Metal ion guided protein-DNA interactions: metal ion dependent molecules with potential applications

(1st October 2016 – 30th September 2021)

Final Report

1. Summary of the project

Cure of diseases with genetic origin would be possible by inducing the cells' own DNA repair mechanisms through DNA cleavage by a designed nuclease. The Nobel prize in chemistry has been awarded for a description of the CRISPR/Cas9 system, an artificial nuclease (AN) of this kind in 2020. The extreme specificity of the nuclease within the target cell is essential for therapeutic purpose. The present artificial nucleases do not comply with this condition yet. In our opinion, only the development of suitable control mechanisms, mimicking those functioning in nature can lead to such versatile and specific artificial nucleases.

Therefore, our goal was to explore an artificial nuclease based on the nuclease domain of colicin E7 (NColE7) with multiple control mechanisms applying a concept of allosteric activation at intra-, and intermolecular levels. We proposed a research project in which we aimed at the better understanding of the structural and thermodynamic aspects of the specific actions of various DNA-binding proteins participating either in the recognition of target nucleobase-sequences or in the induction of cleavage or transcriptional events – with the emphasis on the regulation of these processes. We have to remonstrate that it was not our aim to compete with the existing AN technologies, but to develop regulation mechanisms, which can be generalized to improve the performance of the nucleases for gene therapy.

As bioinorganic chemists we used metal ions at all potential levels of the regulation. The main achievements of the project are the (i) elaboration of the intramolecular allosteric activation modulated through the DNA recognition of the zinc finger domain as well as by shaping the coordination environment of the metal ion in the C-terminal catalytic centre of NColE7; (ii) definition of the specific sections of NColE7 that can be suitable for regulated DNA cleavage when fused to the zinc finger protein - it was found that this process can also be influenced by the zinc(II) concentration through further metal binding motifs; (iii) the findings on the effects of various transition metal ions on binding and cleavage of the DNA by zinc finger proteins; and (iv) description of the regulatory role of the amino acids in the neighbourhood of the active centre of the metal ion dependent CueR protein, which can be applied to control the expression of an artificial nuclease at the level of transcription. Although our project mainly involved fundamental research, the results achieved are expected to contribute to the further development of artificial nucleases potentially applied in personal medicine in the future.

The coronavirus restrictions significantly slowed down the research in the last part of the project period. The participating students, PhD students and researchers were partially or fully prevented from entering the university building either by the regulations or because of infection. The restrictions in the purchase of chemicals and the cancellation of international bioinorganic conferences made the planning of the experimental work and presenting the results difficult and ambiguous. Planned research trips to e.g. Japan and Denmark have been cancelled

due to the coronavirus, and we can still only hope to be able to continue our successful collaborations. Therefore, we focused on finishing and publishing those manuscripts, for which we had enough experimental work. Three manuscripts are still under preparation to be submitted before the end of 2021 while further publications will follow after finishing necessary experiments in collaborating laboratories.

Despite of the above described difficulties, the research throughout the grant period was conducted according to the workplan. 18 papers in total have been published or submitted to international journals and a few more are under preparation; 34 lectures and/or posters were presented at international conferences; 15 lectures at Hungarian conferences (only those conference presentations are included in the publication list at the end of the document, which are related to results not included in the published papers, but are to be published in the near future), 6 lectures at national OTDK (three 1st prizes; prize of Hungarian Chemical Society and prize of the Pro Scientia Society). Three PhD dissertations have been accomplished and successfully defended by the project participants Edit Mesterházy, Ria K. Balogh and Heba Abd Elhameed, while Bálint Hajdu is currently putting together his PhD dissertation based on the results obtained within the frame of this project. 5 MSc and 7 BSc diploma works have been completed in connection with our research.

2. Results

The results which are still to be published are described in more detail, as compared to those already published. The project involved four main work packages.

2.1. Improvement of the allosteric control by understanding the dynamic nature of the effect of the N-terminal sequence on the enzymatic activity of NColE7, i.e. allosteric activation, by NMR

The mechanism of DNA digestion by NColE7 (the nuclease domain of colicin E7) is not perfectly understood yet. The positively charged side-chains at the N-terminus of NColE7 are essential for the nuclease activity and the N-terminal loop extensively interacts with the catalytic centre. NMR measurements provided data on the structure and dynamics of NColE7 and its mutants in the presence and absence of Zn^{2+} and/or DNA.

