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Zinc proteomics and the annotation of the human zinc proteome*

Wolfgang Maret‡

Departments of Preventive Medicine and Community Health and Anesthesiology, The University of Texas Medical Branch, Galveston, TX 77555, USA

Abstract: Sequence databases can be searched for homologies of zinc coordination motifs with characteristic ligand signatures. Ensuing predictions that 3–10 % of the human genes encodes zinc proteins are most remarkable. But they seem conservative when considering that database mining cannot discover new signatures or coordination environments that employ nonsequential binding of ligands and sulfur-ligand bridges. Predictions also fall short for zinc/protein interactions at protein interfaces and for inhibitory zinc sites. Zinc ions transiently target proteins that are not known to be zinc proteins, adding a hitherto unrecognized dimension to the human zinc proteome. Predicted zinc sites need to be verified experimentally. The metal can be absent or sites may bind metal ions other than zinc because protein coordination environments do not have absolute specificity for zinc. The metaphor of the "galvanization of biology" continues to gain prominence in terms of the sheer number of approximately 3000 human zinc proteins and their annotation with new functions. Clearly, description of zinc proteomes cannot be pursued solely in silico and requires zinc proteomics, an integrated scientific approach. Progress hinges on a combination of bioinformatics, biology, and significantly, analytical and structural chemistry.

Keywords: metallomics; zinc; zinc proteins; zinc proteomics.

FROM A SINGLE ZINC PROTEIN TO THE HUMAN ZINC PROTEOME

Zinc, Zn(II), was found to be nutritionally essential for growth of the common bread mold *Aspergillus niger* in 1869, rats in 1934, and humans in 1961 [1–3]. A milestone in establishing the molecular functions of zinc was the discovery in 1939 that erythrocyte carbonic anhydrase contains stoichiometric amounts of zinc, and that zinc is essential for its enzymatic activity [4]. It took another 15 years before a second zinc enzyme, bovine pancreatic carboxypeptidase, was identified [5]. Advances in techniques for protein isolation and metal analyses accelerated the discovery of zinc proteins in all phyla of life and in all classes of enzymes, i.e., oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases [6]. To establish a protein as a zinc protein, the protein has to be isolated and zinc analyzed with a spectroscopic technique to determine the stoichiometry of zinc binding, a procedure that continues to be a challenge in terms of the determination of accurate protein concentrations and stringent control of metal ion contamination. Coordination environments are established by high-resolution structural analysis.

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‡Tel.: (409) 772-4661; Fax: (409) 772-6287; E-mail: womaret@utmb.edu

A major change in the way in which zinc proteins were discovered came with the finding that zinc is a constituent of the *Xenopus laevis* transcription factor IIIA (TFIIIA) [7]. This protein contains nine repetitive sequences of cysteine (C) and histidine (H) residues that were shown, by extended X-ray absorption fine structure (EXAFS) spectroscopy, to bind zinc. The repeats were referred to as DNA-binding domains or fingers [8]. Hence, "zinc fingers" became a generic term for such structural zinc sites. With rapid increases in the number and sizes of sequence databases, searches for homology could be performed and zinc-binding sites could be predicted by database mining. Based solely on the signature of the classical C_2H_2 zinc finger motif, 5092 protein domains containing this motif were identified in the human genome [9]. The number of zinc proteins identified by this zinc finger motif is actually smaller, because many proteins contain several zinc fingers, with a maximum of 36 zinc fingers noted in one particular protein. In addition, there are coordination motifs with four cysteine ligands (C_4), with three cysteine ligands and one histidine ligand (C_3H), and with one cysteine, one aspartate, and two histidine ligands (CDH_2) [10]. The motifs occur as either single zinc fingers or as double zinc fingers with almost any permutation of the order in which the cysteine and histidine ligands occur in the sequence [11]. A triple zinc finger has also been characterized structurally [12]. Not all of the protein domains that are organized by zinc finger motifs interact with DNA or RNA. Many of them interact with proteins, and some of them with lipids [13,14]. In the description of new proteins, it now became customary to assign zinc finger domains without ever performing a chemical analysis of zinc.

