Summary

Zinc ion (Zn^{2+}) is one of the most abundant transition metal found in living organisms. It's major role is to modulate the functions of cellular proteins. However, it is still unknown whether the saturation of proteins with Zn^{2+} ions in the cell is related to their biological function. Many studies, have shown that the Zn^{2+}-dependent folding process is one of the most important factor that influence protein stability and reactivity. One of the most common group of zinc proteins are zinc fingers (ZFs), which are small, compact zinc domains specialized in interactions with other macromolecules. Most of them interact with nucleic acids (DNA, RNA), others with proteins and some with lipids. Their functions are varied, nevertheless, the most important ones are specific DNA recognition, transcriptional activity, regulation of apoptosis and participation in protein folding. Even though, many types of ZFs are known, the most abundant in the human zinc proteome; and thus, frequently described and studied ZF motif is the classical one known as a CCHH motif. This motif has a well-defined and highly conserved sequence containing residues that are responsible for Zn^{2+} ion binding, hydrophobic core formation, as well as variable amino acid residues responsible for interaction with DNA, and residues which function is still unknown. During the metal-coupled folding process of the CCHH type ZF, a characteristic three-dimensional structure is formed, consisting of two anti-parallel β sheets and an α-helix. Such structure is known as ββα. Numerous studies have shown that the presence of the Zn^{2+} ion is crucial for the stability of this unique fold and that artificial mutations or deletions of conserved histidine or cysteine residues affect the zinc binding. Thus disrupt formation of ββα structure and in consequence influence functioning of many ZF proteins (ZFPs).

Both structural and thermodynamic factors influence Zn^{2+} affinity of the ZF causing their diversification to maintain proper functioning of zinc proteins. Therefore, if the pool of available free zinc in the cell is correlated with the Zn^{2+} affinity constants of ZFs then they are activated and can selectively interact with DNA. Although biochemical studies of classical ZFs have a long history, there is a limited knowledge about ZFs sequential, structural and energetic diversity to understand all the rules controlling their stability/instability. Therefore, major goals of this PhD dissertation were to investigate the relationship between sequence-structure-stability of sequentially diverse ZFs, study the biding of silver ions to ZFs in order to determine its impact on their structure, geometry and stability. Lastly, also the biophysical characterization of the cysteine-rich domain derived from the human MTF-1 protein, which is a transcription factor containing CCHH ZF domain, was performed.

The first part of the research is focused on the characterization of structural factors and thermodynamic effects contributing to the various stability in classical CCHH ZFs. For this purpose, two peptide models, based on the consensus peptide 1 (Cp1) ZF sequence originally defined in 1991 (Cp1-1991) and later redefined in 2015 (Cp1-2015), were obtained. The obtain results show that these peptides, despite their high sequence conservation, differ significantly

in terms of zinc complexes stability. Moreover, in the course of this work it was observed that non-conserved amino acids from α-helix fragment are responsible for the major stability loss observed within CCHH ZFs. A complete thermodynamic analysis of zinc binding to sequentially diverse Cp1 ZF peptides has been achieved. The results are consistent with a coupled metal binding-protein folding process. The reactions are primarily enthalpy driven which is associated with formation of strong bonds between the metal ion and the peptide-derived cysteine (Cys) and histidine (His) ligands. In most cases substantially unfavourable entropy contribution associated with peptide folding is balanced by the favourable entropy associated with water release from the metal ions. Nevertheless, some ZF peptides clearly show that particular non-conserved amino acids are responsible for stability loss and that in this case zinc binding process is entropically driven. Such outcome is associated with entropic component derived from conformational reorganization, changes in solvation and/or intramolecular interaction which differ depending on type and position of non-conserved amino acids. This study highlight the role of non-conserved amino acids in zinc affinity modulation.