Most of the planned NMR measurements were carried out, but the collaboration with the NMR laboratory was slowed down due to coronavirus restrictions and we could not finish all experiments and data evaluation. The labelled proteins necessary for the experiments have been expressed in labelled minimal media and purified in suitable amount for the measurements. We have assigned the resonances of the backbone atoms by 3D NMR experiments for the NColE7 and ΔN4-NColE7 proteins. Their secondary structures were estimated and compared to the available crystal structures. We also determined the change in the protein dynamics as a consequence of mutations. The longitudinal relaxation rate (R1), the transverse relaxation rate (R2), and the heteronuclear Overhauser effect (hNOE) for two-spin subsystems of each amide bond provided information about the fast backbone motions, i.e. about the flexibility of the protein. The protein flexibility did not change significantly by the removal of the positively charged N-terminal segment. The zinc(II) binding/removal also did not affect heavily the above mentioned parameters, with the exception of the close surroundings of the metal-binding His573 amino acid, which is also disordered in the single crystal of the apoprotein. This suggests that the difference in the catalytic activity between the native and the mutated protein lies in the fine structure and dynamics of the active centre residues. We plan to carry out the last experiments focusing on the histidine and arginine side-chain nitrogens, which may provide answer on the direct role of the N-terminus. Because of the precipitate formation

in the DNA containing protein solutions we decided to continue our experiments in phosphate buffer mimicking the DNA. These results are needed for publishing a good quality paper. A young researcher, Enikő Hermann working on this part of the project has left our research group to do her PhD in Vienna, which makes the continuation more difficult, but we will put extra effort to accomplish the manuscript as soon as possible. New batches of labelled proteins have been synthesized and purified for these experiments to be carried out in the near future.

Zita Fábian, an MSc student crystallized N-terminal NColE7 mutants in the presence of DNA (Fig. 1), supplementary to the NMR experiments, in collaboration with professor Sine Larsen form the University of Copenhagen.

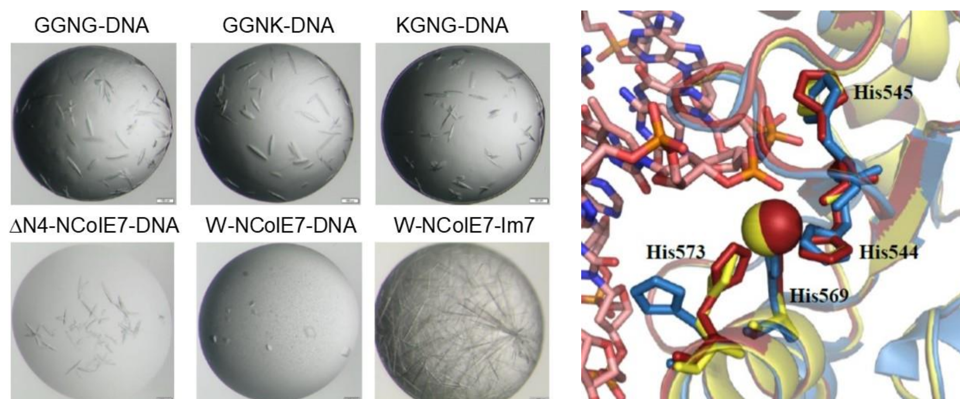


Fig. 1. Right: Results of the crystallization experiments of various NColE7 mutants in the presence of DNA. Left: Comparison of the active centre of the various NColE7 mutants in the presence of DNA. The new GGNK/DNA is in red and the KGNK/DNA is in yellow. They are compared with a zinc(II)ion deficient mutant structure D493Q-NColE7/DNA (blue), published in the literature (PDB id: 3FBD).

