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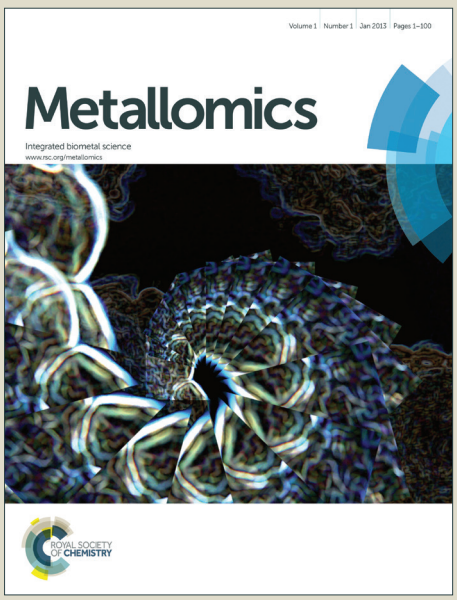

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Relationship between the architecture of zinc coordination and zinc binding affinity in proteins - insights into zinc regulation

Tomasz Kochańczyk, Agnieszka Drozd, and Artur Krężel*

Laboratory of Chemical Biology, Faculty of Biotechnology, University of Wrocław, ul. Joliot-Curie 14a, 50-383 Wrocław, Poland

Corresponding author:

Artur Krężel

E-mail: krezel@biotech.uni.wroc.pl

Tel: +48 71 375 2765

Abstract

Zinc proteins are an integral component of the proteome of all domains of life. Zn(II), one of the most widespread transition elements, serves multiple functions in proteins, such as a catalytic co-factor, structural center and signaling component. The mechanism by which proteins associate with and dissociate from Zn(II) and the factors that modulate their affinity and stability remain incompletely understood. In this article, we aim to address how zinc binding sites present in proteins differ in their architecture and how their structural arrangement is associated with protein function, thermodynamic and kinetic stability, reactivity, as well as zinc-dependent regulation. Here, we emphasize that the concentration-dependent functionality of the interprotein zinc binding site may serve as another factor regulating the relationship between cellular Zn(II) availability and protein function.

1. Introduction

Proteins utilize a large array of cofactors to achieve a variety of structures and functions. Among these cofactors, metal ions perform critical functions and differ significantly from organic cofactors. Of all inorganic cofactors, transition metal ions play a unique role in the facilitation of the enormously diverse functions of proteins. Among all of the transition metal ions present in all domains of life, zinc (formally Zn(II)) is the most widespread, reflecting the utilization of Zn(II) by proteins for a wide variety of biological functions.[1, 2] Bioinformatic analysis of the human genome suggests that up to 3000 proteins participate in Zn(II) binding. This number corresponds to ~10% of all encoded proteins.[3] Interestingly, a similar fraction of Zn(II) binding proteins is found in the structures of human proteins deposited in structural databases. Because bioinformatics-based predictions are ultimately based on homology with known zinc-binding site sequence signature, it is likely that the number of putative zinc proteins will increase over time. Zinc sites located at the interface of protein complexes may be virtually impossible to predict, as the amino acid residues that comprise such sites are separated into two or more different peptide chains. However, there is growing experimental evidence that zinc sites play an important role in cellular regulation, tertiary protein assembly and signaling.[4, 5]

Zinc proteins are diverse in their size, structure, tertiary architecture and function, although Zn(II) predominantly binds to four amino acid residues, adopting a tetrahedral coordination geometry. Among all of the physicochemical properties of zinc proteins, determining the affinity of zinc proteins for Zn(II) is critical for understanding how Zn(II) mediates their function and how proteins regulate its mobility and cellular availability. In his review of zinc coordination dynamics, Wolfgang Maret stated that "in principle, differences in stability constants might simply indicate a certain hierarchy, such that proteins performing the most critical functions …. At present, there is no evidence for such hierarchy".[1] The

affinity of proteins for Zn(II) varies between different classes and localizations of zinc proteins, but there are some similarities within particular classes. For example, zinc enzymes and most small structural zinc domains display a high affinity for Zn(II), which is frequently associated with a slow dissociation rate, thereby persistently facilitating the function of these proteins. Alternatively, other classes of proteins may function in different binding modes which depend on local Zn(II) availability, and their function may be transiently inhibited or activated depending on the levels of available Zn(II).

In this article, we aim to address how zinc binding sites present in proteins differ in their architecture and how their structural arrangement is associated with protein function, thermodynamic and kinetic stability, reactivity, and zinc-dependent regulation. Here, we emphasize that the concentration-dependent functionality of the interprotein zinc binding site may serve as another factor regulating the relationship between cellular Zn(II) availability and protein function.

2. Cellular Zn(II) availability

Cellular Zn(II) availability is a consequence of a number of factors, such as the total zinc concentration in the cell, the affinities of proteins and other molecules for Zn(II), the kinetic properties of the zinc binding sites, the zinc buffering capacity, the redox potential, *etc*. Although the total concentration of Zn(II) in a eukaryotic cell is relatively high (100-300 μM), the actual concentration of free zinc ions ($[Zn(II)_{free}]$) is almost million times lower.[6-9] Currently, it is a consensus opinion that the intracellular $[Zn(II)_{free}]$ ranges from low nanomolar to picomolar range in eukaryotic cells depending on the cell type.[7, 9] Homeostatic $[Zn(II)_{free}]$ is controlled by a number of proteins, Zn(II) sensing and membrane transport proteins, which regulate cellular and vesicular influx and efflux of Zn(II), and by other proteins such as metallothionein, which participate in zinc storage and redistribution.[10]

Changes in the cellular [$Zn(II)_{free}$] occur in relatively a small range, typically between 10^{-9} and 10^{-11} M as both excessively low and high [$Zn(II)_{free}$] is cytotoxic (Fig. 1). Dynamic increase of [$Zn(II)_{free}$] slightly above essential level, which may occur locally (zinc fluxes) may result in transient binding of Zn(II) to different proteins and thus regulate their function by different pathways (Fig 1).[10, 11] Consequently, zinc fluxes results in regulation of cellular zinc sensors, enzymes inhibition, activation of regulatory proteins or induction of protein-protein interactions which may participate in signal transduction (Fig. 1).[12] Excess of [$Zn(II)_{free}$] above physiological level causes irreversible effects such as aggregation which in consequence lead to dysfunction of many proteins (Fig. 1).[13, 14] Similarly, decrease of [$Zn(II)_{free}$] below essential level is also toxic for the cell and results in protein dysfunction mostly due to lack of available metal cofactor and signal transducer (Fig. 1).[12, 13]

Knowledge regarding [$Zn(II)_{free}$] in specific subcellular organelles is considerably less well established than of the cytosolic [$Zn(II)_{free}$]. Classical fluorescent Zn(II)-sensitive probes used for the determination of [$Zn(II)_{free}$] in cytosol do not localize specifically in cellular compartments.[15, 16, 17] Recent progress with development of genetically encoded FRET sensors that specifically bind Zn(II) and are localized in certain compartments shed more light on subcellular Zn(II) distribution. The most explored types of such sensors belong to Zap (introduced by Palmer lab) and CALWY (designed by Merkx group) family of sensors.[9] The application of ZapCY1 and ZapCY2 sensors for the measurements of free of [$Zn(II)_{free}$] in specific organelles indicated sub-picomolar [$Zn(II)_{free}$] in the ER, Golgi and mitochondria (0.2-0.9 pM).[18, 19] Substantially higher levels of [$Zn(II)_{free}$] have been reported using the CALWY sensors as the [$Zn(II)_{free}$] in ER and mitochodndrial matrix was found to be in the range 1.6-7.2 nM and 180-300 pM, respectively.[20] It is unclear why response of both sensor types differ when they are targeted to ER and mitochondrial matrix.[21] However, genetically

encoded sensors uncovered substantial differences in Zn(II) availability that must accounted when considering the function of zinc proteins in particular compartment.