In the next part of the work, bioinformatics analysis carried out using the UniProt database and the ScanProsite tool show that about 10% of the sequences of classical ZF motifs deposited in the UniProt database contain natural substitutions within the conserved metal binding amino acids. Analysis of these results enable to select nine sequences of diverse ZFs with a naturally occurring alteration within coordinating residues (XCHH, CXHH, CCXH and CCHX) present in human and mouse transcription factors identified at the protein and transcript level, which were subjected to further biophysical characterization. This study revealed that XCHH and CXHH ZFs form ML complexes that are 4-5 orders of magnitude weaker in comparison to CCHH ZFs. Nevertheless, spectroscopic studies demonstrate that, depending on the altered position, they may adopt an open coordination geometry with one or two water molecules bound to a central metal ion, which has not been demonstrated in natural ZFs before. Stability data show that both, CCXH and CCHX, peptides have high Zn^{2+} affinity (K_d of 10^{-9} to 10^{-11} M), suggesting their potential biological function. In fact, the analysis of zinc transfer in the thionein/metallothionein cellular buffering system shows that depending on the sequence and affinity these ZFs are fully or partially saturated with Zn^{2+} in the presence of metallothionein. Such outcome suggests a potentially regulatory role for altered ZFs whose affinity constants cover the range of cellular free zinc concentration.

Although natively ZFs are saturated with zinc, they also have the ability to bind other metals that may accumulate and compete with Zn^{2+} leading to irreversibly toxic changes. The latest environmental study have showed that level of silver ions in dust is constantly increasing due to excessive use of silver nanoparticles (AgNPs) in daily use products, including cosmetics, bedding, sports-wear, protective gear (such as masks heavily used during COVID-19 pandemics), and food containers are coated with AgNPs for their antibacterial properties. Because silver ions have a high affinity for thiol groups and their impact on ZFPs has not been described so far. As worked progressed in a further stage of this research project, it was aimed to investigate whether the presence of silver ions may affect the structure, geometry and

stability of ZFs. For this purpose, a number of classical ZF motifs were synthesized, based on the Cp1-2015 ZF sequence. It has been shown that silver can directly replace zinc in a sequentially diverse ZFs (CCHH, CCCH, and CCCC), forming highly stable Ag_nS_n complexes. The cooperative binding of Ag^+ to ZFs leads to thermodynamically irreversible formation of silver clusters perturbing the native ZF architecture and destroying the highly ordered 3D structure of ZFs. Thus, a reported loss of biological function of ZF proteins is a likely consequence of such replacement. To probe structural features of Ag_nS_n clusters found in ZFs, the X-ray absorption spectroscopy (XAS) was used. Selective probing of local environment around silver by XAS showed the predominance of digonal Ag^+ coordination to two sulphur donors, coordinated with an average Ag−S distance at 2.41 Å. No Ag−N bonds were present. These findings provide a chemical fundament for further studies of zinc fingers and other thiolate-rich proteins as targets of silver genotoxicity.

The final stage of the PhD thesis is devoted to the biophysical characterization of a cysteine-rich fragment derived from human metal-responsive transcription factor 1 (hMTF1). The hMTF1 protein participates in the cellular zinc homeostasis by zinc-dependent gene regulation of many zinc proteins. At the C-terminus end, the hMTF1 protein has a unique cysteine-rich motif called a cysteine cluster (CC). This motif is highly conserved in higher eukaryotes, which may suggest its specialized role in the MTF1 activation. In this study, based on the shorter and longer fragment of the cysteine cluster, it has been showed that this motif binds Zn^{2+} ions with affinity similar to that of ZFs, covering the range of cellular free Zn^{2+} fluctuations. Further characterization, shows that the cysteine cluster forms a dimer during Zn^{2+} binding. The presence of this dimer is related to the availability of free Zn^{2+} in the system. Thus, at the picomolar concentration of free Zn^{2+}, only the monomeric metal free form is present, while together with increasing free Zn^{2+} concentration to nanomolar and then micromolar, the dimeric zinc complex (possibly with a binuclear centre) is formed. The conducted research suggests that the cysteine cluster may be involved in hMTF1 activation and its translocation to the nucleus.