COLICIN E7 NUCLEASE DOMAIN, AS A POSSIBLE BASIS OF ARTIFICIAL NUCLEASES

Ph.D. Thesis

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Introduction and the aim of the work

Nucleases cleave the phosphodiester bonds of the nucleic acids at specific or non-specific sites. Enzymes recognizing specific DNA sequences are applied in the recombinant DNA technology, among them the most popular ones are the bacterial restriction endonucleases. Targeted cleavage of the DNA is carried out with these enzymes. These natural nucleases provide a broad scale of the recognized specific sequences, however, it is far from being satisfactory in the case of DNA molecules as large as the genomes of different organisms, including the human genome. The recognized sequences are usually short and occur frequently in a long DNA. In order to perform the hydrolytic cleavage at an arbitrarily chosen unique DNA sequence highly specific artificial nucleases with new recognition sites, different from their natural counterparts are needed. Nevertheless, the study of these enzymes provides useful information for the design of artificial nucleases.

Efficient targeting of genes *in vivo* provides a potential tool for gene therapy. Genes can be regulated, knocked out or in, or the mutated genes can be corrected by means of agents binding to and/or cutting specific DNA sequences. An obvious way to achieve this is to utilize the cell's own repair machineries, i.e. the various recombination processes in a directed manner. Out of these processes the nonhomologous end joining (NHEJ) leads to a targeted mutagenesis, while the homology-directed repair (HR) mechanism functions in the presence of a template DNA of correct sequence or of tandem repeats of DNA.

To obtain artificial enzymes with new sequence specificity the random or systematic mutation of the DNA binding amino-acids in the wild-type enzymes (natural endonucleases) or DNA binding proteins (e.g. the zinc-finger proteins) is performed. The latter proteins may serve, similarly to the independent DNA binding domains of native enzymes for the construction of chimeric enzymes.

A non-toxic artificial nuclease, which is able to recognize specific DNA sequence to cleave that, possesses huge impact in the field of gene therapy. Two strategies are applied in design of such an enzyme: modification of the wild type one recognizing specific sequence or the creation of chimeric enzymes, as a combination of protein domains recognizing specific DNA sequence with a nuclease domain forming an independent functional element. Nowadays applied artificial nucleases consist of zinc-finger proteins as specific-sequence-

recognizing parts and the nuclease domain of FokI restriciton endonuclease. Although these enzymes have been applied successfully in a number of gene targeting experiments, they also have a cytotoxic effect due to the unwanted non-specific cleavages. Therefore, the development of a well-controlled artificial nuclease is still an important issue. Hence my PhD thesis is about finding a nuclease domain that works in a controlled manner. In ideal case such an enzyme stops working as a result of the (partial) degradation within the cell. According to the literature review, the HNH motif as a well conserved catalytic element could be eligible for this type of nuclease. Colicin E7 metallonuclease containing this motif at its C-terminal end was examined.

From the above mentioned reason, in the first part of my PhD thesis the catalytic centre of the nuclease domain of Colicin E7 bacterial toxin (NColE7) was examined. There is a well-conserved, ββα-type metal binding motif (HNH motif) at the C-terminus of this protein, where a Zn^{2+}-ion coordinates to three His residues (**HH**X_{14}**N**X_8**H**X_3**H**). The conserved amino acids are usually supposed to have an important biological role, based our suggestion it is the nuclease activity itself. **That is why the expression of the 42 amino acid long HNH motif was the first goal of my thesis**.

The HNH motif itself did not show nuclease activity. It seemed obvious that it may require the DNA binding protein sequences of the NColE7 to forma n active enzyme. In the second part of my thesis truncated mutants of NColE7 have been expressed to determine the minimal sequence responsible for the nuclease activity. Examination was carried out on the intermolecular interactions, structural network, metal ion and DNA binding ability of the mutants. **The aim of the second part of my thesis was to express ΔN69-NColE7, ΔN45-NColE7, ΔN25-NColE7 and ΔN4-NColE7 proteins in order to examine their metal, - and DNA binding ability with gel electrophoresis, mass spectrometry, circular dichroism,- and fluorescenc spectroscopy.**