3. Zn(II) binding affinities of zinc proteins

3.1. Zn(II) binding affinity – a critical characteristic of zinc proteins

Zinc sites in proteins have been categorized into catalytic and structural classes. Zinc enzymes belong to the best known and characterized class of zinc proteins.[22, 23] The number of identified zinc proteins is constantly increasing. Our current knowledge regarding zinc protein structure and function facilitates the further categorization of zinc proteins into several distinct classes. Because there are many different classes of zinc proteins and Zn(II) serves various functions under different protein architecture and coordination modes, the physicochemical properties of zinc proteins must differ. One critical thermodynamic parameter associated with the stability and functional characterization of metalloproteins is the affinity of Zn(II) to its protein site. The stability of zinc binding sites in proteins reflects their biological functionality and may provide some information about the function and reactivity of zinc binding sites. The binding affinity of Zn(II) to a protein is predominantly determined as a apparent dissociation (K_d) or association constant ($K_a = 1/K_d$) in an equilibrated system. In the case of 1:1 Zn(II):protein stoichiometry, the dissociation constant is expressed in molar units (M) and corresponds to the $[Zn(II)_{free}]$ at which half of the binding sites are bound to the metal. Characterizing proteins that bind more than one Zn(II) requires determining either the step dissociation constants for each binding event or the average dissociation constant if all Zn(II) binding events are quantitatively similar. In the case of 1:2 Zn(II):protein stoichiometry, represented by certain intermolecular zinc sites, the dissociation constant is expressed in molar squared units (M^2), and this value divided by the total protein concentration corresponds to the $[Zn(II)_{free}]$ at which half of the proteins form the Zn(II)-

$(protein)_2$ complex. Similarly, determining the stability of the complex consisting of more than one protein molecule requires consideration of the step dissociation constants, unless the binding of Zn(II) to the protein units is cooperative in terms of ligand coordination. The dimerization constant is a parameter that reflects the of tendency of protein to dimerize and depends on the equilibrium between the monomer and dimer concentrations. Alternatively, if dimerization is induced by a Zn(II) binding event or if the dimer is stabilized by Zn(II), the binding model must consider the equilibrium Zn(II) concentration.[24] However, in studies of protein-protein interactions that occur via Zn(II) binding, these Zn(II) binding characteristics are often neglected.[25]

In many cases, the affinity of Zn(II) to a protein is expressed as inhibition coefficient (IC_{50}) when inhibition of protein activity occurs upon Zn(II) binding. However, such a value rarely reflects zinc complex stability because the IC_{50} value represents the total Zn(II) concentration required for inhibition of 50% of the protein activity. Because the total metal ion concentration cannot be compared with the free metal ion concentration, the IC_{50} is frequently higher than the K_d.[26, 27] An excellent representative example of this property is the inhibition of non-zinc enzymes by Zn(II). IC_{50} values describing inhibition phenomena are frequently determined in pH buffers, whose components participate in Zn(II) binding, increasing the total Zn(II) concentration necessary for 50% inhibition of the examined enzyme.[28] This confound is especially detectable if the affinity of Zn(II) to the enzyme is relatively high. In such cases, the IC_{50} may differ from the K_d by several orders of magnitude.[26, 29, 30]

3.2. Diversity of the data regarding the affinity of Zn(II) to proteins

How strongly proteins interact with Zn(II) is important for understanding overall zinc homeostasis.[1] Free Zn(II) concentration and metal mobility depend on the affinity of the metal

to binding sites in proteins. Some identified Zn(II)-protein interactions do not appear to have a physiologically relevant function due to their weak affinity. The wealth of structural information about zinc binding sites in proteins needs to be analyzed with respect to zinc affinity and subsequently evaluated to determine whether these sites are compatible with the availability of Zn(II) in the intracellular or extracellular environment at which these sites are naturally present. Table 1 presents examples of different protein classes and small protein domains that interact with Zn(II) that were characterized quantitatively in terms of zinc binding affinity. For clarity, the affinities presented in Table 1 are expressed as dissociation constants, and their values vary from the micromolar (10^{-6} M) to femtomolar (10^{-15} M) range, reflecting a vast difference in the binding properties of various proteins to the same metal ion.[31-59] Despite these data, presently, there is a limited amount of information regarding the affinity of Zn(II) to proteins and the associations between zinc protein stability, functionality and biological effects on the [$Zn(II)_{free}$].[60] The available stability data were obtained from proteins from different organisms and localizations and were collected using different methodologies under various conditions. Consequently, comparison of the available data is very difficult. Fortunately, the amount of stability data that has been generated using similar techniques has increased recently.[51, 52, 61-64]

3.3. Relationship between architecture, stoichiometry and structure of zinc binding sites and protein function

Zinc binding domains are typically characterized by Zn(II) bound in a tetrahedral geometry to four protein derived ligands, most frequently nitrogen from histidine (H), sulfur from cysteine (C) and oxygen from aspartate (D) or glutamate (E). Thus, proteins can modulate the properties of Zn(II) affinity using certain combinations of ligands and can utilize Zn(II) for structural or catalytic functions.[65] Based on the concept of hard and soft acids and bases

implies that zinc proteins containing sulfur and nitrogen donors in the coordination sphere are more stable than those containing nitrogen and oxygen donors.[66] However, comparison of the dissociation constants of proteins with significantly different donors and numbers of donors, such as alkaline phosphatase (DDH, DHH),[33] metallothionein (CCCC),[48, 49] and MTF-1 transcription factor (CCHH),[45] revealed similar affinities of these proteins for Zn(II) (10^{-11}-10^{-12} M, Table 1). Despite their similar affinity, the thermodynamics of Zn(II) binding may differ between ligand sets. Studies using naturally occurring Zn(II) binding sequences containing CCHH, CCHC, and CCCC sites that possess similar affinity for Zn(II) revealed distinct enthalpic and entropic contributions to the binding free energy. For example, Zn(II) binding to CCCC sites is primarily entropically driven, which renders this coordination as more susceptible to factors such as pH, temperature and the surrounding dielectric. As fewer cysteines are included in the Zn(II) coordination structure, the entropy becomes less favorable, and the enthalpy of binding becomes more favorable.[67, 68] Alternatively, proteins containing the same donors in the coordination sphere (e.g., HHE) may display significantly different affinity for Zn(II), e.g., prolactin (~10^{-5} M),[69] angiotensin-converting enzyme (~10^{-8} M)[35] and thermolysin (~10^{-11} M).[70] This variability demonstrates that the primary coordination sphere is not a major determinant of zinc protein stability; instead, the protein and ligand environments (secondary coordination sphere) and other structural effects regulate the thermodynamic and kinetic stability and function of the zinc binding site.

