Theses of Ph.D. dissertation

TRANSCRIPTIONAL ACTIVATOR CUER PROTEIN: PURIFICATION, CHARACTERIZATION AND POTENTIAL BIOANALYTICAL APPLICATION

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1. INTRODUCTION

In living organisms, highly specialized processes have been evolved to secure metal ion homeostasis and to protect the cells from harmful effects. In prokaryotes these processes are managed by metal ion selective metalloregulatory proteins at the level of transcription. In this thesis, the metal ion binding features and the structure of the transcriptional activator CueR (Cu efflux Regulator) protein (Figure 1) were investigated. The results presented here contribute to the understanding of the details of bacterial metal ion regulatory mechanisms, and may also forward the design of molecules for selective metal ion binding/accumulation or the development of sensitive metal ion-detection methods.

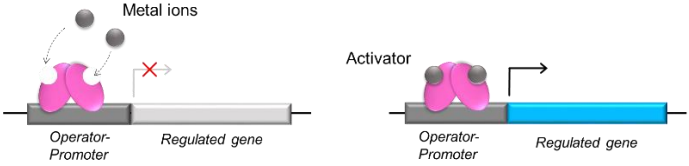

Figure 1: Schematic representation of the prokaryotic activation mechanism: the apo-protein inhibits the transcription, however, the binding of cognate metal ions to the protein allosterically activates transcription of the downstream genes.

The transcriptional activation by CueR is initiated selectively by monovalent transition metal ions, Cu(I), Ag(I) and Au(I) in vitro. The metal ion coordination occurs in a loop-like segment of the protein where the sidechains of two cysteine residues provide linear coordination geometry around the effector metal ion (Figure 2). The metal ion binding loop of the protein is followed by a short, two-turn helix continued in a disordered *C*-terminal region containing two cysteines in a CCHH motif (Figure 2). The *C*-terminal two-turn helix has an allosteric role in the activation process. Upon metal ion binding of CueR it docks into a hydrophobic pocket resulting in a “scissor-type”

movement of the protein and the stabilization of the activator conformation.

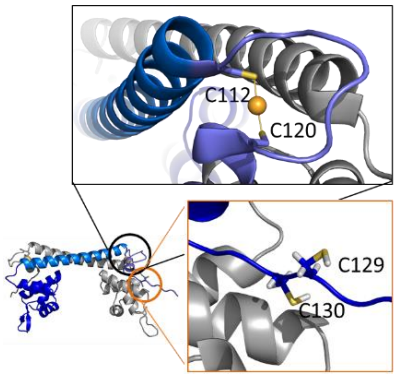

Figure 2: Cysteine residues at the two potential metal ion binding sites (loop and CCHH motif) in the CueR protein (PDB id.: 1Q05).

2. AIMS

Even though CueR is a well described protein in the literature, there are still open questions concerning its transcription regulatory mechanism. Most importantly, how CueR discriminates so successfully between mono and divalent transition metal ions. Furthermore, whether or not the C-terminal CCHH fragment, which has otherwise no direct influence on the transcriptional activity of CueR, plays any role in the operation of the protein. The main goal of this PhD work was to answer these questions by acquiring more data about the details of the selective metal ion recognition of CueR via studying the Wild type (WT) *Escherichia coli* CueR, a *C*-terminally (by 7 amino acid including the CCHH motif) truncated variant (ΔC7-CueR) and an extra cysteine containing truncated CueR variant (S77C-ΔC7-CueR).

In addition to the production and characterization of the proteins, we also aimed to establish a bacterial fluorescent reporter

system where the overexpression of a green fluorescent protein is controlled by the CueR protein.

3. METHODS

The WT CueR and its variants were expressed in *E. coli* BL21(DE3) host cells and purified with fast protein liquid chromatography (FPLC). The protein samples obtained during the purification steps were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The DNA binding capability of the purified proteins was validated with electrophoretic mobility shift assay (EMSA), and the folding of the proteins was monitored by circular dichroism (CD) spectroscopy. The integrity of the protein samples was examined by electrospray ionization mass spectrometry (ESI-MS), and tandem MS (MS^n).

CD spectroscopy was applied to investigate the effect of pH, temperature, and the presence of metal ions and/or DNA on the secondary structure of WT CueR.

The formation of metal ion – protein adducts was followed by ESI-MS. To gain a better understanding of the metal ion coordination of CueR several experiments were carried out with the WT, ΔC7- and S77C-ΔC7-CueR variants via ^{199m}Hg, ^{111m}Cd and ^{111}Ag perturbed angular correlations of γ-rays (PAC) spectroscopies. PAC spectroscopy is a rather exotic spectroscopic method providing information on the local structure and dynamics of the metal binding sites of proteins. Ultraviolet (UV) spectroscopy was also applied to observe metal ion – protein complex formation by monitoring the S^{2-}(thiolate) → $M^{+/2+}$ ligand to metal ion charge transfer (LMCT) transitions.