The purpose of the third part of my project was to determine the sructure of ΔN4-NColE7-C* mutant. X-ray crystallography provides information about the solid phase structure, while NMR spectroscopy provides information about the structure in solution. Production of a protein crystal is a time consuming and difficult task: besides the expression and the succesful purification of the protein many different conditions has to be tried, while

NMR measurement require the expression and purification of ^{13}C- and ^{15}N-isotope enriched protein. **That is why our aim was to crystalize both ΔN25-NColE7 and ΔN4-NColE7-C* proteins and express labelled ΔN4-NColE7* protein for NMR measurement. The structure determination of these mutant compared to the NColE7 published data may provide information about the importance of the missing N-terminal part and its role in the catalytic activity. The obtained knowledge may lead us to a starting point in design of an artificial metallonuclease.**

Applied methods

Recombinant DNA technology (Polymerase Chain Reaction-PCR, gel electrophoresis, ligation of bacterial vectors, transformation of bacteria) was applied for the production of the different NColE7 mutant proteins. The purification of the proteins carried out with ion-exchange, affinity, and gelfiltration chromatographic methods. The purity of the proteins were approved with SDS-PAGE and mass spectrometry. Fluorescence and circular dichroism spectroscopy, mass spectrometry, gel mobility shift measurements were applied to examine the Zn^{2+} and DNA binding ability, as well as, thenuclease activity of these mutants. The structure determination of the truncated mutant lacking only the four N-terminal amino acids was carried out via X-ray chrystallography and the production of ^{13}C and ^{15}N labelled protein for NMR measurements was carried out.

New scientific results

Expression and examination of the HNH motif

1. The HNH motif of Colicin E7 protein was sucessfully expressed as a Ub-HNH fusion protein. The optimized purification process resulted 25 mg protein per one liter LB medium.

2. Weak Zn^{2+}-binding ability of this protein was detected by mass spectrometry and circular dichroism spectroscopy. Not even in the excess of Zn^{2+} the Zn^{2+}-protein complex was quantitively formed.

3. The expression of the HNH motif without fusion protein was carried out. An opimized purification method was elaborated resulting in 2,5 mg protein per one liter LB medium.

4. The study of the HNH motif also showed a weak interaction with the Zn^{2+}-ion (CD, MS measurements were carried out). This suggested that the Ub fusion part did not have any disturbing effect on the metal-binding ability. The structure of the HNH-motif has changed upon addition of the metal-ion, but the structure predicted from the published NColE7 crystal structures was not completely formed – based ont he information obtained from the CD spectra.

5. Neither DNA-binding ability nor nuclease activity was detected with the HNH motif.

Expression and investigation of various NColE7 mutants

6. Four different N-terminally truncated NColE7 mutants were overexpressed in *E. Coli*, showing the fact that none of them have cytotoxic activity.

7. Purification method was optimized for two mutants (ΔN25-NColE7, ΔN4-NColE7), which resulted 8 mg of each protein per one liter LB medium.

8. The Zn^{2+}-binding ability of the mutants showed differences according to MS, CD and fluorimetry. The interaciton of the shorter mutant (ΔN25-NColE7) with the metal-ion was

weaker than with the longer one (ΔN4-NColE7-C*). The latter was able to bind the metal-ion during the whole purification process. These results proved, that the N-terminal loop stabilizes the structure of C-terminal catalytic centre and contributes to the binding of the Zn^{2+}-ion.

9. In their DNA binding abilities these proteins also showed differences. The ΔN4-NColE7-C* protein was able to form stronger iteraction with the DNA molecule than the shorter mutant (ΔN25-NColE7). However, in spite of its strong DNA and metal ion binding it was not able to cleave DNA *in vitro*. Based on these results the N-terminal positively charged amio acids play a key role in the catalytic process of the C-terminal end. This feature could be useful for designing an artificial metallonucleas, which is able to work under allosteric regulation.

Structure determination of ΔN4-NColE7-C* mutant

10. A protein-crystal was grown from the purified ΔN4-NColE7-C* mutant, and X-ray diffraction measuerments were carried out up to a resolution of 1.6 Å.

