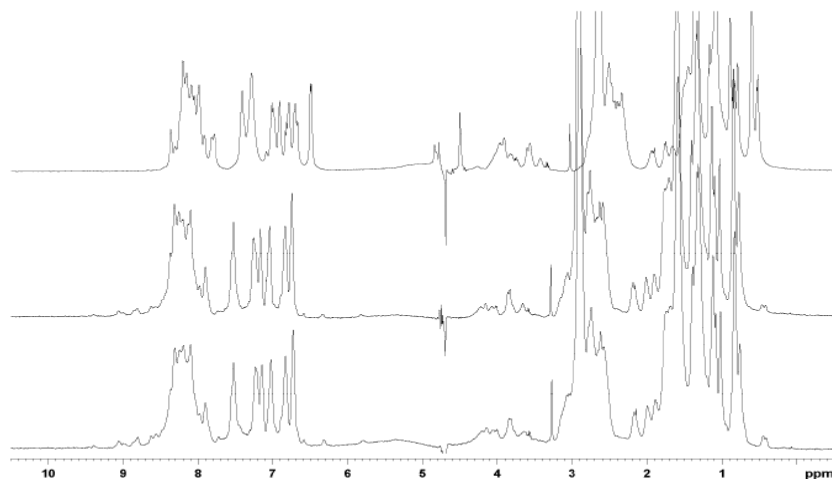

Figure 6. *^{1}H NMR spectra of reduced linear PAF showing spontaneous folding upon oxidation. Upper spectrum: linear PAF under Ar gase. Signals between 8.5-6-5 ppm clearly indicate typical unfolded protein resonances. Middle: spectrum of the same sample after a week of the first measurement. Resonances of the folded PAF starting to appear at 0,5 ppm. Lower spectrum: 9 weeks after the first measurement. The sample was stored at room temperature in an opened vial. In the 9-9,5 ppm region signals of H^N Thr 37, Cys 7 and Cys 14 can be detected as well as methyl signal of valine 45 at 0,5 ppm.*

4. High resolution solution structure of PAF was determined, using new sets of experimentally driven parameters, including NOEs, torsion angle and disulfide constraints.

In the knowledge of the unambiguous disulfide pattern and in possession of uniform ^{13}C-^{15}N enriched protein sample, a more accurate 3D structure of PAF was determined as compared to the previously published model. The model was further refined by the peptide backbone torsion angle restrains derived from ^{13}C backbone chemical shifts. The resulting structure does not differ significantly from the previously determined one with respect to its backbone conformation and secondary structure. However, during the newer structure determination, disulfides were defined explicitly as covalent bonds. These restrains were in agreement with the majority of the NOE the distance restraints. This computational approach also confirmed the 'abcabc' pattern (in which 7-36, 14-43, 28-54 cysteine residues are connected). Furthermore, backbone RMSD values of the new model is 0.40±0.08 which indicates well-determined spatial coordinates (as a comparison, the backbone RMSD of the previously determined structure was 0.65±0.18).

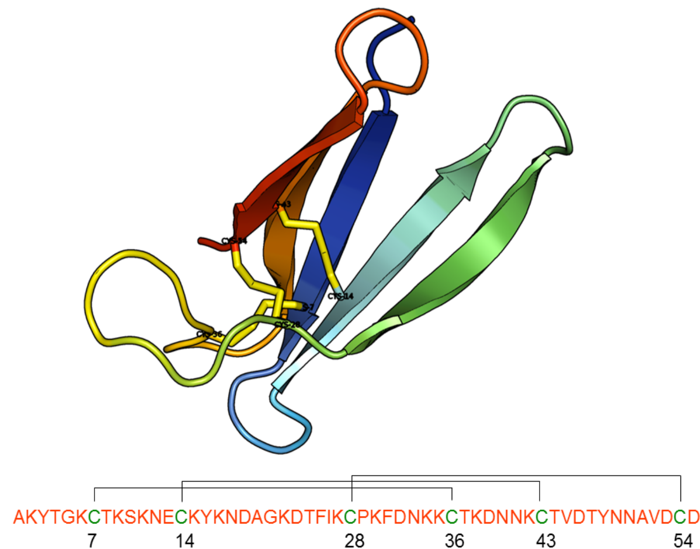

Figure 7. *Ribbon representation of the 3D structure of PAF. Arrows represent β-strandsm disulfide bridges are shown as yellow sticks. Above the structure graphics, the sequence is shown where the cystein pairs are connected with lines.*

