Coordination Dynamics of Zinc in Proteins

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1. Introduction

Biology capitalizes on the specific chemistries of certain transition metal ions. In fact, life depends on transition metal ions as essential trace elements. Biological redox processes, such as nitrogen fixation, photosynthesis, and mitochondrial respiration, rely on the chemistries of molybdenum, iron, manganese, and copper. Zinc (Zn^{2+}) is first among equals in the series of biologically important transition metals. In thousands of proteins, zinc participates in enzymatic catalysis, structural organization, and/or regulation of function.

The great number and variety of zinc proteins has stimulated research on the zinc regulatory and chemical mechanisms that safeguard distribution of zinc to proteins within the cell in a timely and spatially coordinated manner. Zinc concentrations inside cells are strictly controlled to fulfill all the biological function of zinc in proteins and to avoid unwanted side effect of excess zinc ions, such as their influence on the misfolding and aggregation of proteins (Figure 1). Regulatory roles of zinc require transient binding. Therefore, the commonly held view of zinc sites in proteins as permanent fixtures is beginning to change. Many functions of zinc in proteins also require dynamic structures. The usual description of zinc coordination environments as being static neglects a fundamental functional potential of zinc in biology. In this review, we will address the dynamics of zinc coordination and the cellular distribution of zinc, namely, how proteins control zinc (zinc metalloregulation), how zinc controls proteins (zinc signaling), and how zinc concentrations are regulated and buffered intracellularly.

The chemical properties of zinc in enzymes are largely attributed to its function as a relatively strong Lewis acid.[1] Generally, fast ligand exchange, stereochemical flexibility, and redox-inertness are additional characteristics for the selection of zinc ions in the function of so many proteins.

Its physical properties render zinc invisible to most spectroscopic methods of investigation, precluding the application of many techniques that have been instrumental in understanding the functions of other transition metal ions. The coordination spheres in zinc proteins have been probed with various spectroscopies of metal-substituted zinc enzymes, especially cobalt(II), which serves best as a probe, because its coordination is virtually isostructural with that of zinc and it retains catalytic activity while probing metal-dependent steps in the mechanisms of enzymes.[2] However,

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Wolfgang Maret received his diploma in chemistry (1977) and his Ph.D. in natural sciences (1980) from the Saarland University, Saarbruecken, Germany. The mentor for his Ph.D. thesis on metal substitution in liver alcohol dehydrogenase was Prof. Michael Zeppezauer. During his postdoctoral years in Prof. Marvin W. Makinen's laboratory at the University of Chicago (1980−1982), he continued his education in mechanistic enzymology and spectroscopy. He joined Prof. Bert L. Vallee's Center for Biochemical and Biophysical Sciences and Medicine at Harvard Medical School as an assistant professor in 1986 and began to work on the chemical and biochemical mechanisms of cellular zinc homeostasis. Since 2003, Dr. Maret has been an associate professor in the Division of Human Nutrition (Department of Preventive Medicine and Community Health) at the University of Texas Medical Branch in Galveston, TX. His work focuses on molecular mechanisms of cellular metal homeostasis, sulfur redox chemistry, structure and function of metalloenzymes, and functions of micronutrients in chronic and degenerative diseases.

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the metal-substitution methodology has limited use for studying the dynamic properties of zinc(II) ions in cellular biology. Fluorescent chelating agents, in the form of either synthetic dyes or genetically encoded sensors, hold the greatest promise and in fact are now considered the Aladdin's lamp for studies of zinc ions in the biological environment if employed with a proper understanding of their metal selectivity, their physical and chemical characteristics, and how they perturb cellular processes.

During its biological lifetime, every zinc protein binds and releases zinc. How zinc is incorporated into newly synthesized proteins, and the fate of zinc once zinc proteins are degraded, is largely unknown. Dynamic zinc−protein interactions are important in regulating proteins, in sensing zinc, in transporting zinc through cellular membranes, in redistributing zinc intracellularly, and for functions of zinc in proteins in general. Mechanisms of how zinc ions move or are being moved have not been addressed in the literature. The influence of protein dynamics, a fourth dimension in structural biology,[3] on zinc coordination is becoming evident in both structural and catalytic zinc sites, providing new perspectives on the functions of metals in proteins.

After providing a short account of how numerous zinc proteins are, we will begin this article with a general chemical approach to describe zinc coordination dynamics of isolated molecules. Later in the article, we will discuss function and purpose, which are meaningful only as biological concepts. Moreover, in biology, some functions of zinc are specific for the tasks to be performed in a certain organism. These conditional restraints on metal physiology are important, because they make biological inorganic chemistry context-specific: structure and function call for an interpretation with reference to the biological milieu, in which the molecules function. In fact, they may gain different meanings in biology when compared to pure chemistry.

2. Zinc Proteomes

The zinc proteome is the collection of zinc proteins in a given organism. Estimates of the sizes of zinc proteomes are based on mining databases of protein and nucleotide sequences. In this approach, one uses "signatures" of zinc sites that have been established from 3D structures of zinc proteins, so-called structural templates, for homology searches.[4] The advent of sequences of entire genomes offered additional opportunities to examine metalloproteomes. The complexity of zinc proteomes differs among organisms. Between 4 and 10% of the genes encode zinc proteins.[5] An estimated 10% of the human genome encodes zinc proteins, amounting to at least 3 000 proteins.[6] These proteins were annotated as follows: 397 zinc proteins are hydrolases; 302 are ligases; 167 are transferases; 43 are oxidoreductases; and 24 are lyases/isomerases. There are 957 transcription factors; 221 signaling proteins; 141 transport/storage proteins; 53 proteins with structural metal sites; and 19 proteins involved in DNA repair, replication, and translation. In addition, there are 427 zinc finger proteins and 456 other zinc proteins of unknown function. With this estimate in mind, one can appreciate the impact of zinc on human physiology and the extent of regulation necessary to control cellular zinc. Moreover, database mining does not readily identify sites where zinc binds only transiently, where signatures are not yet available from structural templates, where different proteins/peptide chains supply the ligands for binding zinc, and where the order of ligand binding is not sequential in the peptide chain or where ligands bridge metals in multimetal sites.[7,8] A remarkable example of structural complexity is the observation of protein knots in zinc finger domains.[9] Also, zinc ions may not bind to a putative metal-binding site, which can be either devoid of a metal or can bind another metal ion. Strictly speaking, designating a protein domain as zinc binding using sequence data alone is

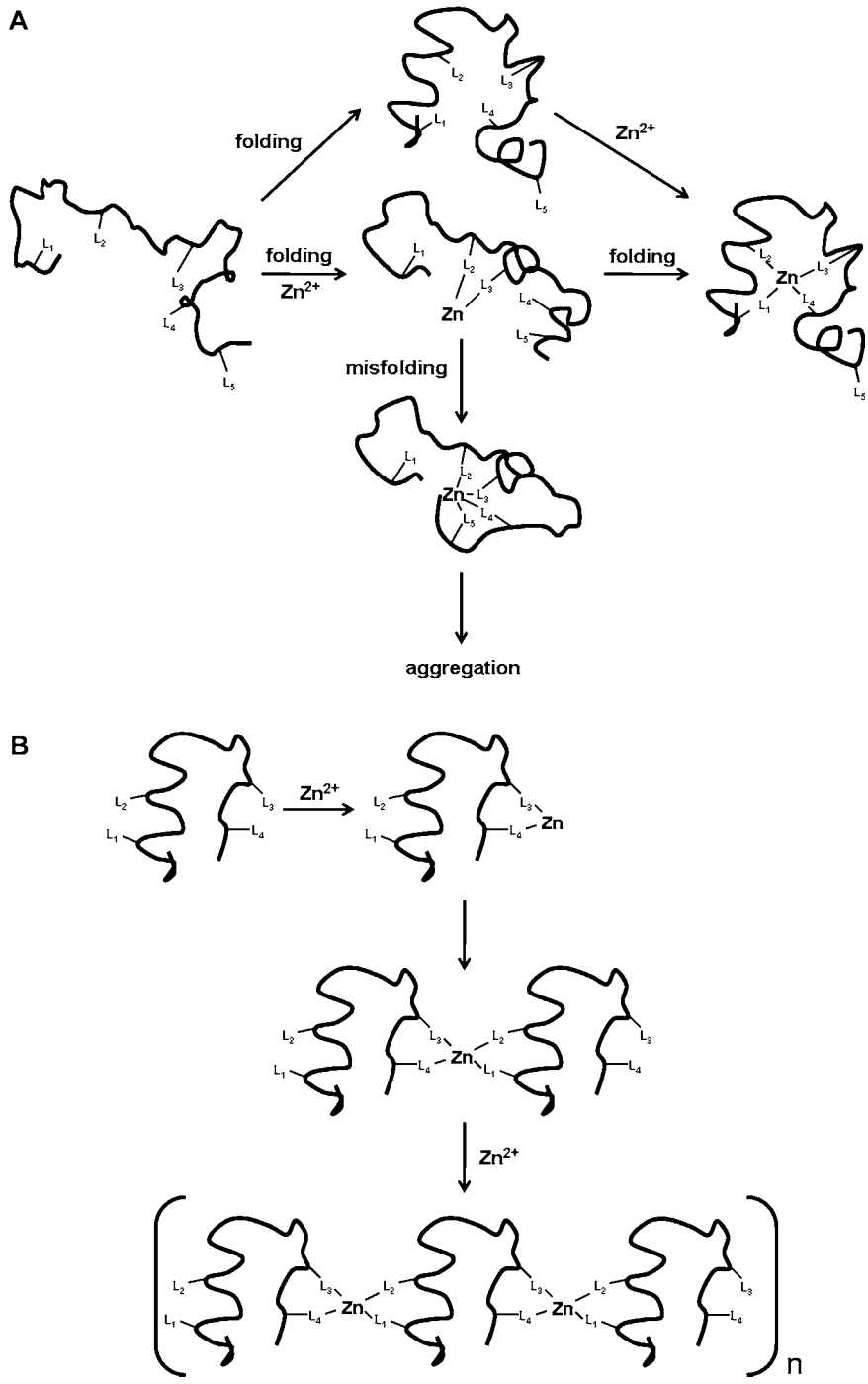

Figure 1. Zinc−protein interactions. (A) Intramolecular. Zinc binds to ligand signatures with the right "bite size" in the unfolded protein and participates in folding to yield either a misfolded protein that may aggregate or a correctly folded zinc protein. Zinc can also bind to the prefolded protein. (B) Intermolecular. At interfacial sites, zinc interacts with several peptide chains to establish homologous or heterologous (not shown) protein−protein interactions. The formation of aggregates can be either a normal or an aberrant process.

inconclusive without additional metal analysis. The bioinformatics approach is further limited, because in vitro various metals can bind to a zinc site, but in vivo biological mechanisms in addition to chemical selectivity define which metal ion is bound to a protein. Biological strategies include the choice of the cellular compartment in which the metal is inserted, thus avoiding competing metal ions to combine with the wrong protein and overriding the binding preference dictated by the Irving−Williams series, and specific molecular recognition of proteins through metallochaperones that keep copper away from zinc sites.[10,11] The abundance of transient zinc−protein interactions suggests that the dimension of the human zinc proteome has yet to be fully explored and that predictions underestimate the number of zinc proteins in zinc proteomes, which could be considerably larger than the estimates given above.[12]

3. Zinc−Ligand Interactions in Proteins

3.1. Interactions with the First Coordination Sphere

Metal−ligand interactions are one aspect of the function of metals in biology. At the extremes, either the effect of the ligands on the metal ion or the effect of the metal ion on the ligands is important for biological activity. In **catalytic**