Typically, zinc in zinc finger motifs is tetracoordinate. The motifs contain a short amino acid spacer (X) between the first two ligands, followed by a longer spacer between the second and third (Y) and again a short spacer between the third and fourth ligands (Z) (Fig. 1A). The proximity of the four ligands and the fact that zinc often organizes relatively small protein domains contributed to the success of this database-mining approach in predicting zinc fingers. Coordination motifs in zinc enzymes are more variable. The majority has only three zinc ligands that are separated by a long spacer (Y) that follows or precedes a short or a long spacer (X) (Fig. 1B). Characteristic ligand signatures in primary structures and zinc coordination motifs were originally established as standards of reference from only about a dozen structures of zinc proteins [15], but are now based on a few thousand structures in the protein database [16,17]. With an increasing number of 3D structures being solved, the number of such structural templates also increased. Database mining contributed significantly to the prediction of structures and functions of zinc in enzymes. Experimental verification of the predictions validated this approach. For example, a zinc-binding site in human LTA_4 (leukotriene A_4) hydrolase was predicted through successful alignment of its putative ligands with the ligands of the catalytic zinc in the bacterial enzyme thermolysin and aminopeptidases, i.e., HExxH….E [15]. The prediction that LTA_4 hydrolase is a zinc enzyme was tested and confirmed by direct analysis [18]. Based on its analogy with thermolysin, the prediction of an activity as a peptidase was also verified [19]. The crystal structure of human LTA_4 hydrolase proved the structural prediction to be correct [20]. The discovery of the relationship of collagenases, snake venoms, and bacterial proteinases was also a triumph for predictions using searches of protein databases before crystal or solution structures of these proteins existed [15]. When collagenases and related endopeptidases are aligned with thermolysin, the third ligand, glutamate (E), is absent. However, when comparing the sequence of the *Astacus* proteinase, or astacin, with some of these proteins and human bone morphogenetic protein (BMP-1), which at that time was not known to have proteinase activity, conserved glycine and histidine residues were recognized and it was suggested that the third ligand could be a histidine in a ligand signature HExxHxxGxxH [21]. The 3D structure of astacin demonstrated that the third ligand indeed is a histidine, thus establishing a founding member of a new family of metallopeptidases [22]. A more extensive search with the above sequence yielded 33 proteinase sequences with the same putative ligands and defined four subclasses for this major proteinase superfamily [23]. Incidentally, the name "metallopeptidases" for an entire clade of proteinases does not foretell that they are zinc enzymes. In contrast to zinc fingers with four ligands, searching with only two out of the three or more ligands makes it more difficult to predict an enzymatic

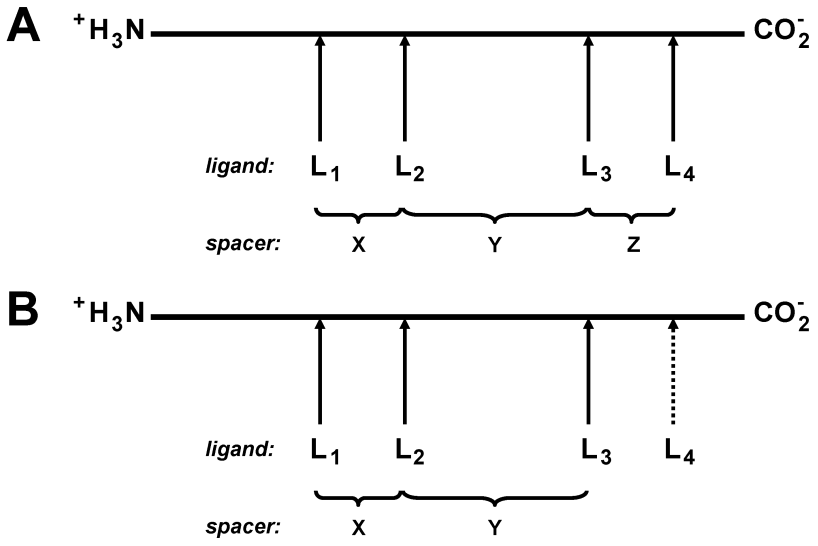

Fig. 1 Ligand (L) signatures and amino acid spacers between the ligands (X,Y, Z) in tetracoordinate structural (A) and catalytic (B) zinc sites in proteins.

zinc site. The difficulties are compounded by the occurrence of multinuclear zinc sites and the occasional presence of long spacers when different domains organize the active site.