5. It was shown in the solution structure of PAF that several "hidden" conformers exist which are not directly observable by NMR, and the proportion of the detectable and not directly detectable forms can be shifted by temperature changes.

It was shown by our thermal protein unfolding experiments that the solution structure of PAF contains a significant amount of conformer which is not directly observable by conventional NMR methods and the proportion of the „visible" and „invisible" fractions could be shifted by varying the temperature. We have estimated the concentration of the unfolded fraction(s) by temperature-dependent quantitative 2D NMR and by modeling of the temperature-induced reversible protein unfolding. The modelling revealed that the local thermodynamic parameters of the unfolding depend from the secondary structure elements: while reversible thermal unfolding of the motile terminal and loop regions could be fitted by two-state model, in case of residues which are positioned within beta-stranded elements (at least) a third conformational state had to be supposed beyond the folded and unfolded states (and this is called the "three state model"). According to our results, the population of the minor „invisible" conformers are not negligible. According to our estimation based on minimized global fit errors, the ratio of the invisible fraction was around 20-30% even at room temperature. Sensitive ^{15}N-CEST–NMR experiments revealed further minor conformations with even lower population (<1%). According to our results, these conformations are in slow exchange with the major form and in case of some residues has significantly different chemical shift which indicates major structural change at these sites.

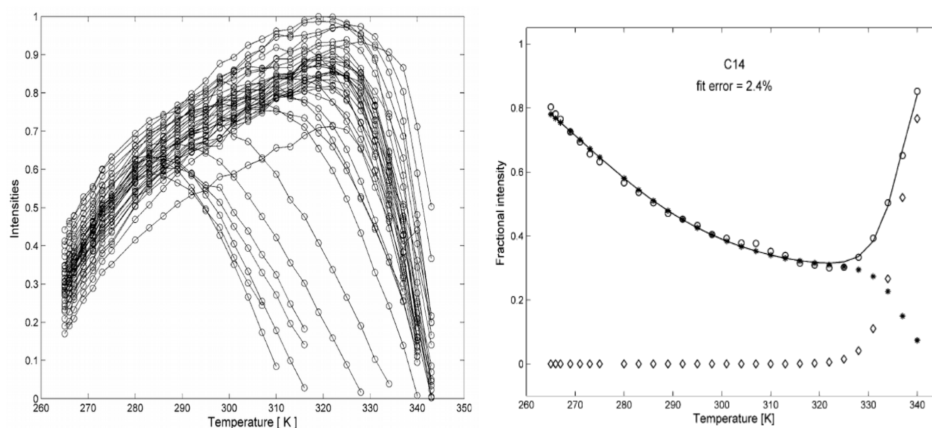

Figure 8. *Left: Integral volumes of ^{1}H-^{15}N-HSQC crosspeaks in the function of temperature. Right: population of the different fractions in function of temperature and fitting of visible and hidden conformational states based on the three-state thermal unfolding model in case of the representative cysteine 14. Folded fraction: □, intermediate state:*, unfolded state: ◊, the two hidden state together: ○.*

6. The detailed structure comparison of PAF and its inactive variant PAF^{D19S} showed that reduced biological activity of PAF^{D19S} can be explained by significant changes in the surface charge distribution and/or changes in thermal unfolding process.