11. The structure determination of this protein proved the steric stabilization effect of the N-terminal amino acids through an extended hydrogen bond network on the HNH motif.

12. Based on the comparison of the crystal structure with that of the NColE7 protein, we suggest that the N-terminal Arg residue could be a flexible proton source in the catalysis as a general acid to protonate the leaving group.

13. For the expression of the ΔN4-NColE7 mutant in order to perform future NMR measurements, we optimized a new protein expression method, which provided 2,7 mgs of ^{13}C and ^{15}N isotope-enriched protein.

14. The measurements of the NMR spectra are under way. The spectra int he presence and absence of Zn^{2+}-ions show stable structure of the ΔN4-NColE7 mutant in solution.

Publications related to the dissertation

Publication in international journals:

1. B. Gyurcsik, A. **Czene**: Towards artificial metallonucleases for gene therapy: recent advances and new perspectives.
Future Med. Chem., 3, 1935–1966 (2011)
IF = 2.522

2. A. **Czene**, E. Németh, I.G. Zóka, N.I. Jakab-Simon, T. Körtvélyesi, K. Nagata, H.E.M. Christensen and B. Gyurcsik: On the role of the N-terminal loop in the function of colicin E7 nuclease domain.
J. Biol. Inorg. Chem., 18, 309-321 (2013)
IF = 3.289

3. B. Gyurcsik, A. **Czene**, H. Barát-Jankovics, N.I. Simon-Jakab, K. Ślaska-Kiss, A. Kiss and Z. Kele: Cloning, purification and metal binding of the HNH motif from colicin E7
*Protein Exp. Pur.*89, 210-218 (2013)
IF = 1.587

4. A. **Czene**, E. Tóth, B. Gyurcsik, H. Otten, J.-C.N. Poulsen, Leila Lo Leggio, S. Larsen, H.E.M. Christensen and K. Nagata: Crystallization and preliminary crystallographic analysis of an E. coli selected mutant of the nuclease domain of Colicin E7 metallonuclease.
*Acta Cryst. Sect F.*69,551-554 (2013)
IF = 0.510

5. **A. Czene**, E. Tóth, B. Gyurcsik, H. Otten, J.-C.N. Poulsen, S. Larsen, H.E.M. Christensen and K. Nagata: The crystal structure of a mutant of the nuclease domain of Colicin E7 metallonuclease exhibits an introverted N-terminus.
Acta Cryst. Sect D. (2013) in preparation

ΣIF = 7.908

Presentations at international and national conferences

Proceeding:

1. B. Gyurcsik, A. **Czene**, E. Endreffy, and K. Nagata: Chimeric metallonucleases for gene therapy
Insights Into Coordination, Bioinorganic, And Applied Inorganic Chemistry, Monograph Series of the International Conferences on Coordination and Bioinorganic Chemistry held periodically at Smolenice in Slovakia, Ed.: M. Melník, P. Segľa, M. Tatarko, ISBN 978-80-227-3085-3, ISSN 1335-308X, Slovak Technical University Press, Bratislava, 2009, 9, 81-88.

Oral and poster presentations:

1. A. **Czene**, B. Gyurcsik, E. Endreffy, K. Nagata: Novel diagnosis of Duchenne Muscular Dystrophy

Graduate School on Metal Ions in Biological systems (MIBS) – Current Trends and Methods in Bioinorganic Chemistry, 7-10 September, Søminestationen – Holbæk, Denmark, (2009) - poster + oral presentation

2. B. Gyurcsik, N.I. Jakab, A. **Czene**, E. Endreffy, K. Nagata: Artificial Metallonucleases For Gene Therapy
10th International Symposium on Applied Bioinorganic Chemistry (ISABC 10). Sept. 25-28, 2009, Debrecen, Hungary (2009) - section lecture

3. B. Gyurcsik, A. **Czene**, E. Endreffy, K. Nagata: Chimeric metallonucleases for gene correction.
The Tokyo Tech-UNESCO Fellows Symposium for Development of Human Resources and Research Network in Science and Technology, 10-11th December, 2009, Tokyo, Japan (2009) - poster

4. B. Gyurcsik, A. **Czene**, A. Jancsó: The effect of metal ions on the SRCD spectral pattern of peptides and proteins.
ISA/ASTRID User Meeting, Aarhus, Denmark, 28th-29th January (2010), - poster.