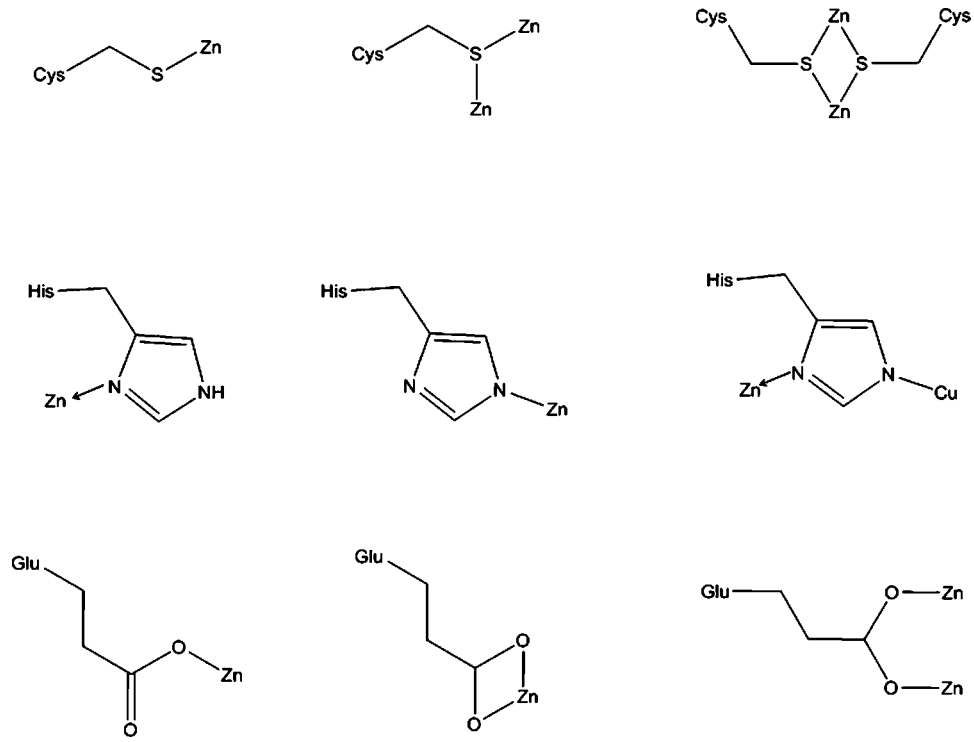

Figure 2. Zinc−ligand interactions. With few exceptions, zinc ligands in proteins are cysteine (Cys) (S-donor), histidine (His) (N-donor), or glutamate (Glu)/aspartate (Asp) (O-donor). For cysteine, there is a single mode of interaction. For histidine, binding occurs with either one of the two nitrogen atoms of the imidazole ring. For glutamate/aspartate, binding modes include one oxygen, which can be syn or anti (not shown), or both oxygens of the carboxylate. All three ligands can bridge one or several zinc ions. For cysteine, there are one, two, or three bridges. An example of three bridges is the Zn_4S_{11} cluster of metallothionein (Figure 6). For histidine, the only bridges known are those between zinc and copper in superoxide dismutase (SOD1) and between zinc and zinc in the zinc transporter CzrB (*Thermus thermophilus*). Aspartate/glutamate can form a bridge with the two oxygens of the carboxylate.

sites of zinc enzymes, the metal activates a nonprotein ligand or a protein ligand, such as the sulfur donor of cysteine. Since there are steric constraints in providing space for substrate binding in enzymes, the zinc ion often has only three protein ligands, an exchangeable water molecule, and/or a position for coordination of another ligand. In **structural** sites, there is rarely space for coordination of an additional ligand, and one purpose of the metal−ligand interaction is to stabilize the folded protein. Another purpose can be making sites reactive and letting zinc come on and off the protein. In this way, zinc sites can become switches for the protein's conformation and influence a large number of interactions with other biomolecules. Thus, in some cases, the chemical stability of a zinc site is transient on a biological time scale. The characteristics of zinc sites involved in other biological processes have not been examined and different requirements of the zinc−ligand interactions would seem to pertain. In **zinc sensors**, the binding energetics is expected to be linked to conformational changes of the protein for transmission of a signal. In sites involved in **zinc transport**, the ligand environment is expected to generate a site, in which the zinc ion is mobile. Further classification of sites with additional functions will hinge upon identification of specific structural characteristics related to such functions.

With few exceptions, the donors from the protein are the imidazole nitrogens from histidine, the carboxylate oxygen(s) from the side chains of glutamate or aspartate, and sulfur from the sulfhydryl group of cysteine (Figure 2). Histidine ligands have two binding modes, but so far it has not been clarified whether the different utilization of Nδ1 and Nε2 is a random event or has functional significance.[13] Carboxylate and sulfhydryl groups in zinc sites and their effects on each other deserve special consideration. For carboxylates, the various binding modes generate different interactions with a considerable spread of metal−ligand distances up to 4.5 Å.[14] Cysteine has a special role because its sulfhydryl group introduces unique reactivities into the coordination environment.[15] It is the combination of the three major ligands, different binding modes (Figure 2), including ligand bridges in multinuclear sites, and variable coordination geometry that are so remarkable, because they accomplish a great number of functions with this rather limited set of ligands.

Traditionally, zinc sites in proteins have been classified functionally as catalytic and structural, but exceptions test the rule. Catalytic sites may have additional structural functions, and structural sites may have a catalytic function. A distinction between catalytic and structural zinc by way of examining their coordination environments may not always be straightforward. For example, zinc in the *E. coli* Ada protein is in a tetrathiolate (S_4) site, like in many structural sites, but one of the ligands is reactive and participates in phosphotriester DNA repair.[16] Also, zinc in the zymogen form of matrix metalloproteinases is tetracoordinate, like in a structural site, but in fact, the thiolate ligand dissociates from zinc and converts the site into a typical catalytic site when the active enzyme is formed.[17] Furthermore, similar zinc coordination, namely, one zinc ion bound to three histidinyl residues, has been identified in carbonic anhydrase (catalytic function), the insulin hexamer (structural function), the serine proteinase tonin (inhibitory function), and the zinc transporter ZnuA (transport function) (Figure 3). Thus, the ligand donor set is not an appropriate indicator of zinc function in a given protein.

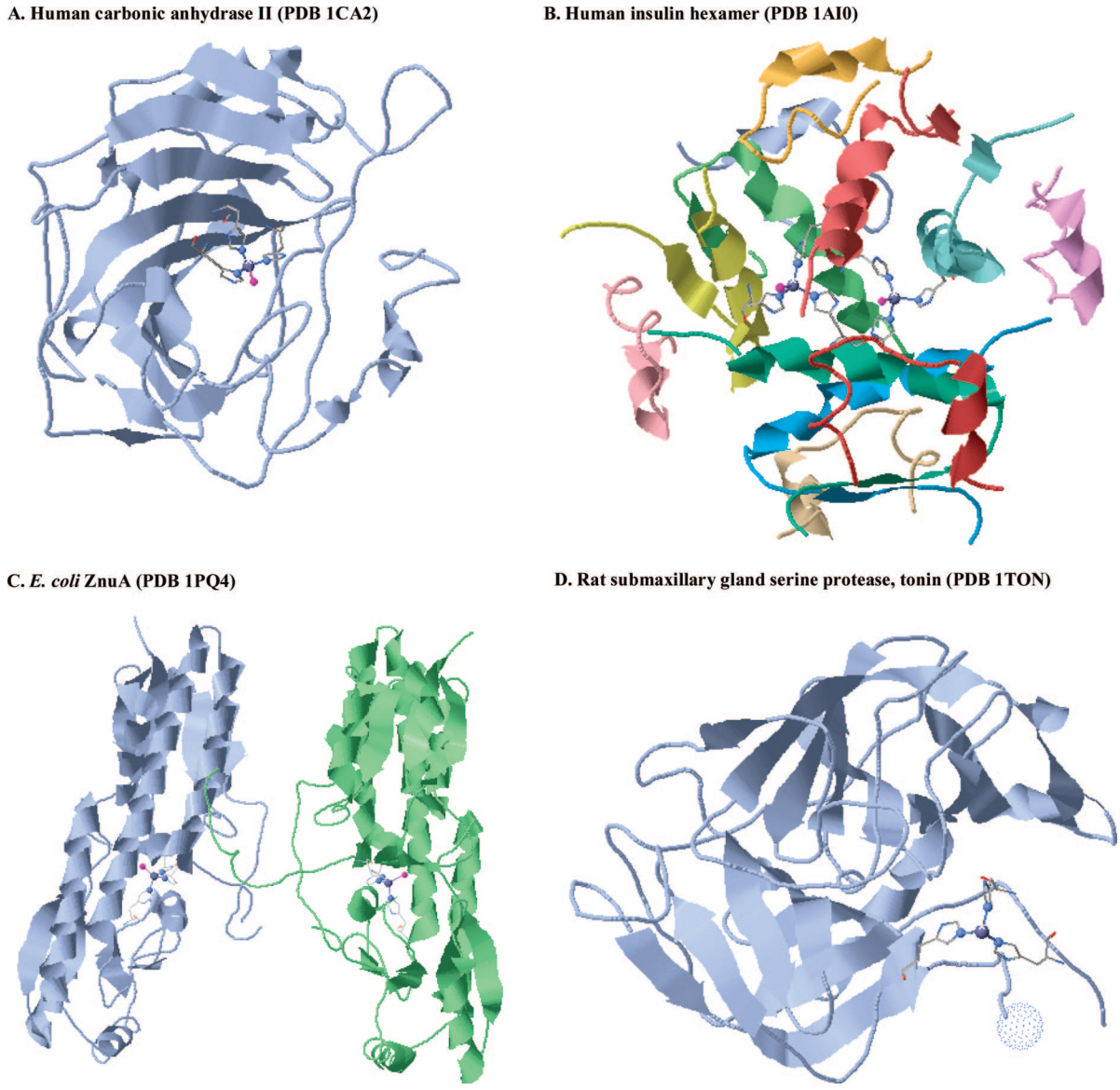

Figure 3. Zinc−histidine interactions. Zinc interactions with three histidines in proteins with different functions. (A) Zinc forms a catalytic site in human carbonic anhydrase II. (B) Two zinc ions stabilize the human insulin hexamer, with each zinc ion bound to ligands from three subunits. In the R(relaxed)-conformation, three water molecules are bound to the zinc ion. Ligand binding to these zinc ions triggers an allosteric transition of insulin to the T(tense)-conformation. During this process, the zinc site changes its coordination number from six to four by expelling two water molecules.[240] (C) The *E. coli* zinc transporter ZnuA is a dimer. Each monomer has a bound zinc ion. (D) An inhibitory zinc ion binds to the rat serine proteinase, tonin. In the crystal structure of tonin, another protomer contributes a glutamate oxygen as a fourth ligand (not shown).

3.1.1. Catalytic Zinc Sites

"Catalytic zinc" covers one area of the importance of zinc. Zinc enzymes have been reviewed extensively.[18−22] They function by using one, two, or three metal ions for catalysis. A special feature of some binuclear zinc sites is the use of a carbamoylated lysine as a bridging ligand.

3.1.2. Structural Zinc Sites

"Structural zinc" covers an equally important area that gained prominence with the discovery of zinc finger proteins.[23] Structural sites generally employ S_4, S_3N, or S_2N_2 coordination. A few mononuclear structural sites do not contain thiolate ligands.[24] Typically, zinc organizes small domains (about 20 amino acids), but when two or three zinc ions are used, much larger domains can be organized. Even though the individual zinc ions are coordinated tetrahedrally, there is a considerable variation in the sequence in which the ligands are employed (Figure 4). Sequential, nonsequential, and clustered, i.e., with sulfur (cysteine) ligand bridges, arrangements contribute to the structural and functional variety. Zinc may also be important for the structure of the entire protein. This occurs not only in small proteins, such as metallothioneins, but also in larger proteins, such as 3-methyladenosine DNA glycosylase I from *E. coli*, where zinc brings the N- and C-termini together.[25] It is an issue, more so for structural sites than for catalytic sites, whether zinc is a permanent constituent of the protein or its binding is transient and modulates the function of the protein. Discoveries of transient zinc binding sites, such as inhibitory zinc sites, zinc sites in sensors and transporters, and protein-interface zinc sites, suggest that a classification of zinc sites based on either structure or function is far from complete.