The possible biological function of the "hidden" exchange partner was investigated with the aid of a point-mutated inactive version of PAF (PAF^{D19S}). According to antifungal activity assays, exchange of one critical negatively charged aspartate to serine by point mutation at the position 19 almost completely eliminated the antifungal activity of the protein. We have investigated the three dimensional solution structure, ps-ns dynamics, amide H^N-H_2O exchange (by CLEANEX), "CEST-detectable" conformers and thermal unfolding of PAF^{D19S} at identical conditions as our previous experiments with PAF. While backbone chemical shifts, NOE-derived structural ensembles at the level of the overall protein fold and ^{15}N-relaxiation parameters showed highly similar properties between the two protein forms, the local parameters of thermal unfolding and distribution of protein surface charge changed significantly in PAF^{D19S} as compared to PAF, due to reshuffling of charged side-chains. The dramatic change of surface charges which relayed further from the site of mutation is able to prevent PAF from binding to its primary target which gives an acceptable explanation to the loss of antifungal activity. However, one can not exclude the possibility that the altered surface charge is not the only factor in the inactivation of PAF by this single residue exchange. According to thermal unfolding experiments, PAF^{D19S} proved to be a two-state folder while the thermal unfolding of PAF is closer to the three-state unfolding model. Therefore it can not be excluded that intermediate "invisible" conformations which are present in PAF but not (or in a less extent) in PAF^{D19S} contribute to the antifungal mechanism.

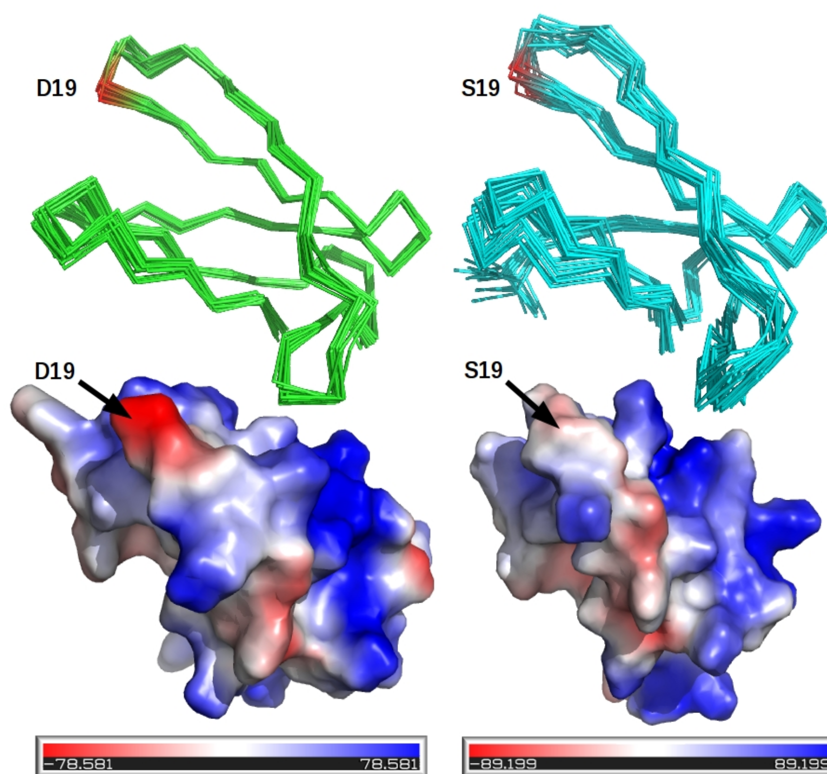

Figure 9. *Upper part: 20 conformer ensemble of PAF (left) and PAF^{D19S} (right) backbones. Aspartic acid 19. is highlighted with red. Lower part surface charge of a representative conformer of PAF (left) and PAF^{D19S} (right) calculated in vacuum. Red: negative charge, white: neutral, blue: positive charge.*

A tézisfüzetben hivatkozott szakirodalom jegyzéke / List of references cited in the Summary of Thesis

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Candidate: Ádám Fizil
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Doctoral School: Doctoral School of Chemistry
MTMT ID: 10032847

List of publications related to the dissertation

Foreign language scientific articles in international journals (3)