5. A. **Czene**, I.N. Jakab-Simon, B. Gyurcsik, K. Nagata, H.E.M. Christensen: New type of chimeric metallonucleases for gene therapy, *DTU Bioinorganic Chemistry Meeting*, Lyngby Denmark, 28th-29th March (2010), - poster

6. B. Gyurcsik, G. Nagy, A. **Czene**, T. Körtvélyesi: Design of a new type zinc-finger chimeric nuclease, *EUROBIC10 - 10th European Biological Inorganic Chemistry Conference*, Thessaloniki, Greece, June 22 - June 26, 2010. - poster

7. B. Gyurcsik, A. **Czene**, N.I. Simon, E. Németh, I.G. Zóka, G. Nagy, T. Körtvélyesi, H.E.M. Christensen, K. Nagata: Study on the HNH motif as a possible component of a controlled zinc-finger nuclease. *International Conference on Gene Targeting*, Vienna, Austria, 9-12th February (2011), - lecture

8. B. Gyurcsik, A. **Czene**, N.I. Simon, E. Németh, I.G. Zóka, G. Nagy, T. Körtvélyesi, H.E.M. Christensen, K. Nagata: Study on the HNH motif as a possible component of a controlled zinc-finger nuclease. *New Trends in Coordination, Bioinorganic and Applied Inorganic Chemistry; XXIII. International Conference on Coordination and Bioinorganic Chemistry*, Smolenice, Slovak Republic, 5-10th June (2011), - lecture

9. B. Gyurcsik, A. **Czene**, I.N. Simon, E. Németh, I.Gy. Zóka, T. Körtvélyesi, H.E.M. Christensen, K. Nagata: Allosteric control in the nuclease domain of colicin E7. *QBIC-3 3rd Quantum Bioinorganic Chemistry Conference*, Cesky Krumlov, Czech Republic, 25-28 June, (2011) - poster

10. A. **Czene**, B. Gyurcsik, E. Endreffy, K. Nagata: Novel diagnosis of Duchenne muscular dystrophy. *4th European Conference on Chemistry for Life Sciences (4ECCLS)*, Budapest, Hungary, August 31- September 3 (2011) - poster

11. B. Gyurcsik, A. **Czene**, N.I. Simon, E. Németh, I.G. Zóka, T. Körtvélyesi, H.E.M. Christensen, K. Nagata: Study on the HNH motif as a possible component of a controlled

zinc-finger nuclease. *4th European Conference on Chemistry for Life Sciences (4ECCLS)*, Budapest, Hungary, August 31- September 3 (2011) - lecture

12. B. Gyurcsik, A. **Czene**, I.N. Simon, E. Németh, I.Gy. Zóka, T. Körtvélyesi, H.E.M. Christensen, K. Nagata: Allosteric control in the nuclease domain of colicin E7, International Workshop on Metal Containing Drugs, Szeged, Hungary, 30-31 August (2011) - lecture

13. B. Gyurcsik, A. **Czene**, N.I. Simon, E. Németh, I.G. Zóka, T. Körtvélyesi, H.E.M. Christensen, K. Nagata: Design of a novel artificial nuclease based on the HNH.
ISABC11 - 11th International Symposium on Applied Bioinorganic Chemistry, Barcelona, Spain, 2-5 December (2011)-poster

14. A. **Czene**, E. Németh, I.G. Zóka, N.I. Simon, B. Gyurcsik, H.E.M. Christensen, K. Nagata: Mutant Colicin E7 proteins reveal the conditions for allosteric control of the enzymatic action.
ISABC11 - 11th International Symposium on Applied Bioinorganic Chemistry, Barcelona, Spain, 2-5 December (2011)-poster