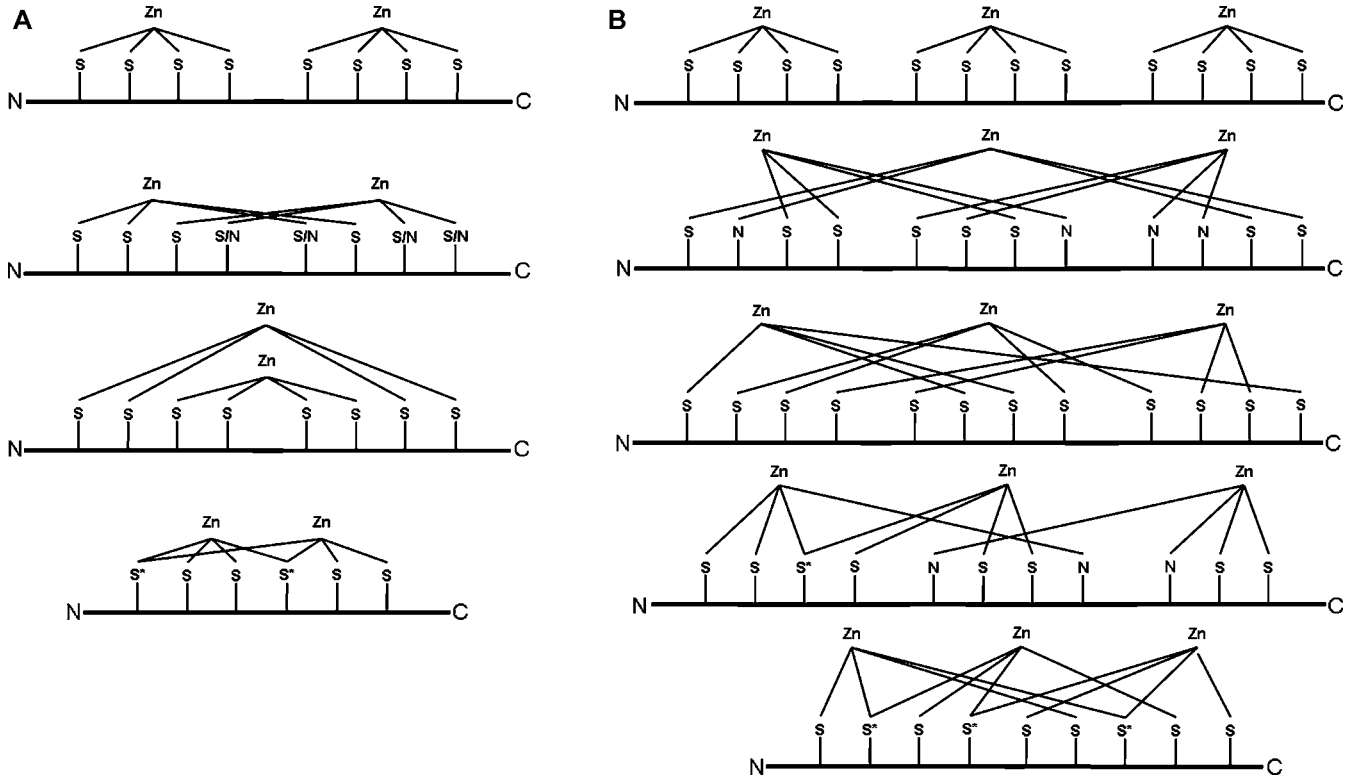

Figure 4. Sequential, nonsequential, and clustered binding patterns of zinc ligands in 2-Zn (A) and 3-Zn (B) sites of proteins. The 3D structures of proteins with multiple structural zinc sites can harbor unexpected levels of organization that are not evident from inspection of their linear sequence.[7] Thus, in addition to their sequential use, the ligands can be used nonsequentially in either an intertwined or interleaved pattern (an example of the intertwined pattern is the zinc-binding domain of RING fingers; an example of the interleaved pattern is the cysteine-rich domain of the protein DnaJ). Another level of organization is the clustered binding pattern when the sulfur donor of cysteine becomes a bridging ligand and is used twice (indicated with S*). In these clustered binding patterns, a tetracoordinate coordination environment is maintained, but the number of ligands per zinc is reduced. The examples given for the 3-Zn sites (B) below the linear arrangement of ligands are the BUZ/Znf-UBP domain,[241] the triquetra knot motif,[9] where the peptide chain actually forms a knot, the zinc-binding domain of the neural inducing factor Churchill,[242] and the N-terminal domain of mammalian MTs.

3.1.3. Inhibitory Zinc Sites

Zinc ions inhibit a great number of proteins. Some enzymes are exquisitely sensitive to zinc inhibition.[26] Phosphorylation signaling, mitochondrial respiration, and neurotransmission are examples where the biological importance of zinc inhibition has been recognized.[27−29] Whether or not inhibition is physiologically significant depends on the availability of zinc ions in the biological environment in relation to the zinc affinity of the protein. This issue will be amplified later in the article when the zinc affinities of proteins and the free zinc ion concentrations have been discussed. Suffice it to say at this point that two types of situations are thought to be physiologically relevant: (i) cytosolic enzymes that are inhibited by nanomolar or even subnanomolar concentrations of zinc ions and (ii) extracellular enzymes that are inhibited by micromolar concentrations of zinc ions.

In the test tube, metal ion concentrations are difficult or impossible to control at nanomolar or lower concentrations without using metal buffers. Using a metal buffer, the zinc inhibition constant of human protein tyrosine phosphatase 1B was determined to be 15 nM.[30,31] This enzyme and many other zinc-inhibited proteins are not zinc proteins themselves. These zinc-inhibited proteins are also part of the zinc proteome, but at present they are not accounted for nor can generalizations about the structures of their zinc-binding sites be made. Mitochondrial aconitase has a zinc site with two histidine ligands and one aspartate ligand.[32] Whether the zinc inhibition constant ($K_i = 2\ \mu M$) reflects zinc binding to this site needs to be further examined. In bovine dimethylarginine dimethylaminohydrolase, zinc binds to only one cysteine ligand in the active site at pH 6.3, but at pH 9.0 it interacts with a histidine ligand in addition to the cysteine.[33] The coordination at pH 7.4, where zinc binds with a K_i of 4 nM, has not been reported.[34]

Biological environments other than the cytosol may have significantly different zinc ion concentrations. For example, the inhibition of some proteinases by zinc is believed to be physiologically relevant because these enzymes are secreted from cells together with zinc. Zinc inhibits carboxypeptidase A, a zinc proteinase, with a K_i of 0.5 μM.[35] Employing a combination of methods, it was predicted that the Glu-270 ligand of the catalytic zinc, a hydroxide that bridges the catalytic and the inhibitory zinc, and a chloride ion are bound to the inhibitory zinc ion.[36] The crystal structure of the zinc-inhibited protein corroborated all of these predictions.[37,38] Zinc also inhibits proteinases that do not depend on zinc as the catalytic metal ion. An example, where kinetic data on zinc inhibition and 3D structures are available, is the human kallikrein (hK) family of serine proteinases. In the kallikrein hK4, a histidine ligand and a glutamate ligand bind the zinc;[39] in tonin, a rat kallikrein, the zinc ligands are three histidines and a glutamate from a neighboring protein molecule; in hK5, the inhibitory zinc has two histidine ligands, and based on homology with tonin, a third histidine ligand is thought to be recruited for zinc binding,[40] a coordination that is also proposed for hK7.[41] Zinc coordination with three histidine ligands in human carbonic anhydrase provides a catalytic

Table 1. Combination of Ligand Sets in Protein-Interface Zinc Sites

ligand combination	ligands	example	PDB
3 + 1	N_3	serine proteinase	1TON
2 + 2	S_4	"zinc hook"	1L8D
2 + 1	N_2O	phosphocarrier IIA^{Glc}	1GLC
1 + 1 + 1 + 1	O_4	trypsin inhibitor	1LU0
1 + 1 + 1	N_3	insulin hexamer	1AI0
1 + 1	S_2	colicin E3	3EIP

site with a dissociation constant of 11.4 (pK_d).[42] However, zinc inhibition constants for kallikreins are only in the low micromolar range. The structural 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.

3.1.4. Protein-Interface Zinc Sites

A special class of zinc sites is located between proteins with ligands provided from different peptides from either the same or different proteins.[43,44] While some of these sites may exist only in the crystallized proteins, with more and more examples of such intermolecular bridging sites being discovered, it is becoming clear that new functions emerge for zinc in higher orders of protein structure and in supramolecular assemblies. The functions include catalysis, inhibition of activity, packaging of proteins for storage, dimerization, formation of protein/receptor complexes, construction of molecular scaffolds, and regulation.[44] Zinc can be essential for the interaction or merely stabilize it. Ligand sets, similar to the ones discussed above, are employed, including ZnS_4 cross-links. How these protein complexes assemble and disassemble is yet another facet of the coordination dynamics of zinc, because different ligand sets must come together to form the site (Table 1). Therefore, at the very low free zinc ion concentrations present in the cell, it is an issue of how sufficient stability can be achieved initially with only two ligands from one interacting protein for an ensuing 2 + 2 ligand interaction. Alternatively, sufficiently high local free zinc ion concentrations could initiate these interactions. Examples that have not been discussed in previous reviews[43,44] include the assembly of signaling complexes, such as the formation of a heterodimer between the Src-type kinase Lck and the T-cell coreceptor CD4/CD8 through a 2 + 2 tetrathiolate zinc site and the formation of a homodimer of this heterodimer through a zinc site with oxygen and nitrogen ligands.[45,46] One wonders whether fluctuations of zinc ion concentrations would differentially affect the formation of the heterodimer and the homodimer. In heterologous interactions, additional factors, such as protein−protein recognition, could potentially operate in order to avoid homologous interactions.

The dimerization of protein disulfide isomerase and its interaction with calreticulin is zinc-dependent,[47] and so is the heterodimerization of the S100A8 and S100A9 proteins to form calprotectin.[48] While zinc affects the associations of these proteins, one would also expect that the association of these proteins affects the availability of zinc ions. Tetramerization to form the active *E. coli* RNase E involves two intermolecular S_4 binding sites.[49] The authors make the intriguing suggestion that the interaction serves as a zinc sensor in which the enzyme loses its activity under zinc-limiting conditions and, in this way, influences the stability of particular RNA transcripts. The involvement of zinc in even larger protein aggregates has been shown in SAM (sterile α-motif) domains of the Shank family of scaffolding proteins.[50] In the presence of zinc ions, the helical fibers assemble to form a protein matrix for organizing the postsynaptic density in neuronal junctions. A glutamate and a histidine from one domain and a histidine from another domain bind zinc. Yet another example of supramolecular assembly is the association of tandem G5 domains in a modular fashion.[51] The G5 domains occur in staphylococcal cell-surface proteins that mediate intercellular adhesion in the formation of biofilms. Tight control of the availability of zinc ions suggests a way of organizing proteins and modulating protein−protein interactions.