Tools of recombinant DNA technology were utilized to construct genes of the bacterial fluorescent reporter system and the functioning of this construct was studied with a fluorescent microscope and a microplate reader.

4. NEW SCIENTIFIC RESULTS

1. After the successful cloning of the pET-21a-CueR, pET-21a-ΔC7-CueR and pET-21a-S77C-CueR plasmids, coding for the wild type CueR (WT CueR) and its variants (ΔC7-CueR S77C-ΔC7-CueR), a chromatographic procedure for the purification of these proteins was designed and optimized.[1] The procedure includes a step of enzymatic digestion of the DNA, an anion exchange, an affinity chromatography a second anion exchange and a gel filtration step. It was evidenced that the induced mutations do not alter significantly the helical-rich secondary structure composition, the DNA binding ability or the specific DNA sequence recognition of the ΔC7- and S77C-ΔC7-CueR proteins as compared to WT CueR.

2. The changes of pH, the presence of DNA and Ag(I) affect significantly the secondary structure of WT CueR, as demonstrated by circular dichroism (CD) spectroscopy.

    2.1. Decreasing the pH from 7.5 to 6.0 induces a transformation of the helix-rich structure of the protein into a β-sheet-rich form, which has not been observed so far for any metalloregulatory protein. This secondary structural switch could be partially prevented by adding Ag(I) ions to the protein and completely prevented by the presence of DNA suggesting that DNA locks CueR in the α-helical-rich form.

    2.2. Temperature dependent CD spectra showed that in the presence of Ag(I) or DNA, the melting pattern of the protein changes in a way which suggests that the stabilization occurs at the DNA binding domain.

    2.3. The CD spectrum of WT CueR recorded in the absence and presence of Hg(II), Zn(II) or Cd(II) are similar, however, the presence of Ag(I) significantly affects the shape and intensity

of the spectrum probably reflecting the stabilization of the metal binding loop and the helices besides the dimerization helix.

3. Series of ESI-MS spectra of WT CueR recorded in the presence of increasing amount of Hg(II), Cd(II) and Ag(I) reflected a significant difference in the coordination characteristics of the protein towards the monovalent and divalent ions.

    3.1. Data revealed that Hg(II) and Cd(II) can bind, with high affinities, to two sites of the protein that are most likely the metal ion binding loop, formed by C112 and C120, and the *C*-terminal CCHH motif.[2]

    3.2. This observation was further supported by the ESI-MS spectra of the Hg(II) - ΔC7-CueR system lacking signals corresponding to a Hg_2-ΔC7-CueR complex.[2]

    3.3. In contrast to Hg(II) and Cd(II), the ESI-MS titration of the WT CueR with Ag(I) showed the formation of only the mono-metallated species indicating that the two potential metal ion binding sites of CueR possess a fundamentally different affinity for Ag(I).

4. Comparative studies on the Hg(II) binding features of WT CueR and ΔC7-CueR carried out in the presence of DNA by UV- and ^{199m}Hg PAC spectroscopies revealed that under subequimolar Hg(II) concentrations and at pH = 8.0, Hg(II) is distributed between two structures with different coordination modes in WT CueR while only one type of binding mode is present in the truncated variant.[2]

    4.1. Under the conditions specified above, Hg(II) ions are coordinated by two and three Cys-thiolates in a linear HgS_2

and a T shaped HgS_3 fashion, respectively, in the two structures formed with WT CueR. The third coordinating thiolate may be recruited from the CCHH motif besides the binding of C112 and C120 of the metal binding loop or vice versa.

4.2. At pH 6.0 and at pH 8.0 in the presence of DNA and 1 or 2 equivalents of Hg(II) per WT CueR only HgS_2 structures are present.

4.3. In contrast to the WT protein, only one binding site is available for Hg(II) ions in the truncated ΔC7-CueR variant and Hg(II) is coordinated exclusively in a HgS_2 fashion.

4.4. Structural data suggest that the formation of the HgS_3 structure of WT CueR is accountable for the selectivity of the protein, since it may sterically hinder the docking of the *C*-terminal helix into the hydrophobic pocket, and consequently prevent the activation of transcription.