Once entire genomes were sequenced and could be mined, predictions were made that at least 3 % of the human genome encodes for zinc proteins [24]. However, it was pointed out that the actual number could be considerably larger because this approach does not account for all zinc/protein interactions [11,25]. A subsequent report using a bioinformatics approach indeed suggested that at least 10 % (about 2800 gene products) of the human genome encodes zinc proteins [26]. As possible zinc proteins were counted: 397 hydrolases; 302 ligases; 167 transferases; 43 oxidoreductases and 24 lyases/isomerases; 957 transcriptions factors; 221 signaling proteins; 141 transport/storage proteins; 53 proteins with structural metal sites; 19 proteins involved in DNA repair, replication, and translation; 427 zinc finger proteins of unknown function; and 456 proteins of unknown function. The authors used three approaches, one based on structures, mining the human proteome with ligand signatures based on known 3D structures, one based on domains, using the libraries of metal-binding domains from the Pfam database [27], and one based on annotations, using any information available about the gene sequences. All three approaches independently identified 1684 zinc proteins.

In summary, starting with the discovery of a single zinc protein in 1939, estimates of the number of human zinc proteins were constantly revised upwards and have now reached the staggering number of a few thousand. It does seem that zinc has galvanized biology [28]. One wonders whether we really have accounted for most of the zinc proteins in the human zinc proteome. The answer is: most likely not. The reasons for this answer are discussed as follows.

A CASE FOR EMPLOYING AN INTEGRATED APPROACH—"ZINC PROTEOMICS"

Undoubtedly, the above predictions set new horizons on the possible scope of the human zinc proteome, they open new dimensions for the role of zinc in biology, and they generate numerous opportunities for experimental exploration of hypotheses, as follows.

1. *Predictions are based on known signatures.* New structures cannot be predicted. Therefore, how complete the coverage is hinges upon whether or not known structures of zinc proteins sufficiently represent all possible signatures. The potential for additional signatures in the human zinc proteome is

high since new motifs continue to be discovered. In a number of domains that are organized by two or three zinc ions, the order of zinc ligands is nonsequential and the sulfur of cysteine can be used twice for bridging different zinc ions in clusters (Fig. 2). Such cross-braced ligand-binding patterns cannot be predicted, and the number of topologies that organize protein architecture through the use of zinc ions is remarkable [13]. An example for the failure of predictions is Churchill, an embryonic neural inducing factor [30]. While it was deduced from the sequence of Churchill that the protein contains two canonical C_4 (tetrathiolate) zinc fingers, the 3D structure demonstrated that it actually contains three zinc ions in a novel topology that includes unsuspected, nonsequential use of ligands, additional histidine ligands, and a binuclear zinc cluster. Thus, predictions about the number of bound zinc ions and their binding modes quite simply can be wrong, even when based on homology with known signatures. A suggestion that metals bind may be put forward, but the inability to predict bi-, tri- and tetranuclear sites with ligands "criss-crossing" to form zinc/thiolate clusters underscores the limitations of predictions. In many cases, 3D structures have been, and will be, the ultimate arbiters for establishing the modes of zinc binding.