3.2. Architecture of Zinc Coordination Environments

Structures of metal sites in proteins are frequently presented by showing only the metal ion and its ligands, although zinc sites in proteins are not isolated units. The discussion in the previous sections has shown that the ligand donors make no clear distinction between the zinc sites in functionally different proteins. Interactions of the ligands with a second shell of amino acids orient the ligands and determine the properties of zinc sites. Amino acids far away from the metal sites that have critical roles in protein tertiary structure can also affect metal coordination. Overall, the secondary and tertiary structure of the protein influences metal binding and so does the dielectric medium surrounding zinc and its ligands. The second shell of amino acids around zinc sites generally contains more polar residues than hydrophobic residues.[13] Carbonic anhydrase will serve as an example of this scaffolding of zinc sites, because extensive studies have probed these secondary interactions experimentally in this enzyme and established the influence of the outer shell of amino acids on the thermodynamics and kinetics of zinc binding (Figure 5). The three histidine ligands (His-94, -96, and -119) of the zinc ion in human carbonic anhydrase II are located on an extended antiparallel β-sheet. Hydrogen bonds exist between His-119 and Glu-117, His-94 and Gln-92, and His-96 and the main chain carbonyl of Asn-244; in addition, the zinc-bound hydroxide forms a hydrogen bond to Thr-199. The energetic contributions of single hydrogen bonds to a zinc ligand have been examined (Table 2). If a glutamate replaces Thr-199 and becomes a fourth ligand, the affinity of the enzyme for zinc increases 40-fold. Additional interactions even farther away affect the affinity of the metal ion (Table 2); for instance, Trp-97 interacts with Met-241. A survey of the interactions between ligands and second shell amino acids demonstrated that peptide−backbone interactions are the most common, followed by Asp/Glu, Lys/Arg, Asn/Gln, and Ser/Thr side-chain interactions.[52] Hydrogen bonding of carbonyl main-chain or carboxylate side-chain atoms with histidine ligands or NH···S hydrogen bonding with cysteine ligands can make a significant contribution to stability. What has been less appreciated, however, is that hydrogen bonding of zinc-bound water molecules to nearby residues also stabilizes the complex. These aspects of the ligands and the protein forcing the zinc ion into a special environment and leading to functionally different states was recognized much earlier when it was noted that the properties of metal ions in proteins differ from those of low molecular weight complexes with similar donors. The presence of unique coordination states of metals in proteins was formalized into the concept of an entatic state, i.e., the energization of a group, in this case the metal cofactor, in a protein.[53]

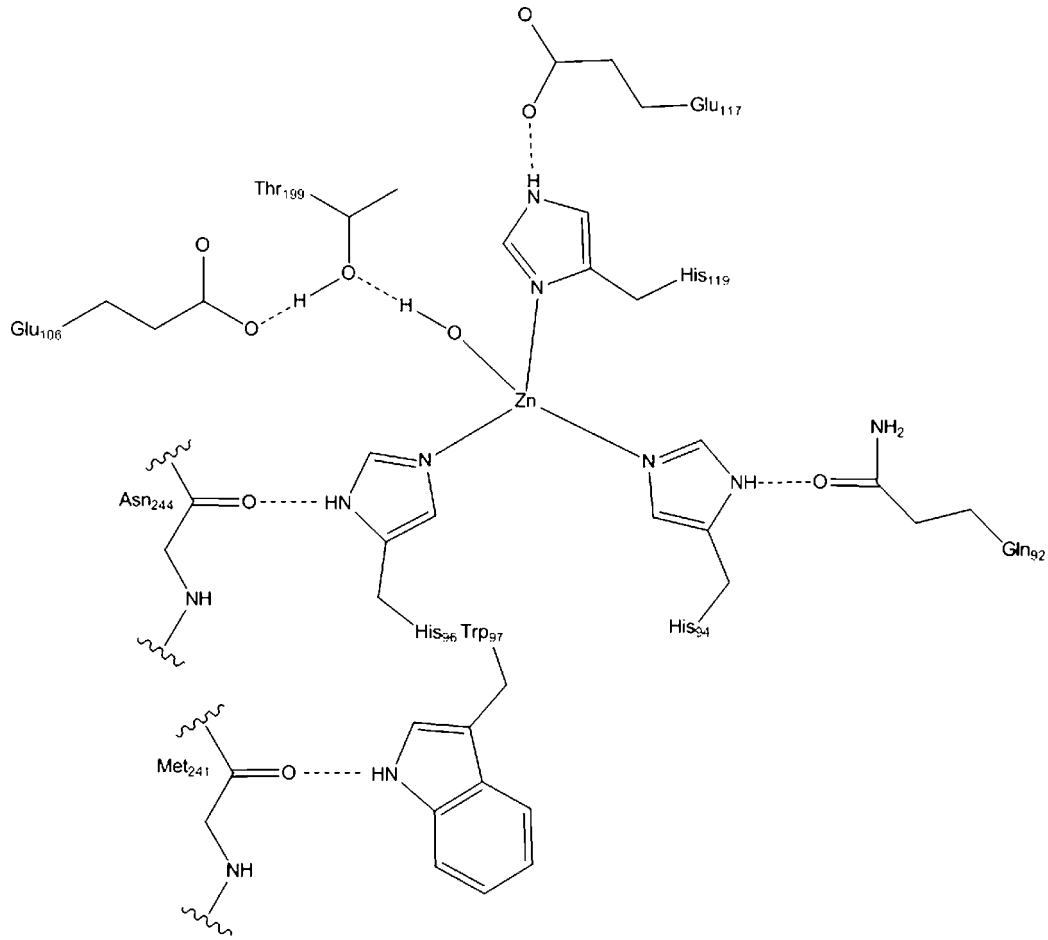

Figure 5. Amino acids in the inner coordination sphere and in the outer shell of the zinc site in human carbonic anhydrase II (modified from Thompson and Fierke[239] to include additional interactions[21]). Histidine ligands and the zinc-bound hydroxide ion interact with other amino acids via hydrogen-bonding, generating a molecular scaffold for the zinc ion.

Table 2. Site-Directed Mutagenesis in the First Coordination Sphere and the Second Shell of the Zinc Site in Human Carbonic Anhydrase II: Zinc Binding Properties

	variant	pK_d	k_{off}, h^{-1}	k_{on}, M^{-1} s^{-1}	ref
ligand	WT	12.1	0.0003	0.1	255, 256
	H94A	6.6	>140	0.1	257
	H94C	7.5	0.5	0.004	255, 257
	H94D	7.8	0.7	0.01	255, 257
	H94E	7.9	30	0.6	257
	H94N	7.4	5	0.03	258
	H94Q	8.1	20	0.7	258
	H119D	7.6	10	0.1	255, 257
	H119Q	7.2	6	0.02	258
2nd shell	T199C	12			259
	T199E	13.7			42
	T199A	10.2	0.002	0.01	260
	Q92A	10.7	0.001	0.02	260
	E117A	10.4	1.5	10	260
	E117Q	8.4	4 680	300	261

The protein exerts different degrees of strain on the coordination environment and, thereby, alters the properties of the metal complex.[54]

4. Physicochemical Properties of Zinc−Ligand Interactions

4.1. Stability Constants

How strongly proteins interact with zinc is a central issue for the availability and mobility of zinc in a cell. In principle, differences in stability constants might simply indicate a certain hierarchy, such that proteins performing the most critical functions retain their zinc most tightly while proteins whose functions can be expended with can give up their zinc when zinc supply becomes limited. At present, there is no evidence for such a hierarchy.

Zinc binding constants for a number of zinc enzymes have been reported (Table 3). The available data, however, are from proteins of different organisms and with different localization, and they were collected under different conditions. Therefore, comparisons have to be approached with these limitations. Despite variability in their coordination environments, *intracellular* eukaryotic zinc enzymes bind zinc very tightly (pK_d values between 10 and 12, i.e., picomolar, pM). Zinc affinities of prokaryotic enzymes are similar (Table 3). *Extracellular* zinc proteins also bind zinc tightly, although some seem to bind zinc less tightly (Table 3). Zinc affinities of the cytosolic eukaryotic zinc proteins, carbonic anhydrase II, superoxide dismutase (SOD1), and sorbitol dehydrogenase, are quite similar,[30] even though these proteins have different numbers and types of ligands. Therefore, it seems that variation of the ligand sphere in cytosolic zinc enzymes does not affect, or affects only minimally, the affinity for zinc. Notwithstanding the scarcity of data and different conditions, a major thermodynamic driving force for zinc transfer or redistribution among these enzymes does not seem to exist.

The pervasive role of zinc as a structural element is one of the reasons why zinc is involved in so many more proteins

Table 3. Zinc Affinities of Zinc Enzymes

enzyme	coordination	pK_d	condition	ref
alkaline phosphatase (*E. coli*)	trinuclear	7.7, 10.2	pH 8.5, 1 M NaCl, 25 °C	243
aminopeptidase (*Aeromonas proteolytica*)	binuclear	9.8	pH 7.2, 0.1 M KCl, 5 mM Hepes, 6 °C	244
angiotensin-converting enzyme (rabbit lung)	N_2O	8.2	pH 7.5, 0.3 M NaCl, 50 mM Hepes, 25 °C	245
carbonic anhydrase II (human)	N_3	11.4	pH 7.0, 15 mM phosphate buffer, 30 °C	42
carboxypeptidase A (bovine)	N_2O	10.5	pH 8.0, 1 M NaCl, 50 mM Tris, 4 °C	246
dipeptidyl peptidase III (rat liver)	N_2O	12.3	pH 7.4, 50 mM phosphate buffer, 25 °C	247
glyoxalase I (human erythrocytes)	NO_3	10.6	pH 8.5, 0.1 M Tris	248
leucine aminopeptidase (bovine lens)	binuclear	9−11	/	249
phosphoglucomutase (rabbit muscle)	not determined	11.6	pH 8.5, 30 °C	250
porphobilinogen synthase (human erythrocytes)	S_3	$K_m = 1.6$ pM	pH 7.2, $I = 0.1$, 37 °C	251
sonic hedgehog (human)	N_2O	<10	pH 7.5, 150 mM NaCl, 100 mM Hepes, 25 °C	252
sorbitol dehydrogenase (sheep liver)	NSO	11.2	pH 7.2, $I = 0.02$, 25 °C	30
stromelysin (human)	N_3	10.7	/	15[a]
thermolysin (*Bacillus thermoproteolyticus*)	N_2O	11.3	pH 7.5, $I = 0.1$, 25 °C	253
		12.6	pH 7.2, $I = 0.02$, 25 °C	254

[a] Personal communication cited in ref 15.

than the other transition metals, which by-and-large have catalytic functions. The description of structural zinc sites is relatively straightforward because, typically, they are tetracoordinate and, for the most part, they do not have nonprotein ligands. Forty percent of all tetrahedral zinc coordination sites in proteins are S_4 sites.[6] Measurements of zinc affinity have been approached almost exclusively by using synthetic peptides. The dissociation constant of zinc for the S_4 coordination sphere is 60 aM;[55] however, the conditional dissociation constant at pH 7.0 is 8 pM. Comparison with S_3N or S_2N_2 coordination suggests that substituting one or two cysteines for histidine does not change the affinity at physiological pH, where all three coordination motifs, S_4, NS_3, and N_2S_2, have an affinity of 2.0×10^{12} M^{-1} for zinc.[55] This value is remarkably similar to that of cytosolic zinc enzymes, again suggesting the absence of thermodynamic gradients. If the affinity for zinc is the same in all three of these coordination types, then something else must account for their differential use in proteins.