Correlation between structural and functional similarities enables categorization of zinc proteins and binding domains into five classes, which are schematically depicted in Figure 2. The first class consists of catalytic zinc binding domains, which are characterized by coordination of Zn(II) using three amino acid donors from a single polypeptide chain, with histidine and aspartate/glutamate as the most common ligands. The most frequent configuration of catalytic zinc binding sites consists of three amino acid-derived donors.[71]

This coordination architecture results in the formation of an open coordination sphere in which exogenous ligands can bind directly to Zn(II) as the fourth ligand (Fig. 2a).[72, 73] The formation of a tridentate zinc binding site is thought to be essential for catalytic activity and is found ubiquitously in enzymes such as alcohol dehydrogenase, carboxypeptidases A and B, neutral protease, thermolysin, β-lactamase, phospholipase C, alkaline phosphatase, and carbonic anhydrase I and II.[73] Studies of the reaction mechanism for zinc enzymes identified water as the fourth ligand and as a critical component of the catalytic zinc binding site. The binding of water molecule to Zn(II) favors either ionization to Zn(II)-bound hydroxide (as in carbonic anhydrase), polarization by a general base to generate a nucleophile for catalysis (as in carboxypeptidase A) or displacement by the substrate (as in alkaline phosphatase).[22, 72] The affinities of intracellular catalytic zinc binding sites for Zn(II) are relatively high, whereas those of extracellular catalytic zinc binding sites are slightly or significantly lower (Table 1).

The second class consists of structural zinc binding sites, which are typically characterized by a mononuclear, tetrahedral closed coordination sphere involving four protein-derived ligands, predominantly sulfur donors from cysteine and nitrogen donors from histidine (Fig. 2b).[71] Most of these sites bind to Zn(II) tightly (thermodynamically stable) and the resulting complex is inert (kinetically stable).[62] In the structural zinc sites, coordination of Zn(II) enables folding of relatively short stretches of the polypeptide chain into well-defined structures that are well-suited to participate in interactions with other domains in the protein or with various macromolecules, such as proteins, DNA, RNA and lipids.[74] These small protein domains that adopt compact structures stabilized by zinc are classically referred to as zinc finger domains. Originally discovered as DNA-binding motifs, zinc finger domains are currently known to be extremely diverse in their structural characteristics and are present in proteins that perform a broad range of functions in various cellular processes, such as DNA replication and repair, transcription, translation, metabolism, signaling, cell proliferation and

apoptosis. Three folding types comprise the majority of zinc finger domains:[75] classical ββα-type (e.g., the transcription factor TFIIIA),[76] treble clef (e.g., the ribosomal protein S14),[77] and zinc ribbon (e.g., the transcription elongation factor TFIIB).[78]

The third class is composed of multinuclear (clustered) zinc binding sites, which are characterized by the presence of more than one Zn(II) per distinct site and disproportionate Zn(II)/ligand ratios due to the presence of bridging ligands, predominantly sulfur donors from cysteines (Fig. 2c).[79, 80] These sites bind Zn(II) very or moderately tightly (thermodynamically stable) while remaining reactive as Zn(II) donors (kinetically labile).[81] These properties enable the unique functions of multinuclear zinc binding sites, which participate in Zn(II) redistribution rather than trapping of Zn(II) in a structurally inert site. A prime example of this type of zinc binding site is metallothionein, to which a up to of seven Zn(II) are bound via 28 intramolecular Zn(II)-sulfur bonds (16 in the α-domain and 12 in the β-domain).[82, 83] Metallothionein displays kinetic lability and high Zn(II) coordination dynamics due to the lack of hydrophobic residues and rigid structure that are typical of structural zinc binding sites and domains. This flexible structure and the presence of different types of Zn(II)-sulfur bonds in metallothionein generate variability in the Zn(II) binding properties resulting in multiple Zn(II) saturation protein species ($Zn_{4\text{-}7}$-protein), critical for the zinc buffering of [$Zn(II)_{free}$] between the nanomolar and picomolar range.[7, 48, 84]

The fourth class contains zinc binding sites in which the ligand environment determines the high mobility of Zn(II) and moderate or low affinities to Zn(II). The affinity and other properties of these zinc binding sites are highly variable and are thought to be modulated by conformational changes in the global protein structure, enabling the mobilization of Zn(II) (Fig. 2d). This class of sites is important for translocation of Zn(II) across plasmatic membranes by specialized proteins such as zinc transporters.[1, 85-88] There are two major families of zinc transporters: the Zrt/Irt-like (ZIP) protein family, which imports

Zn(II) into the cytosol from extracellular or intracellular compartments, and the ZnT protein family, which exports cytosolic Zn(II) to extracellular or intracellular compartments.[89, 90] ZIP proteins share a topology consisting of eight transmembrane domains, a conserved histidine-rich sequence located in a large cytoplasmic loop domain (between transmembrane domains III and IV) and a pair of histidine residues between transmembrane domains IV and V.[91] Presumably, the histidines in the loop are brought into spatial proximity to the histidines in the transmembrane domains to bind Zn(II), facilitating its transport across the membrane.[92] Interestingly, the mechanism of Zn(II) transport by ZIP proteins likely involves an intermolecular exchange of Zn(II) between the transporter and the available low molecular weight zinc-ligand pool rather than the direct uptake of free Zn(II).[93] Compared to ZIP proteins, ZnT proteins, the other family of evolutionally conserved zinc transporters, are more fully characterized, both structurally and functionally. ZnTs transfer Zn(II) using the proton gradient across the membrane as the driving force,[94] with a Zn(II)/proton exchange stoichiometry of 1.[95] Recent structural studies of YiiP, a ZnT protein homolog in *E. coli*, revealed mechanistic insights into zinc mobilization by this type of transporters.[96] YiiP forms homodimers, in which each protomer consists of six transmembrane domains and four zinc binding sites.[97] The conserved zinc binding site within the four-helix bundle transmembrane domain is the primary zinc transport site and, in YiiP, involves tetrahedral coordination of Zn(II) using three aspartates and one histidine. Binuclear zinc binding site in the cytoplasmatic domain is thought to sense cytoplasmatic [$Zn(II)_{free}$] and trigger a conformational change allowing auto-regulation of transporter. Absence of outer-shell constraints in the primary zinc transport site enables rapid transport kinetics whereas the extensive outer-shell interactions surrounding the binuclear zinc binding site allows effective zinc sensing via cytoplasmatic domain.[98] Functional and the structural studies of YiiP support

an alternating-access transport mechanism involving scissor-like movement of the helices in the transmembrane region. [96, 99]