1. Sonderegger, C., **Fizil, Á.**, Burtscher, L., Hajdu, D., Munoz, A., Gaáspári, Z., Read, N. D., Batta, G., Marx, F.: D19S Mutation of the Cationic, Cysteine-Rich Protein PAF: Novel Insights into Its Structural Dynamics, Thermal Unfolding and Antifungal Function.
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2. **Fizil, Á.**, Gáspári, Z., Barna, T., Marx, F., Batta, G.: "Invisible" Conformers of an Antifungal Disulfide Protein Revealed by Constrained Cold and Heat Unfolding, CEST-NMR Experiments, and Molecular Dynamics Calculations.
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3. Váradi, G., Tóth, G. K., Kele, Z., Galgóczy, L., **Fizil, Á.**, Batta, G.: Synthesis of PAF, an Antifungal Protein from P. chrysogenum, by Native Chemical Ligation: Native Disulfide Pattern and Fold Obtained upon Oxidative Refolding.
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List of other publications

Foreign language scientific articles in Hungarian journals (1)

4. Kiss, R., **Fizil, Á.**, Szántay, C.: Az NMR-spektroszkópia szerepe a biologikumok analitikájában.
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Foreign language scientific articles in international journals (6)

5. Huber, A., Hajdu, D., Bratschun-Khan, D., Gáspári, Z., Varbanov, M., Philippot, S., **Fizil, Á.**, Czajlik, A., Kele, Z., Sonderegger, C., Galgóczy, L., Bodor, A., Marx, F., Batta, G.: New Antimicrobial Potential and Structural Properties of PAFB: A Cationic, Cysteine-Rich Protein from Penicillium chrysogenum Q176.
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6. Kiss, R., **Fizil, Á.**, Szántay, C.: What NMR can do in the biopharmaceutical industry.
   J. Pharm. Biomed. Anal. 147, 367-377, 2018. ISSN: 0731-7085.
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7. Sonderegger, C., Galgóczy, L., Garrigues, S., **Fizil, Á.**, Borics, A., Manzanares, P., Hegedűs, N., Huber, A., Marcos, J. F., Batta, G., Marx, F.: A Penicillium chrysogenum-based expression system for the production of small, cysteine-rich antifungal proteins for structural and functional analyses.
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8. Bereczki, I., Mándi, A., Rőth, E., Borbás, A., **Fizil, Á.**, Komáromi, I., Sipos, A., Kurtán, T., Batta, G., Ostorházi, E., Rozgonyi, F., Vanderlinden, E., Naesens, L., Sztaricskai, F., Herczegh, P.: A few atoms make the difference: Synthetic, CD, NMR and computational studies on antiviral and antibacterial activities of glycopeptide antibiotic aglycon derivatives.
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9. Binder, U., Bencina, M., **Fizil, Á.**, Batta, G., Chhillar, A. K., Marx, F.: Protein kinase A signaling and calcium ions are major players in PAF mediated toxicity against Aspergillus niger.
   FEBS Lett. 589 (11), 1266-1271, 2015. ISSN: 0014-5793.
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10. Virágh, M., Vörös, D., Kele, Z., Kovács, L., **Fizil, Á.**, Lakatos, G., Maróti, G., Batta, G., Vágvölgyi, C., Galgóczy, L.: Production of a defensin-like antifungal protein NFAP from Neosartorya fischeri in Pichia pastoris and its antifungal activity against filamentous fungal isolates from human infections.
    Protein Expr. Purif. 94, 79-84, 2014. ISSN: 1046-5928.
    DOI: http://dx.doi.org/10.1016/j.pep.2013.11.003
    IF: 1.695

Informational/educational articles (1)

11. **Fizil, Á.**, Gáspári, Z., Batta, G.: Egy gombaellenes gombafehérje. PAF, a bűvös hatóanyag.
    Termész. világa. 148 (6), 265-268, 2017. ISSN: 0040-3717.

Total IF of journals (all publications): 34,584
Total IF of journals (publications related to the dissertation): 14,273

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

29 March, 2018