In contrast to zinc enzymes, where zinc-binding sites are often preorganized in the absence of metals, zinc binding to some small domains with structural zinc sites is accompanied by folding of the peptide. If ΔG for the folding of the apopeptide is positive, the observed affinity is less than the maximal affinity because some of the energy of metal binding is used for folding. However, when the energy of the folded and unfolded peptide is the same ($\Delta G = 0$) or ΔG is negative (if the energy of the folded peptides is less than that of the unfolded), the maximum affinity is observed.[56] For example, at pH 7.4 there is a 3.9 kcal/mol difference between the conditional dissociation constants of a synthetic peptide (200 fM)[55] and the 37 amino acid zinc finger domain of the Xeroderma pigmentosa A (XPA) DNA repair protein (XPAzf) (158 pM),[57] both with S_4 coordination. These considerations seem to be important for the functions of zinc finger proteins. If maximum affinity is achieved for structure, no energy is borrowed for folding. If, on the other hand, binding is associated with folding, the affinity will be less than maximal, which is one of the reasons why sensing zinc fingers may have lower affinity if the zinc binding energy is transduced into folding of the protein.[58] A spread of values for the conditional association constants for zinc finger peptides from 8.2 to 13.2 is similar to the values reported for zinc enzymes and could reflect the interplay between zinc binding and peptide folding.[59]

The zinc affinities of catalytic sites with three ligands and structural sites with four ligands seem to be similar. Additional interactions of the ligands with a second shell of amino acids enhance the stability of zinc in catalytic sites, presumably to avoid dissociation of zinc from a site with fewer ligands and subsequent binding to a site with more ligands.

However, zinc−protein interactions that are weaker than the ones discussed may also be significant, but only under certain physiological conditions or for different functions. In some proteins, the affinity for zinc is lower compared with other proteins that use the same donor set. Such a destabilization is important for proteins involved in zinc regulation. In the presence of stronger interactions, weaker interactions can be only transient, in particular if zinc is buffered to yield very low concentrations of free zinc ions.

Stability constants can vary among zinc sites within one protein even when zinc is bound to the same types of ligands. The conspicuous features of mammalian metallothionein (MT) are two zinc/thiolate clusters, Zn_3S_9 and Zn_4S_{11}, with characteristic sulfur (cysteine) ligand bridges between the zinc ions.[60,61] The Zn_3S_9 cluster also occurs in histone lysine methyl transferases.[62,63] In the 3-Zn cluster, every zinc ion has two bridging and two end-on ligands. In the 4-Zn cluster, two zinc ions have this coordination while the remaining two have three bridging ligands and only one end-on ligand. All of the seven zinc ions in the two clusters of mammalian MT are in a similar tetrathiolate (S_4) coordination environment (Figure 6). Yet, zinc affinities differ by 4 orders of magnitude.[64] Four zinc ions are bound tightly ($\log K = 11.8$), two with intermediate strength ($\log K \approx 10$), and one zinc ion is bound weakly ($\log K = 7.7$). MT is a prime example for extraordinary coordination dynamics of zinc, because it occurs as Zn_4T, Zn_5T, Zn_6T, and Zn_7T species, depending on the protein concentration and the concentrations of zinc ions. The species Zn_7T does not exist under physiological conditions, because zinc ions are not freely available at high enough concentrations in the cell to saturate the weak binding site. Indeed, biochemical assays always detect a fraction of the protein in the apoform ("thionein").[65,66] The significance for function is that these properties allow MT to participate in cellular zinc redistribution rather than trapping zinc ions in a complex that is thermodynamically more stable than that of other zinc proteins. In spite of the formal S_4 coordination, the coordination environments are fine-tuned regarding their affinities. The zinc/thiolate clusters destabilize

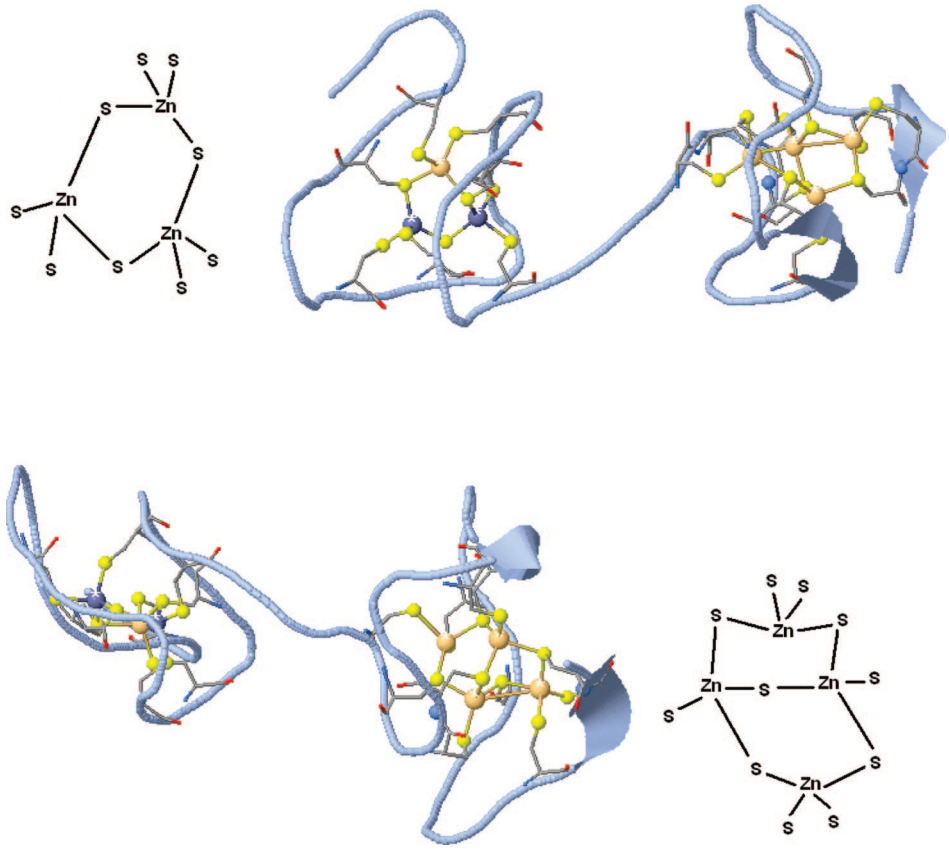

Figure 6. Structures of the metal/thiolate clusters in metallothionein. Two presentations of the same molecule of rat MT (PDB 4MT2) are given for better viewing of the N-terminal Zn_3S_9 cluster (top) and the C-terminal Zn_4S_{11} cluster (bottom).

rather than stabilize zinc sites, compared with mononuclear zinc/thiolate environments. Whether or not the partially zinc-loaded MT species have single defined structures is not known. When ethylenediaminetetraacetic acid (EDTA) removes cadmium from rabbit MT, all three ^{113}Cd-NMR resonances assigned to three cadmium ions in the N-terminal domain decrease uniformly in intensity at the same time, demonstrating that the remaining bound cadmium dynamically redistributes among the three binding sites.[67] When metals are added to thionein, mononuclear metal species form before the sulfur-bridged species are generated.[68]

4.2. Kinetic Constants

Kinetic data are even scarcer than equilibrium data, and if available, studies were mostly performed with chelating agents that have a tendency to form ternary ligand−Zn−protein complexes. With picomolar zinc dissociation constants of zinc proteins and diffusion-limited or slower association rates, dissociation rates must be rather slow ($K_b = k_{on}/k_{off}$).[69] Indeed, the zinc dissociation rate constant from human carbonic anhydrase II is only 3×10^{-4} h^{-1} (Table 2). Second shell ligands have a significant effect on dissociation rates (Table 2). How ligands from other molecules affect metal transfer rates by forming ternary complexes was shown for β-lactamases. Substrates decrease the dissociation rate of zinc, while EDTA increases the association rate of zinc by providing a pool of strongly complexed zinc, which, nevertheless, is available through ligand-exchange reactions.[70,71] In a related experiment, EDTA accelerates zinc transfer between zinc finger peptides by 6 orders of magnitude via its capacity to form ternary complexes.[72] Likewise, chelating agents accelerate the removal of zinc from zinc enzymes. They increase the inactivation of zinc enzymes by thionein, which by itself has only a modest effect on zinc removal.[73] D-Penicillamine increases the dissociation rate of zinc from carboxypeptidase A 420-fold.[74] The term "catalytic chelation" was proposed for this mechanism, in which a chelating agent binds to zinc in the enzyme, dissociates with complexed zinc, and then transfers zinc to a second chelating agent with potentially higher affinity but lacking the capacity to interact directly with zinc in the enzyme.[75] Whether or not small ligands participate in cellular zinc distribution in this way is unknown. The same mechanism of ternary complex formation holds for zinc transfer between two proteins via an associative mechanism. This work provides precedence for how zinc complexes that are inherently thermodynamically stable in proteins have sufficient dynamics to participate in ligand-exchange reactions for zinc redistribution.[72] The relationship between thermodynamic stability and kinetic lability can also be seen in zinc self-exchange rates. Radiozinc (^{65}Zn) exchanges with zinc in zinc proteins, such as carbonic anhydrase II, superoxide dismutase (SOD1), or aspartate transcarbamoylase, in the order of days or longer.[76−78] However, self-exchange rates in mammalian metallothionein, where direct molecular contact is possible, are in the order of minutes.

4.3. Chemical Reactivity of Zinc−Sulfur (Cysteine) Interactions

Among the three types of donors, sulfur is the only one with a special reactivity, and thus it has a unique role in the coordination dynamics of zinc proteins.[15] Thiols can react with many compounds, and the reactivity is maintained when

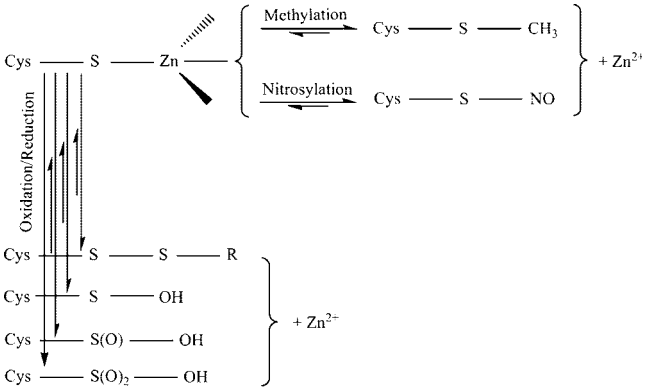

Figure 7. Thiolate-ligand-centered reactivity. Reversible and irreversible oxidation of the sulfur donors in zinc/thiolate sites in proteins with concomitant zinc dissociation or association. Many electrophiles also react with the sulfur donor, covalently modifying it and causing zinc dissociation.

they coordinate zinc (Figure 7). If one considers this reactivity, the deceiving simplicity ("simplexity") of the three main structural zinc motifs (ZnS_2N_2, ZnS_3N, ZnS_4) reveals a rich chemistry. In many cases, considering structural zinc sites as inert elements of protein structure is inappropriate. In the biological environment, these sites can react with other biomolecules, and this reactivity provides pathways for the mobilization of zinc from proteins and the control of protein function.[79]

4.3.1. Redox and Nucleophilic Chemistry

The zinc(II) ion is not redox-active in biology. However, Zn/S(thiolate)-coordination environments are redox-active.[80] The sulfur donor atoms of the cysteine ligands can be oxidized and then reduced again, with concomitant dissociation and association of zinc. The simplest case, in terms of reversibility, in biology is zinc thiol/disulfide interchange, which can be brought about in different ways. Reaction of zinc/thiolate sites with disulfides can lead to mixed disulfides or, when the disulfide is in excess, to an intramolecular disulfide if another cysteine is available in the coordination sphere. The sulfur (cysteine) biochemistry also involves higher oxidation states. Investigators have begun to explore the chemistry of the zinc/thiolate coordination compounds with regard to potential biological implications. Reactive sulfur compounds, such as disulfide-*S*-oxides (thiosulfinates or thiosulfonates), react with Zn/S sites in proteins.[81] Both sulfonates and thiosulfinates coordinate zinc.[82] In β-lactamase from *Pseudomonas aeruginosa*, Cys-221, which is a ligand of one of the two zinc ions, becomes a cysteinesulfonic acid in the oxidized enzyme.[83] Other reactants of Zn/S sites with biological significance are reducible selenium compounds.[84] The differences in the redox potentials of the various valence states of selenium and sulfur are important for reactions between these elements. A remarkable aspect is the catalytic potential of selenol(ate)s in the reaction with Zn/S sites.[85,86] Mixed disulfides, diselenides, selenium trisulfides, and selenyl adducts are all intermediates in the purview of biological inorganic chemistry.