According to this classification scheme, the last, but certainly not least, class of zinc binding sites coordinates Zn(II) intermolecularly, i.e., using ligands supplied from two or more polypeptide chains (Fig. 2e). In this zinc binding architecture, Zn(II) participates in the quaternary structure of the protein complex. These sites, referred to as protein interface zinc sites, share many of the general characteristics of intermolecular zinc sites and perform various functions, such as homo- and heterodimerization, catalysis or regulation.[1, 60] Zn(II) bound at the interface of protein-protein interaction can be either an obligatory component required for formation of the protein complex or stabilize a preformed protein complex.[100] Because the ligands involved in Zn(II) coordination are separated into two (or more) peptide chains, it is virtually impossible to predict the existence of an intermolecular zinc binding site based on an amino acid sequence, in contrast to characteristic zinc binding domains in a single polypeptide chain. Such prediction would be more effective if it involved a pattern search in protein sequence databases (the proteome) and simultaneous processing of protein-protein interaction databases (the interactome).[101] To date, there have been few studies of the affinity of Zn(II) to proteins at interprotein zinc binding sites.[25, 52, 102] The T-cell co-receptor CD4 and the N-terminus of the Src-family tyrosine kinase Lck were identified and characterized to form heterodimers in cytosol via Zn(II) coordination.[25] Another example of interface zinc site is the conserved zinc hook domain present in Rad50 protein. The zinc hook mediates Zn(II)-dependent homodimerization of Rad50 proteins and is required for functional association of Mre11/Rad50/Nbs1 (MRN) complex which plays a key role in DNA damage detection and signaling.[103, 104, 52, 105.]

3.4. Factors affecting the affinity of Zn(II) to a protein

The remarkable similarity of donors in many classes of zinc binding sites of varying affinity for Zn(II) demonstrates that secondary and higher order interactions are crucial factors that determine their stability and function (Table 1). Clearly, these diverse properties depend on other structural effects, such as interactions between the ligands secondary amino acids (the secondary coordination sphere), interactions between non-coordinating residues in the zinc binding site, and the surrounding environment, influencing the tertiary structure of the entire protein. An example of a very stable, both thermodynamically and kinetically, zinc binding site in which Zn(II) is coordinated to only three protein ligands is the catalytic domain of carbonic anhydrase. Its stability is enhanced by secondary interactions of the ligands, which form hydrogen bonds to imidazoles between each of the three Zn(II)-bound histidine residues and protein-derived oxygen atoms.[106] Mutating a hydrogen bond-accepting glutamate to alanine resulted in a 10-fold decrease in the affinity of the protein for Zn(II) and a 200-fold increase in the k_{off} rate. This increase in the dissociation rate decreases the half-time of the zinc complex from 5 days to less than 30 min at 25°C.[107]

Another example in which secondary interactions involve metal-binding residues is the formation of hydrogen bonds between the peptide bond and cysteinate sulfur (-NH···S-), which is found in the HIV-1 nucleocapsid protein (NCp7)[108,109] and in tumor necrosis factor receptor-associated factor (TRAF)-like proteins.[110] These interactions are induced by metal binding, as cysteine undergoes deprotonation upon binding to Zn(II), thereby acting as a hydrogen bond acceptor.[106] The classical ββα CCHH-type zinc finger domains were studied extensively as a model structural zinc binding site to identify the factors that influence their affinity to Zn(II). The CCHH zinc finger motif contains a highly conserved sequence, (F/Y)-X-C-X_{2-4}-C-X_3-(F/Y)-X_5-L-X_2-H-X_{3-5}-H, where X represents a relatively variable amino acid.[111] Binding to Zn(II) via the conserved cysteine and histidine residues is essential for

appropriate folding into the ββα structure and for DNA binding (Fig. 3a).[112] Deleting a single amino acid within the large loop region in the CCHH zinc finger domain exerted significant effects on peptide-metal stability, resulting in approximately 2 and 4 orders of magnitude larger dissociation constants for Co(II) and Zn(II), respectively (Fig. 3b).[62, 113] Studies of a minimalist CCHH zinc finger domain revealed that its stability is largely dependent on a conserved hydrophobic core (Fig. 3c). Alanine mutation of conserved residues that constitute the hydrophobic core result in not only 15-fold weaker affinity to Zn(II) but also a tendency of Zn-(protein)$_2$ complex formation, aside from the canonical Zn-L complex.[111] Furthermore, substitution of the second conserved phenylalanine with leucine in a ββα zinc finger peptide revealed the significant contribution of solvation enthalpy to protein stability.[114] A CCHH zinc finger domain was also used to examine the effect of ligand substitution (Fig. 3d) on its affinity for Zn(II). A systematic study of the second zinc finger domain in Zif268 demonstrated that substitution of a single Zn(II)-binding cysteine with histidine (CHHH), aspartic acid (CDHH) or glutamic acid (CEHH) reduces its affinity by a factor of 16, 440 and 1000, respectively.[115] These results indicate that classical zinc finger domains possess a rigid structure and that any alteration or truncation of their sequence results in a loss of stability.

Although there are a high sequential and structural similarities among all classical zinc finger domains, they differ in terms of their affinity to Zn(II). As introduced by Berg, the consensus zinc finger sequence CP-1, based on 131 ββα zinc fingers, combines all of the sequence similarities in this small domain.[116] Studies performed on the consensus zinc finger showed that its dissociation constant for Zn(II) is as low as ~10^{-16} M.[62] However, this consensus sequence contains many conserved amino acid residues that may stabilize its structure, thus providing high affinity to Zn(II). Therefore, CP1 seems to be not a relevant stability model for natural ββα zinc fingers.[64] Although natural ββα zinc fingers display many major similarities (Fig. 4a), they differ substantially, for example, in the number of amino

acid residues present between the coordinating cysteines or the number of polar and hydrophobic residues. Our recent studies revealed a difference of nearly five orders of magnitude in the dissociation constants (Fig. 4b) of the highly conserved natural ββα zinc finger domains and CP-1, indicating that huge thermodynamic stabilization effects must be hidden in the short amino acid sequence and the 3D structure of these domains.[64] Interestingly, higher dissociation constant can be correlated with the longer amino acid spacer between to coordinating cysteines located in the β-hairpin of the natural zinc fingers (Fig. 4b). Because the dissociation constants of natural zinc finger domains vary between ~10^{-11} and 10^{-13} M, why the dissociation constant of CP1 is significantly lower (~10^{-16} M) remains unclear.[62, 64] The large variation in the stability constants within the same class of natural zinc fingers are in contrast to the common generalization that all zinc finger domains are simply structural zinc binding sites that are always occupied.[62] A portion of zing finger domains may remain unoccupied and may transiently bind to Zn(II) in the cell.[117] However, this hypothesis must be confirmed for multiple different zinc finger domains *in vivo*.