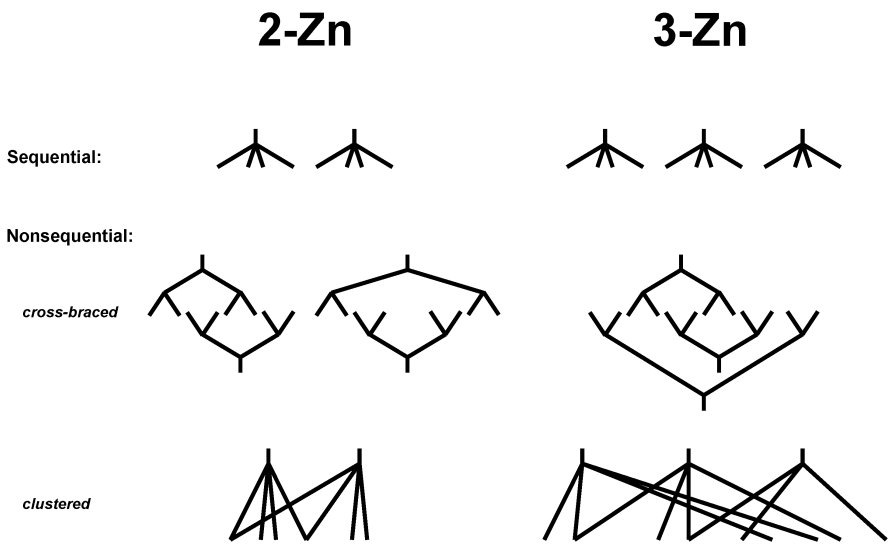

Fig. 2 Zinc-binding ligand signatures in protein domains that are organized by two (2-Zn) and three (3-Zn) zinc ions. The ligands that coordinate zinc in the peptide chain can be sequential (top row) or nonsequential (middle and bottom rows). The nonsequential binding patterns can be further divided into cross-braced (middle row) as shown here, from left to right, for the FYVE domain, the DNAJ cysteine-rich domain (for a review of these binding patterns see [11]), and the Ubp-M BUZ domain [29], and into clustered (criss-crossed) (bottom row) when sulfur-ligand bridges are used as shown here for the zinc/thiolate clusters in fungal transcription factors and for the N-terminal domain of human metallothioneins. Ligand binding in the protein Churchill, which is discussed in the text, is both cross-braced and clustered.

2. *Zinc may not be present because the site does not bind a metal or the site may bind a different metal.* "Zinc finger" became a generic term that is used as if zinc is indeed present. However, the metal ion may not be zinc. While a certain promiscuity of prokaryotic metalloproteins in their choice of metal ions is acknowledged, the issue of metal specificity requires scrutiny for eukaryotic proteins. Their metal homeostatic systems exert strict control over metal availability and distribution. Therefore, the issue may be one of mistaken identity rather than promiscuity. The crystal structure of human histone deacetylase 8 demonstrates a catalytic zinc ion [31]. However, recombinant protein purified from *E. coli* contains eight times more iron than zinc and is more active with iron than with zinc. These observations

led the authors to suggest that Fe(II) may be the catalytic metal in vivo [32]. Also, the CDGSH domain contains iron in MitoNEET, a protein in the outer mitochondrial membrane, despite the previous annotation of this domain as a zinc finger motif [33]. Even if one can exclude the possibility that the wrong metal ion has been incorporated into a metalloprotein because a prokaryotic expression system does not reflect the conditions of metal availability in eukaryotic organisms and because metal acquisition can depend on the composition of the growth media, it remains unknown to which extent zinc signatures overlap with those of other metal ions, in particular, Fe(II). A considerable amount of theoretical work is being pursued to further examine and define the specificity of metal-binding sites in proteins [34,35]. Knowledge from such studies comes to bear on the prediction of metal-binding sites of those proteins for which a 3D structure has been resolved only for the apoform [36].

Location of a protein with potential zinc-binding cysteine ligands is important because the environment may favor disulfide bond formation rather than zinc/thiolate coordination [37,38]. For example, HEDJ, a DNAJ/Hsp40 type of chaperone, localizes to the endoplasmic reticulum. Its zinc-binding ligands are predominantly oxidized to intramolecular disulfide bonds [39]. In addition, many proteins of the intermembrane space of mitochondria have twin Cx_3C or Cx_9C motifs that bind zinc and copper or form disulfides [40,41]. It has been suggested that zinc binding to the reduced form of these proteins keeps them in a conformation that is competent for their import into the intermembrane space [42].