Nitrosylation of thiolates in Zn/S sites can also lead to the formation of disulfides.[87] Metallothionein is a target of nitric monoxide (NO) signaling in vivo.[88] Its nitrosylation causes dissociation of zinc from the protein. Nitrosylating species in biology are *S*-nitrosothiols that react by transnitrosation, i.e., the transfer of NO between thiols. Transnitrosation of Zn/S sites in proteins is likely catalyzed by neighboring charged amino acid side chains.[89] Mechanisms for the nitrosylation of Zn/S sites have been proposed. When *S*-nitrosoglutathione reacts with XPAzfp, nitrosylated cysteine, cysteine-glutathione disulfide, and intrapeptide disulfides are formed.[90] The proposed reaction sequence includes dissociation of the thiolate from zinc, glutathione coordination with its amine and carboxylate to zinc, and then transnitrosation to the free thiolate. Thus, glutathionylation and *S*-thiolation can also occur through reactions with *S*-nitrosothiols.

Some voltage-gated K^+ channels have an interface zinc site that is a redox-dependent zinc switch.[91] One histidine and one cysteine from one subunit and two cysteines from another subunit provide the donors. The interface zinc site is thought to be dynamic, with one weakly bound cysteine that can form either a disulfide in a reaction with NO or a cystine *S*-oxide in a reaction with hydrogen peroxide. Interfacial zinc coordination seems to protect against the formation of the inhibitory disulfide through stabilization of either *S*-nitrosocysteine or the sulfinic acid intermediate via hydrogen-bonding to the imidazole ring of His-104.

The redox chemistry of Zn/S sites is not limited to these oxidants. Many metal and metalloid compounds are oxidants of Zn/S sites. Arsenite and chromate have toxicological significance as reactants.[92,93] Monomethylarsonous acid releases zinc from XPAzfp and forms mono- and diarsenical derivatives.[94]

Multiple functions are linked to these redox mechanisms that allow control of zinc protein activities via their zinc content and redox state and, therefore, have been called redox zinc switches.[79] Zinc dissociation from Zn/S sites can be associated with protein conformational changes, or the dissociated zinc ions can affect other proteins.[95] Redox zinc switches are a general principle for the regulation of proteins. Among the different redox zinc switches, some are *redox sensors* and others are *redox transducers*. In redox sensors, zinc dissociation alters protein function without any additional function of the zinc ion. In redox transducers, the released zinc ions become a "zinc signal" by binding to another protein and affecting its function. Proteins with redox zinc switches serve rather diverse functions, such as controlling chaperone activity, binding interactions of proteins with other proteins or DNA, enzymatic activity, and sensing disulfide stress.[79] Zinc can regulate proteins in structural sites as a molecular switch, i.e., by association/dissociation, with consequences for protein/protein, protein/DNA, protein/RNA, and protein/lipid interactions. Moreover, the redox chemistry of Zn/S sites affects the availability of cellular zinc ions. Conditions that are more oxidative or more reductive increase or decrease, respectively, the availability of zinc ions. This remarkable property integrates some zinc proteins into redox metabolism.

Zinc/thiolate redox chemistry may also be important for reversible zinc binding in several proteins residing in the mitochondrial intermembrane space. These proteins bind zinc with $CysX_3Cys$ or $CysX_9Cys$ motifs.[96] It has been suggested that zinc maintains these nuclear-encoded proteins, such as Tim10 (translocase of the inner mitochondrial membrane), in an import-competent state.[97] Once the protein is in the intermembrane space of mitochondria, zinc can be removed by Mia40 (mitochondrial intermembrane space import and assembly), which then hands zinc over to Hot13 (helper of Tim) while being oxidized by the sulfhydryl oxidase Erv1

(essential for respiration and vegetative growth 1).[98] Alternatively, small Tim proteins may be imported in their zinc-free forms.[99] Whether zinc is indeed associated with these proteins in the intermembrane space has come into question because Mia40 can be isolated as an iron-containing protein.[100] Zn/thiolate proteins, such as metallothionein, also react with aldehydes and ketones with concomitant zinc release.[101]

4.3.2. Chemical Basis of Sulfur Ligand-Centered Reactivity

The observation that enzymes employ zinc-bound thiolates as nucleophiles, and that Zn/S coordination in both catalytic and structural sites of proteins is susceptible to sulfur oxidation and concomitant zinc dissociation, engendered a host of mechanistic studies to examine the role of the remainder of the ligands in sulfur reactivity and how specific factors, such as hydrogen bonding, affect this reactivity. One central issue is whether the zinc-bound or the dissociated thiolate is the reactive species. When the reactivity of Zn/S model complexes toward *S*-alkylation was investigated, it was found that a zinc tetrathiolate complex reacts much faster than either a zinc dithiolate complex or a free thiolate. The results were in agreement with the dissociated thiolate being the nucleophile.[102] Thus, cysteine-ligand dissociation is one mechanism of coordination dynamics (Figure 8A).[103] In studies with other Zn/S model complexes, however, the zinc-bound thiolate is the nucleophile.[104] It has been suggested that the net charge of the complex determines whether alkylation proceeds through an associative or a dissociative mechanism.[105] These studies provide some information for a preferential reaction of tetrathiolate complexes of zinc. NH···S hydrogen bonding and thiolate protonation are important factors in modulating the reactivity of zinc/thiolate complexes. An NH···S hydrogen bond decreases the rate of alkylation more than 30-fold.[104,106] Hydrogen bonding can "deactivate" certain thiolates in complexes containing multiple thiolates. Likewise, protonation of a thiolate decreases its nucleophilicity further. Zinc binding competes with protons and lowers the pK_a value of a thiol/thiolate equilibrium by approximately 2 orders of magnitude.[107] Thus, zinc binding activates a thiolate while protonation deactivates it.

The resolution of structures of metalloproteins is often not high enough to distinguish a coordinated thiol from a thiolate. However, in a high-resolution structure of rubredoxin, with zinc instead of iron in the tetrathiolate site, the ligands are all thiolates.[108] The Zn−S bond will be longer in a bound thiol compared to a bound thiolate. The rather uniform spread of Zn−S distances in zinc proteins would seem to suggest that thiol coordination is rare.[14] A high dielectric medium, such as an aqueous solution or additional polarizable ligands, lowers the pK_a value of a thiol, favoring four deprotonations in the formation of a tetrathiolate complex. This occurs, in particular, at solvent-exposed surfaces in proteins, with lysines or arginines as second shell ligands that are twice as likely as hydrophobic residues to be present in zinc sites.[13,52] We, therefore, continue to refer to sites as tetrathiolate sites, although experimental data and theoretical considerations suggest that thiols can serve as ligands in zinc sites.[109,110] Coordination equilibria in a ZnS_4 model peptide suggest that only 3% are in the thiol state at pH 7.[56] If thiols are present, the word "tetrathiolate" is not correct. Certainly, the protonation state of sulfur in thiolate coordination environments is critical for their reactivity.

5. Dynamics of Zinc Coordination

5.1. Coordination Dynamics of Zinc Sites

Protein structure has a key role in the coordination dynamics of metal sites. It determines whether a site is rigid or flexible and whether the metal remains bound or is mobile. Mobility of zinc can be induced by specific thiolate ligand-centered reactions in sites that, overall, do not seem to differ in their coordination environments from sites with a purely structural role, as discussed for ZnS_4 sites. Examination of the effects of electrostatic screening and protein packing in zinc fingers demonstrated a continuum from purely structural to reactive sites and identified reactive zinc fingers as those with poorly screened scores.[111] In addition to the modification or dissociation of sulfur (cysteine) ligands, glutamate or histidine ligands can associate reversibly with zinc in proteins (Figure 8B,C). Whether or not transient zinc sites have additional characteristics that induce mobility of zinc has not been investigated.

5.1.1. Zinc−Metalloprotein Synthesis

One example of coordination dynamics is the delivery of zinc in the biosynthesis of zinc metalloproteins. The question of when and how proteins obtain their metal cofactor is answered differently for different metal ions.[112] The process can involve metallochaperones for Ni^{2+} or Cu^{+}, or enzymes, such as chelatases, for Fe^{2+} insertion into porphyrins. Zinc insertion into zinc proteins could occur via zinc-mediated protein/protein interactions, i.e., without zinc ever being free (associative mechanism), or via free zinc ions (dissociative mechanism) (Figure 9). It has been argued that zinc redistribution cannot be based on specific metallochaperone proteins because too many would be required to supply all of the many zinc proteins.[113] Yet, the redistribution must occur with tight control of free zinc ions to avoid nonspecific reactions and cytotoxic effects.

Since in vitro the catalytic metal ion can be removed from the active site of several zinc enzymes and then reinserted again, it has been argued that in vivo zinc is inserted into enzymes once they have folded completely. In the test tube, efficient reconstitution can be achieved with zinc ions, but a clear difference between a chemical and a biological experiment is that, in the cell, zinc ions are not necessarily freely available for this purpose.[114] For some enzymes, the structure of the apo-form (the metal-free form of the protein) was investigated and found to be essentially the same as that of the holo-form (the metal-bound form of the protein).[115−118] Minor changes are restricted to the positions of the metal ligands in the active site. They may be important, though, for the way the protein handles the metal ion. For the reconstitution of manganese superoxide dismutase, a conformationally gated uptake of the metal into the active site was proposed.[119] In this mechanism, amino acids outside of the active site participate in a swinging door mechanism involving one of the histidine ligands. When the catalytic zinc in horse liver alcohol dehydrogenase (HL)ADH is removed, one of the two cysteine ligands (Cys-46) also swings out and comes into hydrogen-bonding distance with a glutamate (Glu-68).[115] In the apoform of carboxypeptidase A, the zinc ligands, Glu-270 and His-196, move away from their original positions.[116] It is not known whether these changes simply reflect repositioning of the ligands as a result of compensating for the charge of the missing metal ion or