Another example of a significant contribution of secondary interactions to the stability of a zinc binding site was recently identified for the LIM domain. An individual LIM domain contains a conserved binding sequence, C-X_2-C-X_{16-23}-H-X_2-C-X_2-C-X_2-C-X_{16-21}-C-X_2-(C/H/D/E).[118] The eight highly conserved amino acids required for Zn(II) coordination form two zinc fingers in a tandem topology.[119] Individual zinc fingers from the LIM domain of the PDLIM1 protein display moderate affinity for Zn(II) when examined separately (Fig. 5a). Interestingly, when they are coupled to form a consensus tandem pair, their Zn(II) dissociation constant decreases by 50-fold (Fig. 5b). Elongation of the C-terminus by three amino acid residues from the native sequence results in an additional 5-fold decrease in the dissociation constant (Fig. 5c). Additionally, elongation of the LIM domain by 14 amino acids

at the C-terminus of the protein decreases Zn(II) dissociation constant to a lesser extent (Fig. 5d).[51]

Although many zinc binding domains remain uncharacterized in terms of their affinity for Zn(II), intermolecular zinc domains most likely remain the most enigmatic. Our recent study of zinc hook peptides revealed that intermolecular coordination can display very high affinity due to the formation of a hydrophobic groove in the dimer interface by two pairs of conserved hydrophobic residues (Fig. 6).[52] The alanine scanning analysis showed that the single substitution of valine or leucine to alanine increases dissociation constant by 2-fold and 8-fold respectively, whereas double mutation of both residues resulted in increase of dissociation constant by 62-fold. Substitution of a proline to alanine, resulted in increase of dissociation constant by 47 fold, suggesting that formation of β-hairpin structure is another factor responsible for stabilization of the complex.[52]

4. Properties and architecture of zinc sites – insights into zinc regulation

Zinc regulation is a process in which binding to Zn(II) is thought to induce structural changes that, in turn, modify the functionality of the protein. The factors that determine Zn(II) regulation, which are influenced by the physicochemical properties of protein, include the stability of interaction, the on and off kinetics, the stoichiometry of the complexes, Zn(II) ligand reactivity, and the protein structure. Notably, the mechanisms that alter the Zn(II)-protein binding properties mutually influence one another (Fig. 7).

The protein affinity for Zn(II) is a critical determinant of whether the binding of Zn(II) to the protein is permanent, transient or nonexistent in a physiological protein environment. Although the data from the literature on the affinities of Zn(II) to proteins remain limited, the available data demonstrate large diversity in zinc protein stability (Table 1). For example, the affinity of Zn(II) to intercellular catalytic and structural sites in proteins is generally high, as

proteins containing Zn(II) in catalytic and structural sites utilize different inter- and intraprotein interactions to increase their affinity to Zn(II). As for regulatory zinc binding sites, biologically relevant regulation of protein function is expected to be reversible; therefore, Zn(II)-regulated proteins exhibit lower affinity to Zn(II), facilitating transient Zn(II) binding.[11] Lower affinity to Zn(II) is attained by decreasing the number of protein derived ligands bound to metal ion, distortion from ideal geometry, increased number of hard base atom donors (according to the HSAB concept), or decreasing the number of interactions with the secondary coordination sphere and the global protein structure (Fig. 7).[66]

Proteins that perform transient Zn(II)-dependent activities frequently display lower affinity to Zn(II) than typical catalytic and structural zinc binding sites to appropriately respond to amplitude of physiological $[Zn(II)_{free}]$ fluctuations (Fig. 1).[10, 26] A study of protein tyrosine phosphatase (PTP 1B) showed that the activity of this enzyme is inhibited by $[Zn(II)_{free}] < \sim 10^{-8}$ M and thus may also be controlled by metallothionein (MT) as a source of Zn(II). Whether or not Zn(II) ions are indeed available for interaction with the enzyme depends on the apoprotein thionein (T) to MT ratio and its redox state.[26] A recent study showed that receptor protein-tyrosine phosphatase β displays an even lower dissociation constant $\sim 10^{-11}$ M demonstrating that transient inhibition of this receptor occurs in the picomolar range of the $[Zn(II)_{free}]$.[8] Although the range of the $[Zn(II)_{free}]$ fluctuations is relatively narrow ($\sim 10^{-8} < [Zn(II)_{free}] < \sim 10^{-9}$) it may be adequate to regulate the activity of various proteins (Fig. 1).[26, 11]

Zinc binding sites with similar affinities can bind and dissociate Zn(II) at different rates. For example, many zinc enzymes, such as carbonic anhydrase, display high affinity for Zn(II) and a very slow dissociation constant (k_{off}) of the metal complex.[120] A notable exception is metallo-β-lactamase IMP-1, in which two tightly bound Zn(II) ions in the active site are amenable to facile displacement by exogenous Zn(II).[121] Studies performed on Zn(II)

peptide complexes have revealed that a thermodynamically stable zinc protein may become kinetically unstable in a competitive environment and directly transfer their Zn(II) via intermediate ternary complex formation rather than via an association/dissociation mechanism.[122] Tetrathiolate ZnS_4 sites are more reactive in metal transfer reactions than ZnS_2N_2 sites despite their similar affinity for Zn(II). This finding is explained by the electrostatic repulsion between thiolates, which promotes the temporary breaking of individual coordinative bonds.[122] The opposing properties of thermodynamic stability and kinetic lability of the Zn(II)-bound protein are intrinsic to metallothioneins and are thought to be related to the their unique Zn(II)-thiolate clusters. Kinetically labile metallothioneins are capable of rapidly transferring their tightly bound Zn(II) to other acceptor proteins and exchanging with exogenous heavy metal ions.[123] As mentioned above, metallothionein does not possess hydrophobic residues, and the lack of a hydrophobic core simplifies Zn(II) exchange (Fig. 7).

Chemical modification of the ligand residue typically impairs its coordination properties, thereby influencing the affinity, binding kinetics and structure of the zinc binding site. Among all of the principal proteins ligands that bind to Zn(II), cysteine thiolates exhibit the highest reactivity due to their nucleophilicity. Therefore, ZnS_4 sites (-2 charge) are expected to be most reactive, followed by ZnS_3N (-1 charge) and Zn-S_2N_2 (neutral).[124] In addition to the ligand composition of a zinc binding site, its local protein environment is an apparent determinant of reactivity. Analysis of zinc finger domain structures revealed an increasing number of steric and electrostatic screening interactions around the coordination sphere as the anionic property (reactivity) of the coordination sphere increases. These interactions include NH···S hydrogen bonds and core/charge interactions.[124] It was also found that more flexible, i.e., less structured, zinc binding sites are more reactive.[125] Chemical reactions of cysteine may include oxidation to form various species, alkylation or S-

nitrosylation.[126,127] The DNA repair protein ADA, which removes methyl groups from DNA backbone phosphoesters, is a notable example in which a Zn(II)-coordinating cysteine is methylated.[125] Several other proteins that exhibit similar transfer of an alkyl group to a Zn(II)-coordinating cysteine have been identified.[128] Interestingly, S-nitrosylation of a cysteine residue can act as a molecular switch that disrupts the intermolecular zinc binding site, leading to the dissociation of the Zn(II)-bridged dimer.[129] Susceptibility to oxidation of cysteine-containing zinc sites depends on several factors such as their structure, zinc affinity and presence of hydrophobic residues around the zinc binding site.[130, 51] Metallothionein is an example of protein with very fast oxidation rates and can be converted to partially oxidized MT (MT_{ox}) and oxidized T (T_{ox}) forms in monomeric or oligomeric species.[131, 132, 133] Oxidation of metallothionein results in Zn(II) release from binding sites and increase of [$Zn(II)_{free}$].[133, 134] The highly dynamic equilibrium between oxidized and reduced species of metallothionein/thionein with various zinc affinity makes this protein central in Zn(II)/thiolate redox biochemistry.[133, 131, 135] Recently, an interplay between cysteine oxidation and formation of intermolecular zinc site has been reported for the mCRY1/mPER2 protein complex, responsible for circadian rhythm regulation. Interestingly, the dissociation constant for zinc binding to the mCRY1/mPER2 complex has been found in the lower nanomolar range which suggests a dynamic, regulatory character of the zinc site (Fig. 7).[5]