As shown in Fig. 1, the metal ion binding residues of the N-terminal mutants are closely situated around zinc(II). The His545 side chain is responsible for the nucleophile generation by deprotonating a water molecule. The plane of this imidazole ring is changed in the N-terminal mutants lacking the arginine at the N-terminus. Thus, its orientation may be fixed by the N-terminal arginine, which can be crucial for the efficient catalytic activity. These experiments were included in BSc and MSc diploma works, OTDK works and presented at scientific conferences [4,12]. We plan to publish the NMR and X-ray results with protein/DNA complexes in an international journal, as well. During the crystallization experiments we have monitored the behaviour of the protein DNA or Im7 immunity protein complexes in solution, too. At low concentrations, needed for circular dichroism spectroscopic studies, the solubility issues did not disturb the experiments. We demonstrated that under cocrystallization conditions the results reflect induced protein folds instead of the (partially) disordered original structures. The analysis of synchrotron radiation circular dichroism spectra revealed that the Im7 immunity protein stabilizes the native-like solution structure of unfolded NColE7 nuclease mutants via complex formation. This is consistent with the fact that among the several available crystal structures with its inhibitor or substrate, all NColE7 structures are virtually the same. Our results draw attention to the possible structural consequence of protein modifications, which is often hidden by compensational effects of intermolecular interactions. These results were published in [1].

2.2. Optimization of the allosteric activation by the N-terminus of NColE7 to enhance the nuclease specificity

The active centre and the N-terminal regulating parts in NColE7 are separated by the non-specific DNA binding site, which can be replaced or complemented by a specific DNA binding array in ANs. A precise molecular design is needed to achieve the proper relative steric position of the catalytic and regulator sequences. Thus, the latter can activate the enzyme exclusively upon binding to the selected DNA sequence. We have constructed the genes of three artificial nucleases (ANs) and synthesized the proteins based on the initial computational design. These enzymes showed low, but specific nuclease activity, performing single strand DNA cleavage. The detailed investigation of the designed artificial nucleases has been carried out and the primer prolongation assay was optimized for the efficient study of the nicking activity of the enzymes. These results were published [6] and several lectures have been presented at OTDK (first prize for Zita Fábián MSc student) and research conferences [16]. We have continued the study of the NColE7-based ANs to improve the regulation of the enzymes. The genes of the so called straight models have been constructed. This means that the catalytic centre is placed at the N-terminus of the zinc finger while the regulatory part is now at the C-terminus, as they were computationally designed. During this procedure the length of both sequences were varied in a way that one amino acid has been removed from the whole NColE7 sequence at various positions. We obtained several Cx-ZF-Ny constructs where x+y=130 amino acid residues (Fig. 2).

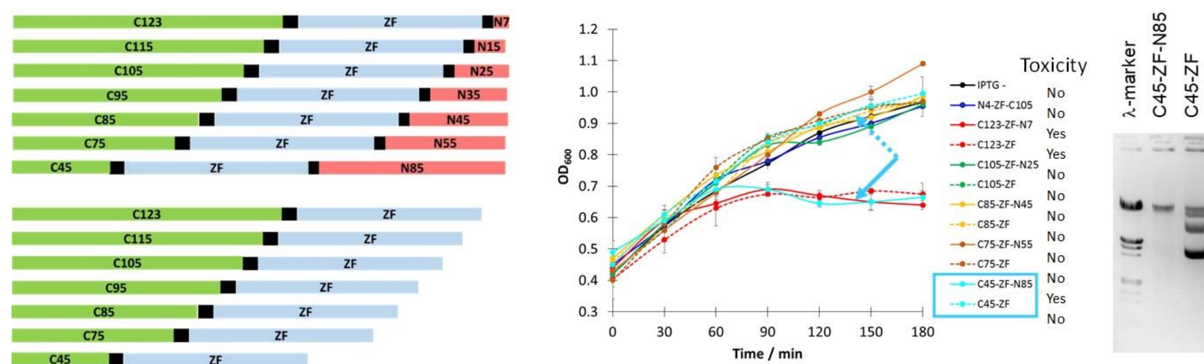


Fig. 2. Right: Scheme of the straight model AN constructs. Middle: bacterial growth during the AN expression. Left: agarose gel electrophoresis investigation of the effect of ANs on the plasmid DNA within the bacterial cells.

Bacterial cells served as detectors of the toxicity of the designed artificial nucleases. The decreased growth is related to increased toxicity. Among several constructs we could either detect the toxicity even without the regulatory part, or the ANs were inactive. The only difference in the activity among the nuclease pairs with or without the regulatory sequence was found for the C45-ZF-N85 / C45-ZF pair. The C45-ZF protein lacking the regulatory part could not cleave the DNA supporting that the allosteric activation in this protein is functional. Furthermore, we could demonstrate that the toxicity of the C45-ZF-N85 construct is indeed related to the DNA cleavage within the bacterial cells. However, the DNA cleavage of the C45-ZF-N85 proved to be nonspecific. We have carefully optimized the purification and the conditions of the catalytic experiments to understand the requirements for the specific action. It was very hard to achieve an acceptable purity of the protein without using any affinity tag.