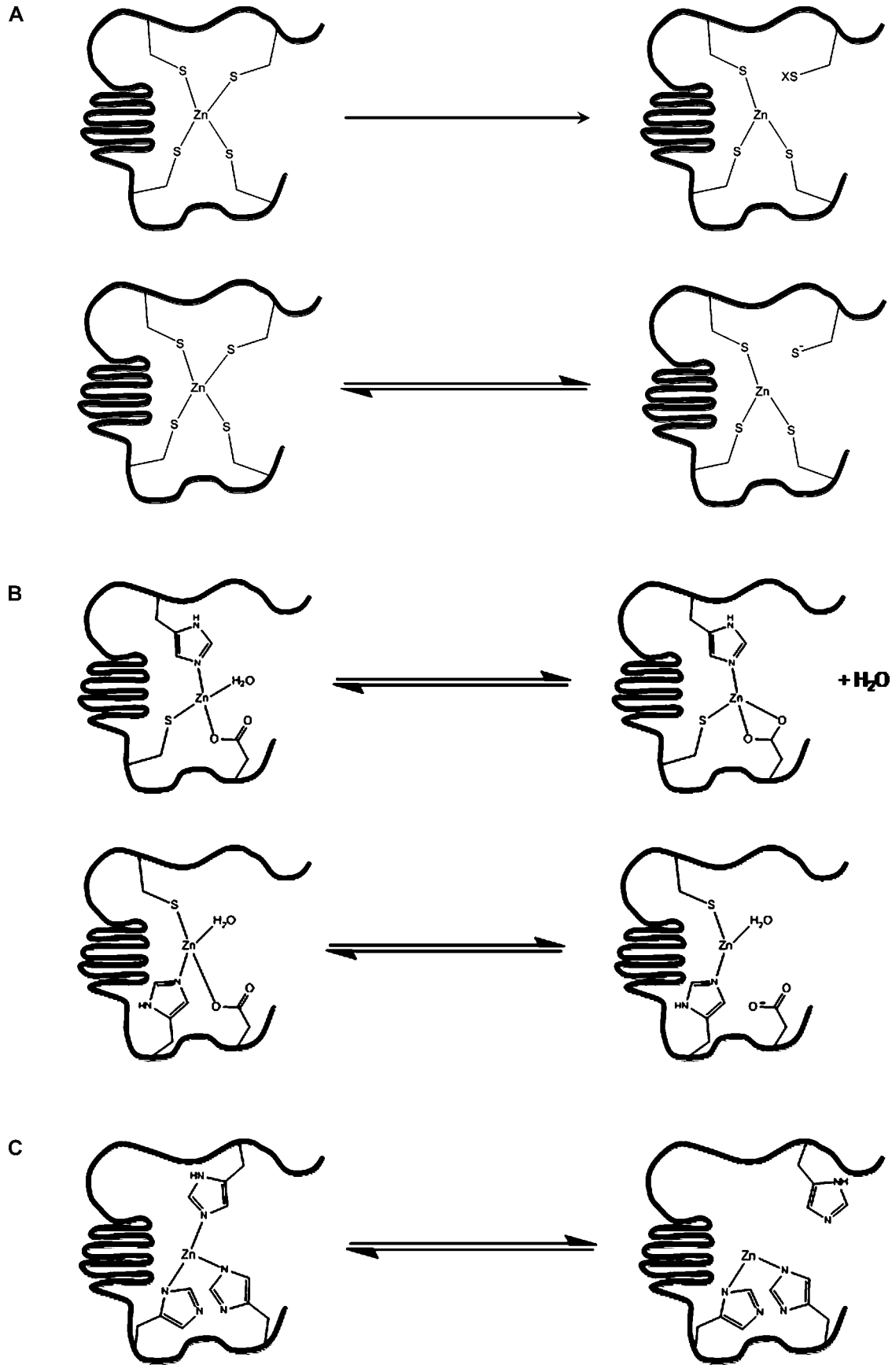

Figure 8. Coordination dynamics of zinc sites with specific reference to dissociation of a single ligand. (A) Cysteine modification or dissociation, examples: oxidation of zinc/thiolate clusters in metallothionein and activation of matrix metalloproteinases. (B) Carboxylate shift mechanism and carboxylate dissociation, examples: farnesyl transferase and some alcohol dehydrogenases. (C) Histidine dissociation, example: zinc inhibition of kallikreins.

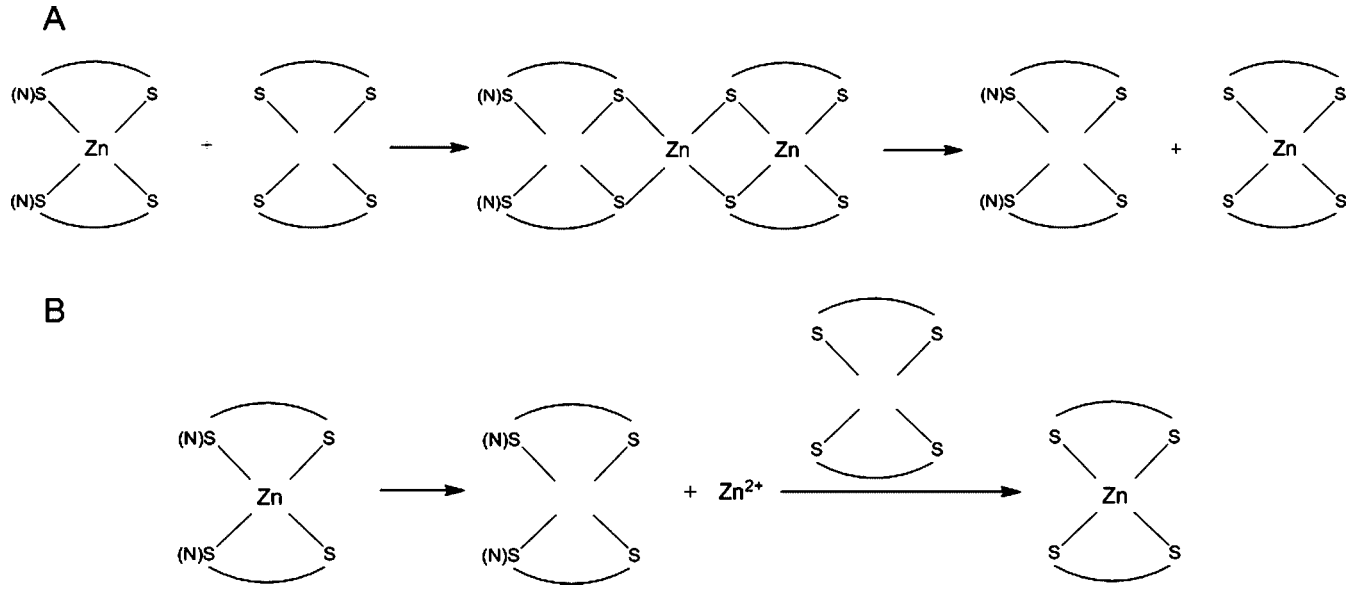

Figure 9. Associative and dissociative mechanisms of zinc transfer. (A) In an associative mechanism, a conduit of ligands transfers the zinc ion that is never free in the process. (B) In a dissociative mechanism, zinc ions are free before they associate with another partner.

whether the movement of the metal ion along a special path of amino acids is a general mechanism of how metal ions reach their final destination in a protein. A limitation of all these studies is the use of apoproteins that had already been folded before the metal ion was removed or inserted. It remains largely unknown whether zinc has a role in the folding of these proteins. In metallo-β-lactamases, a zinc-induced domain organization is thought to participate in the acquisition of zinc for the active site.[120]

The folding of many zinc finger domains depends on zinc.[69] If zinc is present while the protein folds, there is a high likelihood of non-native structures being formed. A study of the solution structures of peptides corresponding to S_4, S_3N, and S_2N_2 zinc fingers under conditions of substoichiometric amounts of zinc demonstrates different ligand compositions of the zinc sites and zinc ions bridging different binding sites.[121] This structural adaptability generates a significant conformational space that could be exploited for biological recognition if underloading with zinc ions occurs under limited zinc supply. In zinc finger proteins with multiple fingers and with nonsequential binding patterns of ligands, site selection has been addressed. TRAF (tumor necrosis factor receptor-associated factor) has a tandem array of zinc finger domains.[122] Zinc affinities for alternative structures were comparable to those of the correct structures. The specific factors that lead to the correctly folded and zinc-ligated peptides are not fully understood. They may include cooperativity of metal binding, as shown for the RING (really interesting new gene) finger domain of BRCA1 (breast cancer 1), where zinc binding is sequential and anticooperative,[123] or for another tandem zinc finger in Zap1, where zinc binding is not sequential and cooperative.[124]

If zinc ions were available freely, it would have major implications for protein folding and function. Unfolded proteins have many donor ligands of the right "bite size" for interaction with zinc. For example, the CxxC motif is very common in proteins serving not only as a metal-binding motif but also those with redox functions.[125] When the effect of zinc on the folding of the DNA-binding domain (DBD) of the tumor suppressor p53 was investigated, even a small excess of zinc ions trapped p53 in a misfolded state.[126] The DBD of this transcription factor contains 10 cysteines and 9 histidines, out of which only 3 cysteines and 1 histidine coordinate zinc in the native conformation. Zinc binds to the non-native state with subnanomolar affinity. The sites for this metal misligation are not available when the protein is in its native state, however. Whether such misligated, non-native states are obligatory intermediates in a folding pathway or are formed randomly is not known. Thus, on one hand zinc ions can provide a kinetic trap in the folding of the protein, but on the other, the apo-DBD aggregates in the absence of zinc. Unless other parts of the protein or chaperones assist folding, zinc ions should be available only at concentrations that do not cause misligation (<1 nM) and they should be delivered fast enough to the correct binding site to avoid aggregation. Clearly, protein folding must occur in an environment of low zinc ion concentrations, and for zinc proteins, in an environment of controlled zinc ion availability. MT has the properties to provide zinc ions in a controlled way.[30,114] Metallothionein/p53 interactions have been discussed. MT modulates p53 conformation in vitro and, when overexpressed, modulates p53 transcriptional activity, presumably through zinc transfer reactions.[127,128] Thus, a critical question in zinc biology is the following: At which concentration are zinc ions present and do specific processes provide zinc during protein folding?

Protein misfolding is a step in the pathobiochemistry of degenerative diseases.[129] A surplus or a deficiency of zinc is implicated in several amyloid diseases. In the absence of its metals copper and zinc, human superoxide dismutase 1 (SOD1) is prone to oligomerize through formation of intermolecular disulfides.[130] Amyloid SOD1 fibrils are found in the spinal cord of patients with amyotrophic lateral sclerosis. A surplus of zinc ions interacts with the amyloid beta protein Aβ1-40 and leads to its aggregation and the stabilization of harmful nonfibrillar, amyloid forms rather than Aβ fibrils, a hallmark of the plaques in Alzheimer's disease.[131] Zinc ions also promote amyloid formation of mutant transthyretin.[132] To which extent zinc/copper interactions have a role in the aggregation of other peptides, such as α-synuclein in Parkinson's disease, prion protein in transmissible spongiform encephalopathies, or β_2-microglo-

bulin in a particular type of amyloidosis, will depend on the local relative concentrations of these and other metal ions.[133,134]

5.1.2. Coordination Dynamics in Zinc Enzymes

The following sections will address coordination dynamics of sites in which the zinc remains bound to the protein and from which it dissociates.

Zinc Does Not Leave the Coordination Sphere in the Protein. Lessons on how zinc coordination spheres change can be learned from the flexibility of zinc sites in zinc enzymes. The first evidence for coordination dynamics in zinc enzymes stemmed from studies of zinc-dependent horse liver alcohol dehydrogenase ((HL)ADH, the EE isozyme), a member of the family of medium-chain dehydrogenases/reductases. The protein ligands of the catalytic zinc ion are two cysteines and one histidine. In addition, four cysteines bind a second, structural zinc ion relatively far away from the active site. In ADH, coenzyme (NAD^+/NADH) binding induces conformational changes of the protein and alters the structure of the catalytic metal ion.[135−138] The function of the metal differs in the two directions of the reaction. Activation of an aldehyde/ketone for hydride transfer requires an "electron pull", whereas activation of an alcohol requires an "electron push." At least three states of the metal ion need to be considered: in the enzyme in the absence of coenzyme, in the enzyme complex with NAD^+, and in the enzyme complex with NADH. The ligand-sphere transitions during coenzyme binding involve changes of both the geometry and the coordination number of the zinc ion.[139,140] In different ADHs, the coordination number can vary between four and six.[141] For (HL)ADH, the highest resolution structure (0.9 Å) has been achieved for the complex of the enzyme with NADH. In this structure, the zinc-bound water molecule occupies two alternating positions.[142] X-ray diffraction studies of class III ADH (ADH3), spectroscopic studies of cadmium(II)-substituted ADH, and theoretical calculations[143−146] are in agreement with a glutamate (Glu-68) associating reversibly with the catalytic zinc ion. Whether or not this step is obligatory in the catalytic cycle of liver ADHs remains an unresolved issue. On the basis of sequence alignments and interpretation of spectroscopic data, it was predicted that the ligands of the catalytic zinc in sorbitol dehydrogenase differ from those in ADH.[147−149] In sorbitol dehydrogenase, a secondary alcohol dehydrogenase, the ligands of the catalytic zinc are indeed different: one cysteine, one histidine, and one glutamate.[150] Coordination changes during the catalytic cycle involve glutamate association and dissociation.[150] High-resolution crystal structures of binary and ternary complexes of glucose dehydrogenase from the halophilic archaeon *Haloferax mediterranei* demonstrate considerable dynamics in zinc coordination and in the location of the zinc ion in the active site.[151] In the coenzyme (NADP(H))/enzyme complex, the protein ligands of the catalytic zinc are the Nε2 of His-63, the Oε2 of Glu-64, and the Oε1 of Glu-150. Glucose binding induces a loop closure that results in a movement of zinc away from the two glutamate ligands, resulting in Oδ2 of Asp-38 becoming a ligand. In this process, the positive charge on the zinc ion increases. As proposed for the mechanism of sorbitol dehydrogenase, only two protein ligands (Asp, His) bind to zinc in this complex. Further along the reaction coordinate in the ternary complex, zinc moves again and the ligands are now Asp-38, His-63, and Glu-64.