Ultimately, all zinc protein complexes are driven by the free energy change resulting from their association with Zn(II) and the concentration of the substrates, both Zn(II) and the protein. The protein concentration factor appears to negligible for 1:1 complex formation as long as it is within a range in which it does not significantly perturb the [$Zn(II)_{free}$]. Furthermore, this rule appears to be applicable for complexes in which one protein molecule binds to more than one Zn(II) ion. In contrast to this result, formation of protein complexes that bind Zn(II) at the interface between subunits may be regulated by Zn(II) affinity of the

Zn(II)-bridged ternary complex, [$Zn(II)_{free}$] and concentration of protein subunits. Therefore, protein concentration appears as a key factor determining the fraction of Zn(II)-bridged ternary complex. Thus, intermolecular zinc binding sites provide a variety of roles depending on the protein concentration (Fig. 7). The potential function of the regulation of intermolecular zinc binding sites is that there is an extremely large dynamic range of protein abundance in cells, from less than 50 to more than 10^6 molecules per cell while only a few high-copy proteins contribute to most of the total protein mass.[136, 137] Moreover, the local and global concentrations of proteins can vary widely and display dynamic characteristics. Some specific proteins are transiently localized to the membrane and neighboring regions or in cellular compartments. Quantitative analysis of the proteome dynamics, with particular attention to cellular compartments, remains a holy grail.[138]

5. Concluding remarks

Cellular zinc proteins must bind to Zn(II) with sufficient affinity to perform their function under a low level of tightly controlled [$Zn(II)_{free}$]. The stability of zinc proteins is regulated by many structural and physiochemical factors. Some of these structural factors permanently determine the protein affinity for Zn(II), whereas others may change dynamically and regulate the Zn(II) coordination environment in a manner that reversibly modulates the zinc protein function. We are beginning to understand the underlying principles of the Zn(II)-dependent regulation of protein functions, revealing their great diversity. In this article, we primarily focused on how zinc binding sites present in proteins differ in their architecture and how their structural arrangement is associated with protein function, Zn(II) binding affinity and zinc-dependent regulation. We suggest that the concentration-dependent functionality of the

interprotein zinc binding site serves as another factor regulating the relationship between cellular Zn(II) availability and protein function.

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Table 1. The affinities of zinc proteins and zinc binding domains for Zn(II).

Protein	Architecture: intermolecular/intramolecular and mononuclear/multinuclear	Binding amino acid residues	Function (Localization)[a]	-logK_d	Method of determination[d]	Ref.
Alpha-fetoprotein (*Thunnus alalunga*)	intramolecular mononuclear	HHH	S (E)	9.4	Equilibrium dialysis	32
Alkaline phosphatase (*Escherichia coli*)	intramolecular multinuclear	2 sites: DDH, DHH	C (I)	11.3, 11.7	Equilibrium dialysis	33
Aminopeptidase-B (*Rattus norvegicus*)	intramolecular mononuclear	HHE	C (I)	12.4	Competition with 2-PA	34
Angiotensin converting enzyme (*Oryctolagus cuniculus)*	intramolecular mononuclear	HHE	C (E)	8.2	Competition with OP	35
Antitoxin MqsA (*Escherichia coli*)	intramolecular mononuclear	CCCC	S (I)	≥17	Competition with TPEN, PAR	36
Dipeptidyl peptidase III (*Rattus norvegicus*)	intramolecular mononuclear	HHE	C (I)	12.3	Competition with 2-PA	37
HIV nucleocapsid (NCp7)	intramolecular mononuclear	CCHC	S (I)	10.2	Spectrophotmetric reverse titration of Pb(II) complex with Zn(II)	38
Hsp33 (*Escherichia coli*)	intramolecular mononuclear	CCCC	C (I)	17.4	Competition with PAR	39
Human carbonic anhydrase (*Homo sapiens*)	intramolecular mononuclear	HHH	C (I)	12.0	Competition with PAR	40
Human estrogen receptor α (*Homo sapiens*)	intramolecular mononuclear	2 sites: CCCC	S, K (I)	9.3, 10.0	Spectrophotmetric reverse titration of Co(II) complex with Zn(II)	41
Human serum albumin (HSA) (*Homo sapiens*)	intramolecular mononuclear	HDHD	S (E)	7.5	Equilibrium dialysis	42

Human serum transferrin (*Homo sapiens*)	intramolecular mononuclear	[c]	S (E)	7.4	Competition with NTA and TETA	43
Keap1 (*Mus musculus*)	intramolecular mononuclear	CCCC	S (I)	11.0	Competition with PAR	44
MTF-1 (*Mus musculus*)	intramolecular mononuclear	6 sites: CCHH	S (I)	10.5	Spectrophotmetric reverse titration of Co(II) complex with Zn(II)	45
GAGA (*Drosophila melanogaster*)	intramolecular mononuclear	CCHH	S (I)	8.3	Gel mobility	46
Glucocorticoid receptor α (*Rattus norvegicus*)	intramolecular mononuclear	2 sites: CCCC	S, K (I)	9.7, 9.5	Spectrophotmetric reverse titration of Co(II) complex with Zn(II)	41
Mammalian serum retinol-binding protein (RBP) (*Sus domesticus*)	intramolecular mononuclear	HHH	S (E)	11.7	Equilibrium dialysis	47
Metallothionein MT2a (*Homo sapiens*)	intramolecular multinuclear	7 sites: CCCC	S/R (I)	11.8 (4 sites), 10.5, 9.9, 7.7	Competition with FluoZin-3	48
Metallothionein MT3 (*Homo sapiens*)	intramolecular multinuclear	7 sites: CCCC	S/R (I)	11.5 (7 sites)	Competition with 5F-BAPTA	49
Neural zinc finger factor 1 (*Rattus norvegicus*)	intramolecular mononuclear	CCHC	S (I)	9.9	Competitive metal binding spectrophotometry titration: Co(II) displacement using Zn(II)	50
PDZ and LIM domain protein 1 – LIM domain (*Homo sapiens*)	intramolecular mononuclear	2 sites: CCHC, CCCH	S (I)	14.5	Competition with EDTA	51
Rad50 protein (*Pyrococcus furiosus*)	intermolecular mononuclear	Peptide fragment 440-453 2×CC	S (I)	19.2[b]	Competition with EDTA	52
Retroviral gag gene-encoded core nucleic acid binding protein	intramolecular mononuclear	CCHC	S (I)	12.0	Spectrophotmetric reverse titration of Co(II) complex with Zn(II)	53

(Rauscher murine leukemia virus)						
Superoxide dismutase (*Homo sapiens*)	intramolecular mononuclear	HHHD	S (I)	13.4	Competition with PAR	54
Thermolysin (*Bacillus thermoproteolyticus*)	intramolecular mononuclear	HHE	C (E)	11.3	Competition with OP	55
Transcription factor Sp1-3 (*Homo sapiens*)	intramolecular mononuclear	CCHH	S (I)	9.2	Spectrophotmetric reverse titration of Co(II) complex with Zn(II)	56
TFIIIA (*Xenopus laevis*)	intramolecular mononuclear	CCHH	S (I)	8.5	Equilibrium dialysis	57
Tristetraprolin (*Mus musculus*)	intramolecular mononuclear	2 sites: CCCH	S (I)	10.2	Spectrophotmetric reverse titration of Co(II) complex with Zn(II)	58
Xeroderma pigmentosum group A complementing protein XPAzf (*Homo sapiens*)	intramolecular mononuclear	CCCC	S (I)	9.8	Spectrophotmetric reverse titration of Ni(II) complex with Zn(II)	59

[a] C, S, R, I, and E denote catalytic, structural, and regulatory protein function and intra- and extracellular localization, respectively.