As a continuation of these experiments, based on the results of molecular dynamic simulations carried out in collaboration with professor Chris Oostenbrink, we identified mutations, which could lead to an optimal artificial nuclease in terms of specificity. Young researchers, Enikő Hermann and Réka Csáki, established two mutant enzymes (R447G and W464A). The new proteins have been purified and characterized, and the catalytic studies

revealed promising results. We have optimized the conditions of the DNA cleavage by the C45-ZF-N85 protein that was able to carry out DNA cleavage within a plasmid DNA containing the recognition sequence, while keeping the nonspecific DNA intact (Fig. 3). The preparation of a manuscript from these results is in progress.

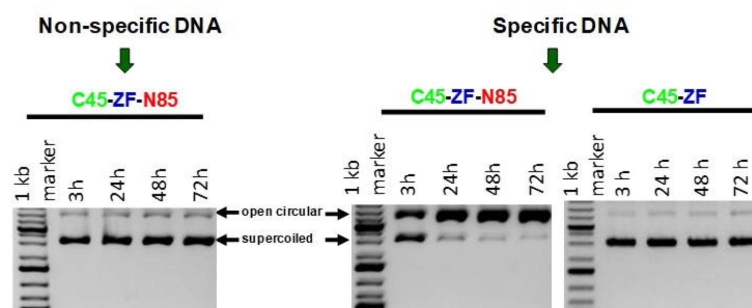


Fig. 3. Agarose gel electrophoresis monitoring of the plasmid DNA cleavage by the C45-ZF-N85 / C45-ZF pair artificial nuclease.

In the above experiments it was essential to obtain the precise amino acid sequences of the proteins, as they were designed. However, protein purification is usually carried out by affinity chromatography using tags, which can not be removed without some amino acids leftover in most of the cases. These may seriously affect the activity of the designed artificial nucleases. The Ni(II)/Cu(II)-inducible cleavage of the peptide bond formed the basis of a new method for protein purification, which we elaborated and published [13]. We described a method for affinity purification of an NColE7 mutant metallonuclease displaying its precisely determined native termini without any remnant amino acids. The gene encoding the target protein was inserted into a newly designed cloning site containing two self-eliminating BsmBI restriction enzyme sites. As a consequence, the engineered DNA code of the Ni(II)-sensitive Ser-X-His-X motif is fused to the 3′-end of the inserted gene followed by the gene of an affinity tag for protein purification purpose. Then the C-terminal segment, starting from Ser, is cleaved off from the purified protein by a Ni(II)-induced protease-like action. The success of the purification and cleavage was confirmed by gel electrophoresis and mass spectrometry, while the structural integrity of the purified protein was verified by circular dichroism spectroscopy. Our new protein expression DNA construct is an advantageous tool for protein purification when the complete removal of affinity or other tags, without any remaining amino acid residue is essential. The new procedure can easily be generalized and combined with various affinity tags at the C-terminus for chromatographic applications.

We have also discovered that the C-terminal metal ion affinity tag fused to the AN protein could influence the catalytic activity. The C45-ZF-N85 type artificial nuclease activity was inhibited by the 6×His tag, whereas the nuclease was activated when the tag was cleaved off, providing a new tool for the multiple regulation approach. PhD students Bálint Hajdu and Heba Alaa Eldeen [23] contributed to the studies presenting and discussing this phenomenon with the NColE7 and its mutants, published in J. Inorg. Biochem. [18] and presented at international conferences. The spectroscopic, mass spectrometric and catalytic experiments revealed that the effect of the C-terminal affinity tag is multiple. The inhibitory effect partially arises from the 6×His-tag that binds to the metal ion in the catalytic centre, thereby preventing its interaction with the substrate. We have found that this effect can be regulated by the level of zinc(II) concentration. The catalytic activity showed a maximum at around 2-3 equivalents of the added zinc(II) ions, when the 6×His tag is saturated by the added zinc(II) and residue H545, responsible for the nucleophile generation, is still mostly in its free state.