15. B. Gyurcsik, A. **Czene**, E. Németh, I.G. Zóka, E. Endreffy, H.E.M. Christensen, K. Nagata: Targeting the breakpoint in duchenne muscular dystrophy.
Nobel75, 75th Anniversary of Albert Szent-Györgyi's Nobel Prize Award, Szeged, Hungary, 22-25 March (2012)-lecture

16. A. **Czene**, B. Gyurcsik, E. Endreffy: A simple carrier detection method in Duchenne muscular dystrophy affected families.
IIIrd HuRo conference - Genetic diagnostic method developments, Szeged, Hungary, 27th April (2012) - lecture

17. B. Gyurcsik, A. **Czene**, E. Németh, I.G. Zóka, E. Endreffy, H.E.M. Christensen, K. Nagata: Targeting the breakpoint in Duchenne Muscular Dystrophy
ISMEC 2012, International Symposia on Metal Complexes, Lisbon, Portugal, June 18th – 22nd, ISMEC Acta, Vol. 2, p.111 (2012)-poster

18. B. Gyurcsik, A. **Czene**, E. Németh, I.G. Zóka, E. Endreffy, H.E.M. Christensen, K. Nagata: Targeting the breakpoint in Duchenne Muscular Dystrophy by an artificial metallonuclease.
EUROBIC11, 11th European Biological Inorganic Chemistry Conference, Granada, Spain September 12-16 (2012) - poster

19. B. Gyurcsik, A. **Czene**, E. Németh, I.Gy. Zóka, E. Endreffy, H.E.M. Christensen, K. Nagata: Design of a novel artificial metallonuclease for targeting the breakpoint in Duchenne Muscular Dystrophy.
The 3rd Leading Graduate Schools International Conference, Tsukuba, Japan, November 1-2 (2012), invited lecture

20. E. Endreffy, A. **Czene**, B. Gyurcsik: Carrier diagnostics with different molecular genetic methods in dystrophinopathy.
Newborn screening and molecular diagnostics of inherited disorders - HuRo conference Szeged, January 25, (2013)-Lecture

21. **Czene** A., Gyurcsik B.: A Duchenne féle izomdisztrófia újfajta diagnosztikája
XXXI. Kémiai Előadói Napok, 2008. október 29-29, Szeged.

22. Németh E., Zóka I.Gy., **Czene** A., Jakab-Simon I.N., Gyurcsik B., Körtvélyesi T., Nagata K., Christensen H.E.M.: A colicin E7 fehérje nukleáz doménjének vizsgálata.
MKE, 1. Nemzeti konferencia, Sopron 2011. május 22-25 - előadás

23. **Czene** A., Gyurcsik B.: A Duchenne-féle izomdisztrófia újfajta diagnosztikája
MKE, 1. Nemzeti konferencia, Sopron 2011. május 22-25 - poszter

24. Zóka I. Gy., Gyurcsik B., **Czene** A., Németh E.: A colicin E7 nukleáz N-terminális végén rövidített mutáns fehérjéinek vizsgálata.
XXXIV. Kémiai Előadói Napok, Szeged, 2011. November 2-4 - előadás

25. Gyurcsik B., **Czene** A., Németh E., Tóth E., Zóka I.Gy.: Aktiváló allosztérikus szabályozás kialakításának lehetősége egy Colicin E7 alapú mesterséges metallonukleázban.
46. Komplexkémiai Kollokvium és az MTA Koordinációs Kémiai Munkabizottság ülése, 2012. május 21-23, Mátrafüred - előadás

26 **Czene** A., Gyurcsik B., Christensen H.E.M.: Colicin E7 metallonukleáz fehérje mutánsainak előállítása szerkezetmeghatározás céljából.
46. Komplexkémiai Kollokvium és az MTA Koordinációs Kémiai Munkabizottság ülése, 2012. május 21-23, Mátrafüred - előadás

27 Tóth E., **Czene** A., H. Otten, J.-Ch. Navarro-Poulsen, S. Larsen, Gyurcsik B.: A ΔN4-NColE7 metallonukleáz mutáns kristályszerkezete.
46. Komplexkémiai Kollokvium és az MTA Koordinációs Kémiai Munkabizottság ülése, 2012. május 21-23, Mátrafüred - előadás