[b] K_d values determined for ZnL_2 stoichiometry.

[c] No data available.

[d] 5F-BAPTA, NTA, OP, 2-PA, PAR, TPEN, TETA denote: 1,2-bis(2-amino-5-fluorophenoxy)ethane-N,N,N‘,N‘-tetraacetic acid, nitrilotriacetic acid, 1,10-phenanthroline, 2-pyridinecarboxylic acid, 4-(2-pyridylazo)resorcinol, N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine, respectively.

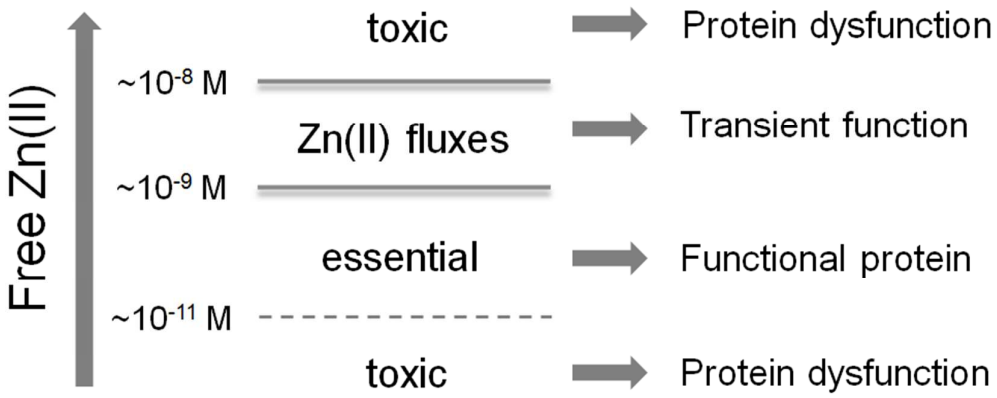

Figure 1. Impact of free Zn(II) concentration [$Zn(II)_{free}$] on protein functionality in the cell.

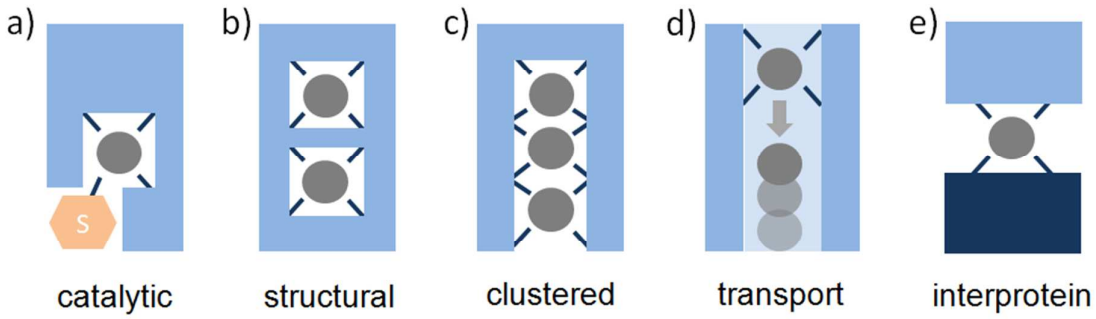

Figure 2. Schematic representation of zinc coordination architectures in proteins.

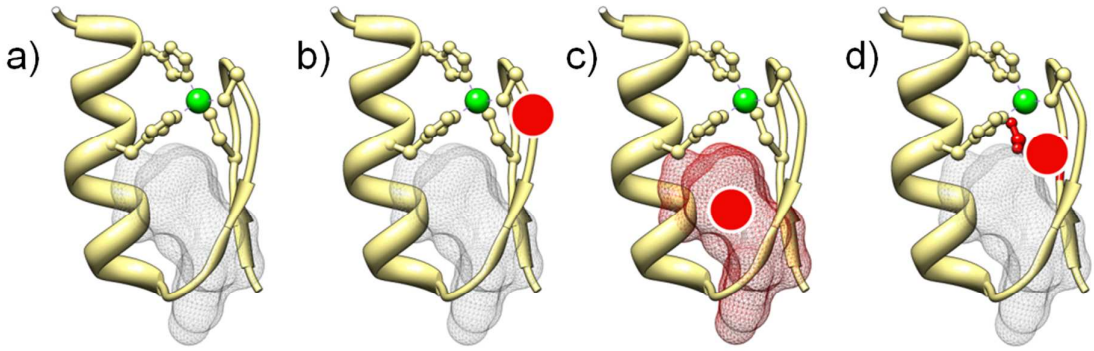

Figure 3. Influence of alterations in the amino acid sequence of the classical zinc finger domain on its thermodynamic stability. a) Typical ββα zinc finger domain containing its conserved hydrophobic core (gray); b) Deletion of an amino acid following the second cysteine residue in the large loop; c) Alanine substitution of conserved residues responsible for formation of the hydrophobic core; d) Substitutions of the first Zn(II)-coordinating cysteine. Red indicates a change in the zinc finger domain.

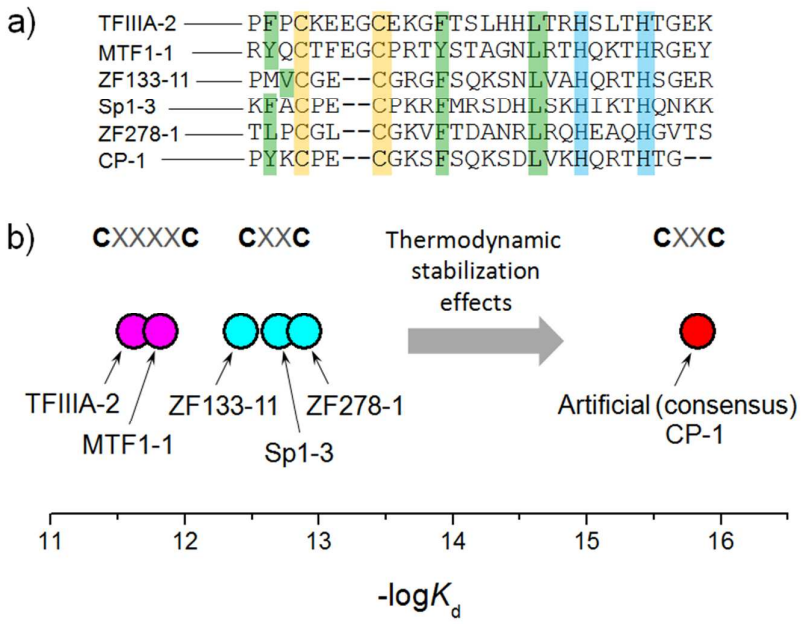

Figure 4. Comparison of ββα zinc finger domains. a) Amino acid sequences of the examined natural and consensus ββα zinc finger domains;[62, 64] b) Comparison of the dissociation constants of the natural and consensus zinc finger domains. CXXC and CXXXXC denote the number of amino acid residues present in the β-hairpin loop between the Zn(II)-binding cysteines.