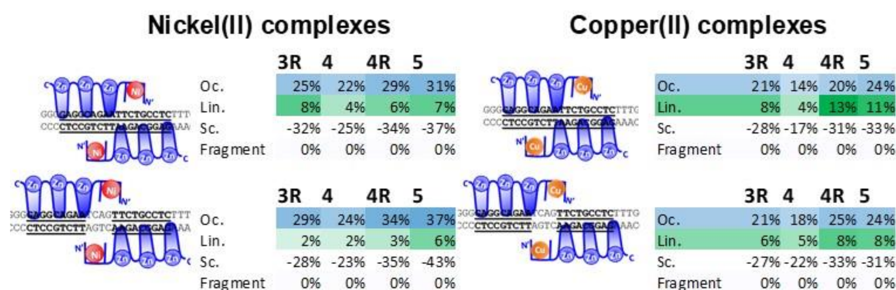
2.3. Optimization of the DNA recognition specificity of the ANs including primarily designed zinc finger (ZF) proteins

ZF proteins can be designed to specifically recognize target DNA forming the basis of a modular AN platform. We modified the ZF recognition sequences to optimize the interactions between the terminal segments and to act cooperatively in a dimer-like construct, providing enhanced regulation and specificity. We mapped the competition phenomena between the specific/non-specific DNA binding sites, as well as between the DNA binding proteins participating in packaging the DNA (e.g. histones) and ANs. An important notice was that histone proteins efficiently supressed the non-specific DNA binding of the zinc fingers in the test-tube experiments. This suggests that the binding and thus, the nuclease activity within the cells containing packaged DNA are more specific than under the ‘sterile’ conditions including the ‘naked’ DNA and the artificial nucleases.

As the first step leading to the study of the zinc finger specificity without compromising the recognition domain by an additional protein string from the NColE7 was to elaborate an artificial nuclease based on the N-terminal ATCUN motif, which can be established in situ by a specific cleavage of the protein sequence at the N-terminus by nickel(II) ions. Such motif will mark the DNA upon DNA binding of the zinc finger. A manuscript has been published in Metallomics from this topic [7] and the results have been presented at OTDK (first prize for Bálint Hajdu) and research conferences [17].

We have monitored the DNA cleavage activity of the new zinc finger artificial nucleases based on the in situ obtained N-terminal ATCUN motif. The DNA damage was minor under various conditions. Therefore, we have designed new proteins varying the length and amino acid composition of the linker sequence between the DNA binding and catalytic domains. A library of new genes were constructed and the new proteins have been expressed and purified by metal ion affinity chromatography. While trying to exchange the metal ion to copper(II) within the ATCUN motif we faced difficulties with slow reactions. Therefore, to avoid nickel(II) binding in the ATCUN motif we optimized the purification procedure to be based on the interaction of cobalt(II) with His tag. In this way, we could carry out metal ion exchange and establish a stable zinc finger protein with copper(II) containing ATCUN motif, as well. The CD spectroscopic study of the metal ion binding of the new proteins revealed that the zinc finger structure is characteristic for the zinc(II)-bound form. CD spectroscopy is an essential tool of studying the formation of metal complexes. It can also provide closer insight into the neighbourhood of the metal ion. Such a CD study on the copper(II) His peptide complexes as active site models revealed multiple metal ion complexation isomerism [2].

Catalytic experiments were carried out by PhD student Bálint Hajdu and two young researchers, Réka Csáki and Gábor Tóth using the zinc finger nucleases with nickel(II) or copper(II) in their active centre (Fig. 4).



Nickel(II) complexes (upper):

	3R	4	4R	5
Oc.	25%	22%	29%	31%
Lin.	8%	4%	6%	7%
Sc.	-32%	-25%	-34%	-37%
Fragment	0%	0%	0%	0%

Copper(II) complexes (upper):

	3R	4	4R	5
Oc.	21%	14%	20%	24%
Lin.	8%	4%	13%	11%
Sc.	-28%	-17%	-31%	-33%
Fragment	0%	0%	0%	0%

Nickel(II) complexes (lower):

	3R	4	4R	5
Oc.	29%	24%	34%	37%
Lin.	2%	2%	3%	6%
Sc.	-28%	-23%	-35%	-43%
Fragment	0%	0%	0%	0%

Copper(II) complexes (lower):

	3R	4	4R	5
Oc.	21%	18%	25%	24%
Lin.	6%	5%	8%	8%
Sc.	-27%	-22%	-33%	-31%
Fragment	0%	0%	0%	0%

Fig. 4. Selected promising nuclease activity test by the series of ATCUN motif containing zinc fingers. 3R, 4, 4R and 5 are zinc finger proteins with different linkers between the protein and the ATCUN motif. Four different DNA target sequences were applied.