5. A combined use of ^{111}Ag and ^{111m}Cd PAC spectroscopies provided novel information on the possible mechanism of the metal ion selection of CueR by demonstrating the high coordinational flexibility of the metal binding loop in the binding of mono- and divalent metal ions.[3]

5.1. The ^{111}Ag PAC spectrum of the WT CueR displays two nuclear quadrupole interactions at pH = 8.0 and $T = -196$ °C representing two coordination geometries: a distorted linear structure with two coordinating thiolates and a possible longer distance contact to the S77 backbone carbonyl oxygen and another, higher coordination number species, either as a result of coordination by a nearby amino acid residue or an extraneous ligand such as a solvent water molecule.

5.2. ^{111}Ag PAC data obtained for the Ag(I)-bound ΔC7-CueR variant reflect the formation of very similar structures to those of the WT protein, which confirms that the *C*-terminal CCHH motif does not participate directly in Ag(I) binding.

5.3. The ^{111m}Cd PAC spectrum recorded in the Cd(II)-WT CueR system is distinct from the ^{111}Ag spectra and indicates a symmetric coordination possibly in a distorted tetrahedral CdS_4 species.

5.4. These results highlight the great potential of PAC spectroscopy providing information on the metal site dynamics at the nanosecond timescale. With such data in hands, we propose that the functional linear metal binding site structure of CueR may be disrupted upon binding of the non-cognate metal ions, which hinders the activation of transcription.

6. A Cu(II)-responsive bacterial fluorescent reporter system was established and optimized. Fluorescent microscopic imaging revealed that 1 mM or 10 μM ambient Cu(II) induce the expression of EGFP in LB and M9 minimal media, respectively, whereas, ambient Zn(II) ions, even in a concentration of 10 mM, do not induce the expression of the fluorescent protein owing to the selectivity of CueR.

5. List of Publications

Id. number in the Hungarian Collection of Scientific Publications (MTMT): 10054977

Publications related to the dissertation

[1] R.K. Balogh, B. Gyurcsik, É. Hunyadi-Gulyás, H.E.M. Christensen, A. Jancsó: Advanced purification strategy for CueR, a cysteine containing copper(I) and DNA binding protein. *Protein Expression and Purification,* 123, 90–96 (2016).
IF = 1.29

[2] R.K. Balogh, B. Gyurcsik, É. Hunyadi-Gulyás, J. Schell, P.W. Thulstrup, L. Hemmingsen, A. Jancsó: C-terminal cysteines of CueR act as auxiliary metal site ligands upon Hg(II) binding - A mechanism to prevent transcriptional activation by divalent metal ions? *Chemistry – A European Journal*, 25, 15030-15035 (2019)
IF = 5.16

[3] R.K. Balogh, B. Gyurcsik, M. Jensen, P.W. Thulstrup, U. Köster, N.J. Christensen, F.J. Mørch, M.L. Jensen, A. Jancsó, L. Hemmingsen: Flexibility of the CueR metal site probed by instantaneous change of element and oxidation state from Ag^{I} to Cd^{II}. *Chemistry – A European Journal,* Accepted (2020)
IF = 5.16

ΣIF = 11.61

Further publications

[4] E. Németh, R.K. Balogh, K. Borsos, A. Czene, P.W. Thulstrup, B. Gyurcsik: Intrinsic protein disorder could be overlooked in cocrystallization conditions - An SRCD case study. *Protein*

Science, 25, 1977-1988 (2016). DOI: 10.1002/pro.3010
IF = 2.42

[5] K. Kowalski, J. Skiba, Q. Yuan, A. Hildebrandt, H. Lang, D. Trzybiński, K. Woźniak, R.K. Balogh, B. Gyurcsik, and V. Vrček: Ferrocenyl GNA nucleosides: a bridge between organic and organometallic xeno-nucleic acids. *ChemPlusChem*, 83, 77-86 (2018) DOI: 10.1002/cplu.201700551
IF = 3.44

[6] N. Ivošević DeNardis, J. Pečar Ilić, I. Ružić, N. Novosel, T. Mišić Radić, A. Weber, D. Kasum, Z. Pavlinska, R.K. Balogh, B. Hajdu, A. Marček Chorvátová, B. Gyurcsik: Algal cell response to laboratory-induced cadmium stress: a multimethod approach. *European Biophysics Journal*, 48, 231-248 (2019). DOI: 10.1007/s00249-019-01347-6
IF = 2.53

[7] H.A.H. Abd Elhameed, B. Hajdu, R.K. Balogh, E. Hermann, É. Hunyadi-Gulyás, B. Gyurcsik: Purification of proteins with native terminal sequences using a Ni(II)-cleavable C-terminal hexahistidine affinity tag. *Protein Expression and Purification*, 159, 53-59 (2019) DOI: 10.1016/j.pep.2019.03.009
IF = 1.29