3. *Zinc may bind only transiently.* Isolation of a protein as a zinc protein requires that the binding be so tight that zinc does not dissociate during isolation. In this way, a distinction between metalloproteins and metal-protein complexes was made [43]. Metal-protein complexes have significantly lower affinities for the metal and hence may lose it during purification. Whether zinc-protein complexes with lower affinities than those of zinc metalloproteins are physiologically significant is a matter of their binding affinities and the concentrations of zinc ions available in the cell. Based on fluctuations of cytosolic free zinc ions in the picomolar to low nanomolar concentration range, it was suggested that sites with zinc affinities in this range may represent regulatory zinc sites [44,45]. Indeed, some enzymes that are not recognized as zinc metalloenzymes are tightly inhibited by zinc [46]. For example, the zinc affinity of human protein tyrosine phosphatase 1B, which regulates the phosphorylation state of the insulin receptor, is 15 nM [45]. Corrected for free zinc ion concentrations in the assay, the K_i value for zinc inhibition of human erythrocyte Ca^{2+}-ATPase is 80 pM [47]. Given that the free zinc ion concentration in erythrocytes has been estimated to be 24 pM [48], such binding of inhibitory zinc ions may be physiologically significant.

Structures are known for some inhibitory zinc sites. In some cases, they have coordination environments similar to those of typical zinc enzymes. However, while cytosolic zinc enzymes have subpicomolar zinc affinities, transient sites bind zinc much weaker. Specific structural features seem to destabilize these zinc sites, either thermodynamically or kinetically, for functions that do not depend on the permanent presence of zinc. What these features are and how they make affinities different from those of zinc enzymes is unknown. Even for proteins with similar tetrathiolate coordination environments, such as human metallothionein, the affinities vary over orders of magnitude. At least one of the seven zinc-binding sites in human metallothionein-2 is thought not to be occupied with zinc under normal physiological conditions [49].

Some inhibitory zinc sites have affinities in the nano- to micromolar range. Again, knowing the location of the protein is important for interpreting data because some cellular and extracellular compartments may allow higher free zinc ion concentrations than those in the cytosol. Zinc ions are stored in vesicles ("zincosomes") that localize to specialized neurons in the brain [50]. They are secreted from such vesicles and affect the postsynaptic neuron. One target of zinc ions is the *N*-methyl D-aspartate (NMDA) receptor, where zinc binds with nanomolar affinities to the NR1a-NR2A receptor and with micromolar affinities to the NR1a-NR2B receptor [51]. The relatively high synaptic zinc concentration after secretion suggests physiological significance of this inhibition.

Mitochondrial aconitase has an inhibitory zinc site ($K_i = 2$ μM) with two histidine ligands and one aspartate ligand [52]. In bovine dimethylarginine dimethylaminohydrolase, zinc binds to only one cys-

teine ligand in the active site at pH 6.3. An additional histidine ligand participates in binding at pH 9.0 [53]. The coordination at pH 7.4, where zinc binds with a K_i of 4 nM, remains unknown [54].