From these results we could conclude that the most efficient DNA damage was obtained using short linkers, with increasing number of positively charged amino acid residues between the ZF DNA recognition domain and the ATCUN motif [20].

While investigating the zinc finger proteins and their interactions with DNA, we have found that fundamental data about the metal ion binding of these ligands are scarce in the literature. Therefore, we have introduced a new fluorescent method of measuring the metal ion binding of the new proteins, besides applying CD spectroscopic experiments. For these fluorescence measurements we could efficiently use a new plate reader instrument purchased within the frame of the GINOP-2.3.2-15-2016-00038 project. We have observed that non-cognate metal ions compete with zinc(II) for the zinc fingers, which can contribute to the control of the zinc finger-based artificial nuclease action. The results obtained using e.g. silver(I) ions (which may originate from silver nanoparticles frequently applied recently in e.g. medical devices or bone implants and even daily used products, for their antibacterial properties) has been included in a manuscript to be submitted in the next days. (K. Kluska, G. Veronesi, A. Deniaud, B. Hajdu, B. Gyurcsik, W. Bal, A. Krężel: Resolving the structure of silver fingers by high-resolution X-ray absorption spectroscopy – new insight into functional damage of zinc fingers.) We have demonstrated that silver(I) ions can replace zinc(II) from the active centre of our zinc finger protein, thereby ceasing the protein DNA interactions. Bálint Hajdu PhD student has applied various methods (ITC, fluorescence, UV-Vis absorption and CD spectroscopy, mass spectrometry) to study the zinc(II) binding affinity and competition in metal ion binding with various toxic metal ions, such as Ag(I), Hg(II), Cd(II), Ni(II). He is currently constructing his PhD dissertation from the results of the above experiments and in parallel, we are in the process of preparing at least two more manuscripts in the close future.

In the meantime Zeyad Nafaee PhD student has made a progress on his experiments with another specific DNA binding protein, nuclear factor I, which promotes the replication of Adenovirus, and thus can be converted into an antiviral artificial nuclease as the proof of principle. He has optimized the protein expression conditions and the purification by HPLC.

Experiments with CRISPR/Cas9 were carried out by PhD student Heba Alaa to provide a comparison for the newly designed NColE7-based ANs. Based on the results of these experiments, we have also modified the zinc finger DNA binding domains to target a selected DNA sequence within the ErbB1 oncogene. From the bacterial cell growth experiments including several C45-ZF-N85 and C45-ZF genes (with various ZF sequences arising from the stepwise change of each zinc finger motif), we could conclude that the C45-ZF-N85 type artificial nuclease construct is robust against the changes in the zinc finger motifs. Thus, it can be easily redesigned for various target DNA sequences, while keeping the catalytic efficiency and specificity. During the experiments with the CRISPR/Cas9 system, we have recognized that the cargo of the artificial nuclease into the cancer cells has to be optimized concerning the efficiency and cytotoxicity. In collaboration with our colleagues from colloid chemistry and biochemistry fields we have developed a new water-soluble cationic lipopolymer carrier, which proved to be more efficient and less cytotoxic than the commercially available control transfection agent. These results have been published [21]. Next we are going to compare the two artificial nuclease systems. The best performing NColE7-based ANs will be subjected to assays monitoring their controlled and specific behaviour in cells and their ability to induce DNA repair in the laboratory of our Japanese collaborator, prof. Kyosuke Nagata, once research trips will be allowed after the coronavirus restrictions.

2.4. Exploring the possibility of additional control of the nuclease action by mapping the metal ion selectivity of MerR and ArsR transcriptional regulators

In line with the workplan, it was investigated whether an additional control of the nuclease action by utilizing metal ion regulated transcriptional switches is possible. This would allow the promotion of nuclease action in a strictly controlled manner e.g. by increasing the cellular level of a specific metal ion. In order to develop such a controlling route, we studied the metal ion binding to native and modified metalloregulatory proteins and their model peptides. These results led to the better understanding of the selective signal transmission from the metal ion binding domain though the protein to DNA, allowing for the modulation of the regulation processes.