ΣIF = 21.29

Poster presentations at international conferences

1. R. Balogh, A. Jancsó, B. Gyurcsik, H.E.M Christensen: Synthesis, purification and metal ion binding studies on native and modified

CueR metalloregulatory proteins. REGIONAL CONFERENCE: "Heavy metal as contaminants of the environments"- HuRo conference, Timisoara, Romania, 17 May 2013

2. R. Balogh, A. Jancsó, B. Gyurcsik, H.E.M Christensen: Synthesis, purification and metal ion binding studies on native and modified CueR metalloregulatory proteins. 13th Edition of Academic Days Timisoara, Timisoara, Romania,13−14 June 2013

3. R.K. Balogh, K. Borsos, A. Czene, E. Németh, B. Gyurcsik: Induced Folding of NColE7 Metallonuclease Monitored by SRCD. 590. WE-Heraeus-Seminar on Synchrotron Radiation Circular Dichroism (SRCD) Spectroscopy, Bad Honnef, Germany, 17−20 May 2015

4. R.K. Balogh, A. Jancsó, B. Gyurcsik, H.E.M. Christensen, É. Hunyadi-Gulyás: Strategies for purification of a cysteine containing DNA binding protein. 13th European Biological Inorganic Chemistry Conference, Budapest, Hungary, 28 August – 1 September 2016

5. R.K. Balogh, A. Jancsó, B. Gyurcsik, E. Németh: A study on the secondary structure of the metalloregulatory protein CueR: effect of pH, metal ions and DNA. 14th International Symposium on Applied Bioinorganic Chemistry (ISABC), Toulouse, France, 7−10 June 2017

6. R.K. Balogh, A. Jancsó, B. Gyurcsik, E. Németh: pH induced structural switch of CueR metalloregulatory protein. 19th International Union of Pure and Applied Biology (IUPAB)

Congress and 11th European Biophysical Societies' Association (EBSA) Congress, Edinburgh, Scotland, 16−20 July 2017

7. R.K. Balogh, E. Mesterházy, K. Kato, K. Nagata, A. Jancsó ,B. Gyurcsik: Detection of toxic metal ions by the CueR metalloregulator. 14th European Biological Inorganic Chemistry Conference, Birmingham, United Kingdom, 26−30 August 2018

8. R.K. Balogh, E. Mesterházy, K. Kato, K. Nagata, A. Jancsó ,B. Gyurcsik: Detection of toxic metal ions by the CueR metalloregulator. COST-sponsored ARBRE-MOBIEU plenary meeting, Zagreb, Croatia, 18−20 March 2019

9. R.K. Balogh, Béla Gyurcsik, Éva Hunyadi-Gulyás, Peter W. Thulstrup, Lars Hemmingsen, Attila Jancsó: Investigation of Hg(II) binding of CueR protein: a possible role of C-terminal cysteines in selective operation of the protein, Interlaken, Switzerland, 11−16 August 2019

Oral presentations at international conferences

1. R.K. Balogh, H.E.M. Christensen, M.N. Asaka, K. Kato, K. Nagata, B. Gyurcsik, A. Jancsó: The potential application of CueR protein in selective detection of toxic metal ions. XXV. International Conference on Coordination and Bioinorganic Chemistry - The 50th Anniversary of ICCBIC, Smolenice, Slovakia, 31 May – 5 June 2015

2. R.K. Balogh, Béla Gyurcsik, Attila Jancsó: Transcriptional activator CueR protein: purification and characterization, 9th Chemistry Towards Biology (CTB), Budapest, Hungary, 24−27 September 2018

Oral presentations at Hungarian conferences

1. Balogh R.K., Mesterházy E., Gyurcsik B., Jancsó A., H.E.M. Christensen, M.N. Asaka, K. Kato, K. Nagata; Réz(I)ionok szelektív kimutatása a CueR fémszabályzó fehérje segítségével. XXXVIII. Kémiai Előadói Napok, Szeged, 2015. október 26-28.
2. Balogh R.K., Gyurcsik B., Jancsó A.: A CueR fehérje másodlagos szerkezetének vizsgálata cirkuláris dikroizmus spektroszkópiával. XXXIX. Kémiai Előadói Napok, Szeged, 2016. október 17-19.
3. Balogh R.K., Jancsó A., Gyurcsik B., Németh E.: Egy transzkripciós aktivátor fehérje pH indukált szerkezetváltozásának tanulmányozása. 51. Komplexkémiai Kollokvium, Balatonvilágos, 2017 május 29-31.