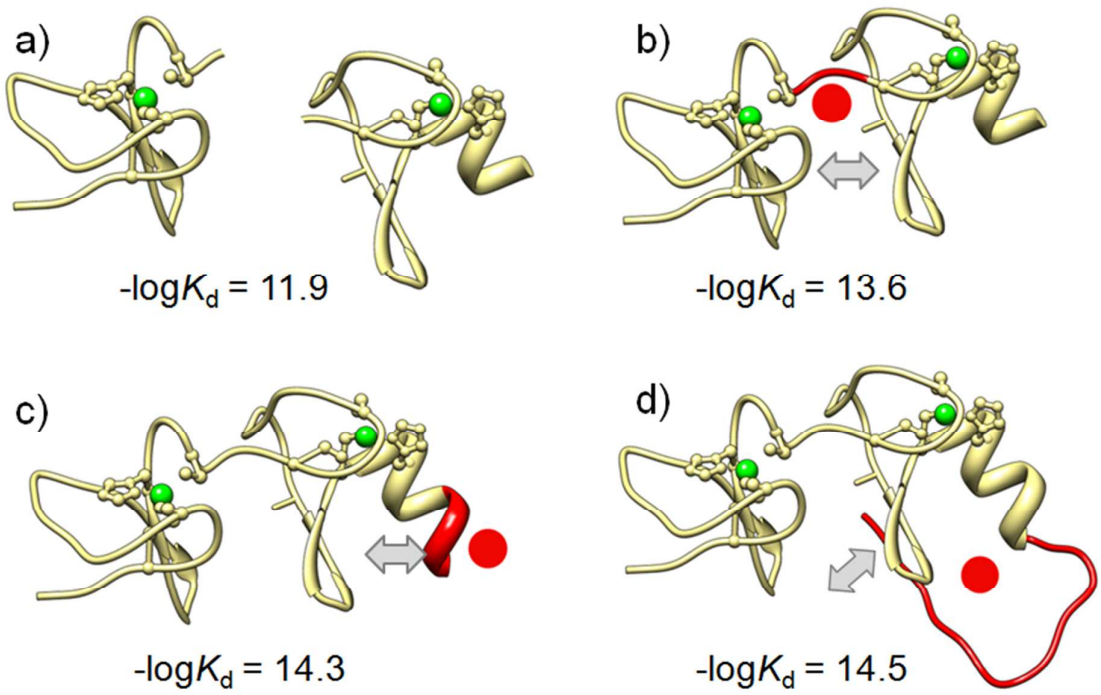

Figure 5. Influence of truncation of the PDLIM1 protein LIM domain on its average affinity for Zn(II).[51] a) The separate zinc finger domains in the LIM domain; b) The LIM domain; its length is based on the consensus of the literature; c) The LIM domain elongated three amino acids at its C-terminus (newly suggested full length of the LIM domain); d) The full length LIM domain of the PDLIM1 protein elongated by additional 14 amino acid residues at the C-terminal tail. The gray arrows indicate the interaction regions responsible for the stability changes. Red demonstrates a domain truncation.

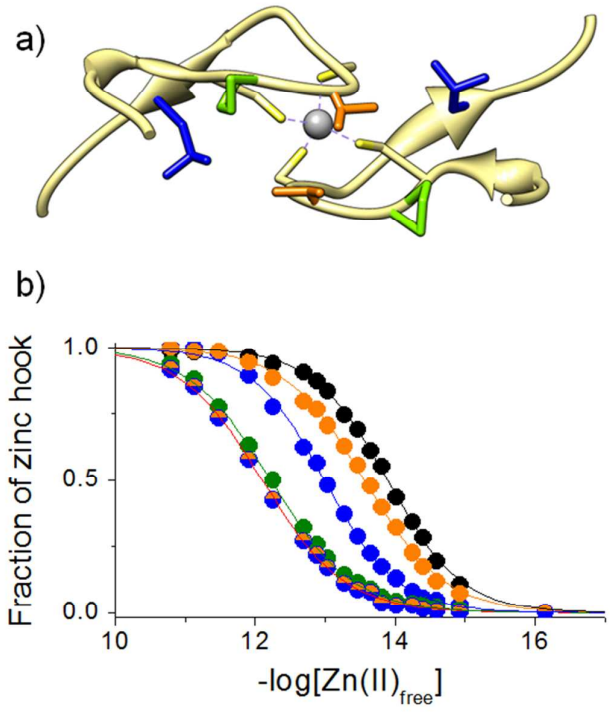

Figure 6. Influence of particular amino acids on the affinity of Zn(II) to a zinc hook domain fragment.[52] a) Structure of the zinc hook domain based on the crystal structure of a Rad50 protein fragment from *Pyrococcus furiosus* (pdb: 1L8D). Orange, blue and green represent valine, leucine and proline; b) Isotherms of zinc hook complex formation for the wild type (black circles) and alanine-substituted valine, leucine and proline zinc hook peptides. The orange-blue circles denote the valine and leucine double mutant.

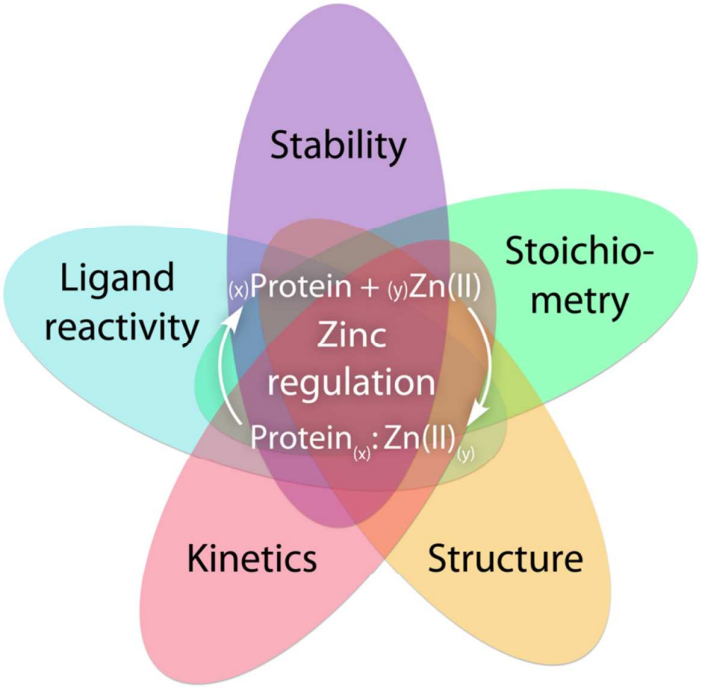

Figure 7. Schematic representation of the discussed factors that mediate zinc protein regulation in the cell.

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