Systematic studies on the structure of a MerR family protein CueR and its interaction with DNA and/or metals by various methods, including e.g. gel shift assays, ESI-MS, UV-Vis, CD- and PAC (Perturbed Angular Correlation of gamma-rays) spectroscopies were carried out, both through the investigation of the protein itself and through its model compounds. PAC spectroscopy has inevitable values in investigation of protein metal sites. The knowledge about its applications in bioinorganic chemistry was reviewed in [3]. CD experiments with the CueR protein indicated an unexpected phenomenon, i.e. a dramatic structural change of the protein upon the change of pH from 8.0 to 6.0. In parallel we observed that the presence of metal ions and DNA has a large influence on this process [25]. We explained these results by the pH sensitivity of the histidine side-chains that alter significant structure stabilizing electrostatic interactions upon their protonation. Crystallization experiments were conducted in collaboration with Prof. Beáta Vértessy. The promising protein crystals were subjected to X-ray crystallography at a synchrotron source. The evaluation of the results was hindered by the coronavirus situation, but it is in progress. In order to forward the understanding of the regulatory mechanism governed by CueR we have constructed mutants of the protein and optimized their purification procedures. The properties of the CueR protein and its mutants were investigated focusing on the role of the C-terminal CCHH motif. CD spectroscopic experiments revealed that the ΔC7 mutation did not significantly affect the secondary structure composition of the protein. MS experiments showed that up to two Hg(II) ions can bind to CueR, while ΔC7-CueR accommodates only one Hg(II). UV absorption and ^{199m}Hg PAC spectra indicated HgS_2 structure at both the functional and the CCHH metal site. However, at sub-equimolar concentrations of Hg(II) at pH 8.0, the metal binding site displays an equilibrium between HgS_2 and HgS_3 structures, the latter involving cysteines both from the metal ion binding loop and the CCHH motif. Form these results we could hypothesize that the C-terminal CCHH motif provides auxiliary ligands that coordinate to Hg(II) and thereby prevents activation of transcription by the non-cognate divalent metal ion, such as Hg(II) [15]. The properties of Hg(II) in binding to Cys-thiolate residues were studied in more details via other systems to contribute and support the results obtained with the CueR variants [14,24]. These experiments were expanded to investigating the binding of the cognate metal ion Ag(I) to CueR and its mutants. ^{111}Ag PAC measurements revealed that the flexibility of the metal binding site is crucial in the activation of the transcription by this protein. We have already published the new results in a paper [19] and another one has been submitted [26]. By this we could contribute to the better understanding of the metal ion selectivity of the regulatory mechanism of CueR. Ria Katalin Balogh participated mainly in these studies, and accomplished her PhD in this topic [22].

Next we are going to apply the collected knowledge to regulate the expression of the nuclease enzymes by CueR in a two plasmid systems. Eszter A. Fodor, a PhD student, continued these experiments after Ria K. Balogh finished here PhD studies and left to Denmark.

We have also applied model compounds in this project. The complex forming behaviour of model peptides of the metal ion binding domain of CueR were studied directly with copper(I) ions by spectroscopic methods and the results were published [8,10,11]. Edit Mesterházy PhD student participated in these experiments and has accomplished her PhD in this topic. Studies on the interaction of As(III) with ArsR model compounds have also been carried out and published with the participation of Levente I. Szekeres PhD student [5,9]. The above results have also been presented at several scientific conferences.

3. Personnel

During the whole time interval of the project we have been supporting the carrier of young researchers by employing them until they found an appropriate funding or job, helped them to improve their network building capabilities by supporting their participation at national and international conferences, as well as by arranging numerous study trips abroad. The following young researchers participated in the research project: Abd Elhameed Heba Alaa Eldeen Hosiny, Balogh Ria Katalin, Csáki Réka, Czene Anikó, Hajdu Bálint, Hermann Enikő, Mesterházy Edit Éva, Nafae Zeyad, Németh Eszter, Szekeres Levente István, Szunyogh Dániel Mihály, Tóth Eszter, Tóth Gábor, Wéber Edit, Fodor Anna Eszter. Besides, a large number of MSc and BSc students has also joined our research group.

4. Publications acknowledging the funding within the K16_120130 project

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