Carboxypeptidase A, a zinc proteinase, is inhibited by the binding of an additional zinc ion with a K_i of 0.5 μM [55]. Employing a combination of kinetics, visible spectroscopy with cobalt(II) as a probe for the catalytic zinc, and chemical modification, it was predicted that the inhibitory zinc would be bound by glutamate-270, a hydroxide that bridges the catalytic and inhibitory zinc, and a chloride ion [56]. All of these predictions proved to be correct when the crystal structure of the protein with the inhibitory metal ion was solved [57]. Significantly, zinc also inhibits proteinases that do not depend on zinc as the catalytic metal ion. An example where data on both zinc inhibition and 3D structures of the zinc-protein complexes are available is the human kallikrein (hK) family of serine proteinases (Table 1). Low micromolar concentrations of zinc inhibit these enzymes. The kallikrein hK4 has a zinc-binding site with a histidine ligand and a glutamate ligand [60]. In tonin, a rat kallikrein, the zinc ligands are three histidines, including a glutamate from a neighboring protein molecule. In hK5, two histidine ligands coordinate the inhibitory zinc. Based on its homology with tonin, a third histidine ligand is thought to be recruited for zinc binding [61]. A similar coordination is proposed for hK7 [62]. Zinc coordination with three histidine ligands in human carbonic anhydrase provides a site with a dissociation constant of 11.4 (pK_d) [64]. The factors that determine the 5-order-of-magnitude difference in stabilities between the catalytic zinc in carbonic anhydrases and the inhibitory zinc in kallikreins remain largely unknown. One reason for this difference may be the fact that the affinity of zinc in catalytic sites of enzymes, such as carbonic anhydrase, is not determined only by the direct zinc ligands but also by the residues that form hydrogen bonds with these ligands and additional secondary interactions [16,65]. For those of the kallikreins that function in prostate physiology, such as hK3 (prostate-specific antigen or PSA) and hK4 (prostase), a case for the physiological significance of zinc inhibition can be made because zinc concentrations in seminal plasma and prostate fluid are around 10 mM, and thus, they are about 1000 times higher than those in blood. However, it is not the total zinc concentration alone, but the zinc buffering capacity and the free zinc ion concentration that are important for relating the availability of zinc ions to the zinc affinities of proteins. It is unknown whether inhibitory zinc sites have unique signatures that could be used for database mining. Hence, the number of physiologically important inhibitory zinc sites cannot be predicted and remains unknown.

Table 1 Zinc inhibition constants for human kallikreins.

Human kallikrein	$K_{i\ (app)}$, μM	Ref.
2	3–5 (pH 7.5)	[58]
3	6 (pH 7.5)	[59]
4	16 (pH 7), IC50	[60]
5	8 (pH 7); 2 (pH 8)	[61]
7	10 (pH 7.5)	[62]
14	0.01 (pH 8)	[63]

Protein interface sites are another class of zinc/protein interactions, where an assessment of how frequently they occur has been impossible [66,67]. Ligands can stem from two, three, or even four protomers, and there can be homologous and heterologous protein/protein interactions. Obviously, predictions of zinc binding cannot be made if the binding partner is not even known. A striking example is the interaction of the *Src*-type kinase Lck with the T-cell co-receptor CD4/CD8 through a C_4 tetrathiolate zinc-binding site [68]. Furthermore, this heterodimer forms a homodimer through the SH3 domains of the kinase, using a zinc-binding site with oxygen and nitrogen ligands [69]. One would assume that changes in the availability of cellular zinc ions affect the functions of such large signaling complexes.

CONCLUSION

An integrated approach, zinc proteomics, is necessary to describe the zinc proteome. Among other disciplines, it includes bioinformatics, structural biology, and computational and analytical chemistry. In order to determine whether the affinities of proteins for zinc are biologically meaningful, or whether zinc binding is simply a chemical phenomenon, the affinities of proteins for zinc, the availability of zinc ions, the zinc-buffering capacity, homeostatic mechanisms, and the biological context in which a protein functions need to be known.

Definition of the zinc proteome also has a quantitative dimension. It can be considered complete only if it accounts quantitatively for the zinc present. Assigning all the zinc in the cell to the zinc proteome, however, assumes that proteins are the only biological molecules that interact with zinc. No attempt has been made to quantify zinc proteins and calculate the resulting zinc concentrations, which should add up to about 200–300 μM for human cells. Yet such an approach, consisting of adding up the amounts of zinc, may not be satisfactory because a small amount of zinc can be distributed among a relatively large number of proteins of low abundance. As the example of zinc in transcription factors demonstrates, low-abundance zinc proteins are functionally very significant. To account for all the zinc, it may be necessary to define the zinc metallome [70], which is the sum of the zinc proteome, the zinc ionome, and zinc that is possibly bound to any other biomolecules, and to practice a branch of science that has been termed "metallomics" [71–73].

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