Coordination changes in catalytic zinc sites indeed may be a more general aspect of enzymatic catalysis. Time-resolved studies of reaction intermediates also point at considerable coordination dynamics in zinc proteinase catalysis. In the enzymatic activation of the zymogen form of matrix metalloproteinase-9 by tissue kallikrein, transitions of the coordination sphere of the zinc ion have been observed.[152] Before the inhibitory cysteine ligand in the pro-metalloproteinase dissociates, Glu-402 enters the coordination sphere as a fifth ligand. The sulfur ligand then dissociates while the glutamate carboxylate remains initially bound until it is replaced by a water molecule entering the coordination sphere.

Thus, the flexibility in coordination of the catalytic zinc ion in these enzymes is expressed in different ways: variation of zinc/ligand bond lengths, flexible glutamate coordination, and displacement of the metal itself from its original position.[141] A so-called carboxylate-shift mechanism was discussed for other zinc enzymes, such as farnesyl transferase.[153] In this mechanism, the bidentate binding mode of a carboxylate changes to monodentate and vice versa. A combination of cysteine, glutamate/aspartate, and histidine ligands is most favorable for this mechanism. In both cobalamine-dependent (MetE) and cobalamine-independent (MetH) methionine synthases, there is a displaceable glutamate/asparagine ligand.[154] In these enzymes, dissociation of the endogenous ligand is accompanied by a considerable shift in the position of zinc (1.9−2.0 Å) and an inversion of its geometry

Modulation of the properties of the zinc ion without ligand dissociation is central to the concept that sulfur ligands of enzymatic zinc sites provide a valence buffer.[155] In cytidine deaminase, an enzyme with S_3 coordination of the catalytic zinc, changes in Zn−S distances of one of the three cysteine ligands were observed and are thought to be significant in modulating the Lewis acidity of the zinc ion, such that the Zn−S bond is shorter in the ground state but longer in the transition state for maximizing the charge on the hydroxide ion. The ZnS_4 site in DNA primase of bacteriophage T7 changes its structure considerably during catalysis. Two of the zinc−sulfur bonds become elongated from 2.3 to 2.7/2.8 Å. The increased electron density at the central zinc atom is thought to be important for recognition of specific DNA sequences.[156]

These examples demonstrate zinc coordination dynamics without the zinc ion ever leaving the coordination environment. It illustrates that zinc or a protein ligand move, and that the ligand can come on and off the metal, which are necessary steps toward metal dissociation and association. One would presume that the stability of the complexes changes in this process, though there are no data at present. Changes may be small, localized, or transient, posing no immediate threat for the protein to lose its metal.

After having been involved in long and sometimes heated discourses about the interpretation of spectroscopic studies versus crystal structures during my graduate and postgraduate years (W.M.), and now finding more evidence that metals indeed move in the catalytic sites of zinc enzymes, I am often reminded of Galilei's famous words: Eppur si muove! (And yet it moves!)

Zinc Leaves the Coordination Sphere in the Protein. Studies on the zinc sites in (HL)ADH also provided information on metal dissociation. Using manganese(II) ions as an NMR probe, solvent proton magnetic relaxation studies

detected a metal binding site in addition to the one in the catalytic site.[157] When zinc ions are added to the enzyme, they first bind to this site before they migrate into the active site. This mechanism is supported by kinetic studies that are consistent with a 2-step mechanism of metal binding to form the active enzyme.[158] Metals could reach the active site by "jumping/leaping" or through a conduit of amino acids without ever dissociating from the protein surface.

When cadmium(II) ions were added to apo-(HL)ADH, i.e., the derivative in which the catalytic zinc is removed but the structural zinc remains in place, cadmium initially binds to the active site, but then, in a slower process, migrates to the structural site and displaces zinc, which in turn migrates to the active site.[159] This process is driven by thermodynamics because four thiolates in the structural site provide higher affinity for cadmium than two thiolates in the catalytic site.[160] A remarkable finding is the jumping of cadmium between the two sites in *Bacillus cereus* β-lactamase at a stoichiometry of one cadmium per binuclear zinc site. The process is intramolecular and occurs on a microsecond time scale.[161] An intramolecular zinc transfer has also been observed for human porphobilinogen synthase. Once a disulfide in the active site is reduced, zinc migrates from a distal site to the active site.[162]

5.2. Zinc Transfer and Trafficking

5.2.1. Metallothioneins

A review of the coordination chemistry of metallothioneins (MTs) is beyond the scope of this article. The considerable variation in the primary structures of MTs suggests a corresponding variation of their metal-binding motifs, as is now borne out by the structure of a plant zinc metallothionein.[163] The name metallothionein was proposed for a metal-containing and sulfur-rich protein isolated from equine renal cortex.[164] Proteins and peptides with such characteristics were found in many organisms. Accordingly, a classification based on the similarities of primary sequences was proposed:[165]

- Class I: polypeptides with locations of cysteine closely related to those in equine renal MT;
- Class II: polypeptides with locations of cysteine only distantly related to those in equine MT; and
- Class III: metal−thiolate polypeptides that are not genetically coded.

A focus here will be on those MTs that participate in zinc homeostatic mechanisms, and where coordination dynamics have been investigated in detail.

The structure with 20 reduced cysteinyl residues binding the 7 zinc ions is the accepted model for the mammalian protein (Figure 6). However, it is not the physiologically important structure, because the different zinc ions have considerably different affinities.[64] In tissues and cells, MT occurs in the holo-form, "metallothionein"; in the apo-form, "thionein"; and in the oxidized form, "thionin".[65,66,166] In this regard, MT differs from many zinc proteins, the zinc sites of which are generally fully occupied. In rat liver, 20% of MT is in the apo-form and 7% is oxidized.[66] This distribution of the different forms changes when zinc ions become available or when the cellular thiol/disulfide redox state changes. MT is neither fully loaded with zinc ions nor are all of its cysteines in the reduced state. It is this variation of the structure that is important for the mechanism of action of MT and its biological activity. Thus, structural studies of the isolated molecule have provided one answer, but the biochemical environment, including zinc ion availability and redox state, determines the significance of structures other than the one with 7 zinc ions and 20 bound cysteine ligands.

The metals in the clusters of MT are quite dynamic. Cd-NMR spectroscopy of rabbit MT demonstrated that cadmium ions interchange their positions *intra*molecularly more rapidly within the 3-Cd cluster than within the 4-Cd cluster and between clusters.[167] *Inter*molecular exchange of zinc ions occurs through an associative mechanism with two kinetic phases ($k_1 = 5000\ \text{min}^{-1}\ \text{M}^{-1}$, assigned to the 3-zinc cluster, and $k_2 = 200\ \text{min}^{-1}\ \text{M}^{-1}$, assigned to the 4-zinc cluster).[168]

Zinc proteins were not considered to be redox proteins. However, the redox capacity of Zn/S sites makes MT a redox protein, the zinc-binding and redox state of which depend on the biological environment in which it functions. Thus, a novel and general mechanism of action for MT entails the oxidoreductive characteristics that the redox-active sulfur-donor group of cysteine confers on zinc sites in the clusters.[80] In a biological context, this mechanism links the reducing power of a cell and the relative mobility of zinc and its control.

In test tube experiments, MT activates the apo-forms of zinc enzymes.[169] The activation is a function of either the redox state[114] or the zinc load of MT, which is expressed as the T/(MT + T) ratio.[30] For example, MT can be a zinc donor to the apo-form of sorbitol dehydrogenase ($\log K = 11.2$ for zinc), or it can be a zinc acceptor to remove the inhibitory zinc from protein tyrosine phosphatase 1B (PTP1B) ($\log K = 7.8$ for zinc).[30] T/(MT + T) molar ratios, expressing the metal load of the protein, are in the range of 0.08−0.31 in tissues and cells. Under these conditions, picomolar concentrations of free zinc ions are made available from MT for reconstituting apo-enzymes. Higher ratios can be obtained under more oxidizing conditions, making nanomolar concentrations of free zinc ions available from micromolar MT concentrations.[30] In this case, enzymes that are not zinc metalloenzymes, such as PTP1B, are inhibited. The properties of MT as a redox-dependent zinc donor and acceptor and its extensive gene regulation suggest ways of tightly controlling (i) zinc ion fluctuations to avoid unspecific reactions, such as misfolding of proteins, (ii) a variety of zinc-dependent biological processes, and (iii) a signaling network, in which redox signals are converted into zinc signals.[79,170] In MTs, the redox and coordination chemistry of the thiols are linked: MTs are redox proteins affecting zinc availability, and they are zinc proteins affecting the redox state of other proteins. Taken together, the existence of at least four major isoforms of mammalian MT, the tissue-specific expression of these isoforms, the regulation of their expression by multiple signaling pathways, and the chemical properties just described signify new functions of these proteins that are specifically adapted for controlling zinc ions.[171] In addition to its intracellular function, extracellular functions of MT are now acknowledged.[172,173]

5.2.2. Albumin

Albumin is a major zinc transport protein in the blood. Human serum albumin has a K_d of 45 nM for zinc. The zinc-binding site is thought to involve N donors of His-67 and 247 and O donors of Asn-99 and Asp-249, as well as a water molecule.[174] Fatty acid binding to albumin triggers a spring-lock mechanism to move two of the ligands, His-247 and Asp-249, both located on one domain, relative to the two ligands on the other domain, and thereby, to release zinc.

This example shows how an effector molecule is involved in extracellular zinc delivery. MT could be the acceptor of zinc. Albumin binds MT with a K_d of 15 μM.[175]

5.3. Zinc Sensing

Zinc sensing in eukaryotes involves occupancy of zinc finger domains and their interactions with DNA. The metal response element (MRE)-binding transcription factor-1 (MTF-1) senses cellular zinc ion concentrations in multicellular eukaryotes and activates the expression of proteins involved in zinc homeostatic mechanisms, such as thionein (T, the apo-form of MT). MTF-1 has six canonical S_2N_2 zinc fingers (F1−F6) toward the N-terminus, which is the DNA-binding region of the molecule.[176] The six zinc fingers are connected by the typical linker, TG(E/Q)(K/R)P, with the exception of F3 and F4, where the linker is one residue shorter, namely, TGKT. The zinc sensor in yeast is called the zinc-responsive activator protein 1 (Zap 1). *Saccharomyces cerevisiae* Zap1 has seven S_2N_2 zinc finger motifs (F1−F7).[177] It is a transcriptional repressor and activates gene expression in zinc-limited cells by binding to a zinc-responsive element.[178] The DNA-binding domain of Zap1 was mapped to a C-terminal segment of 174 amino acids with five canonical S_2N_2 zinc fingers (F3−F7).[179] Zap1 also contains two transcriptional activation domains, AD1 and AD2, which are the zinc-sensing domains.[180]

In contrast, zinc finger domains are not used in sensing zinc ions in prokaryotic cells. Metal sensor proteins have been studied relatively well in bacteria, especially members from three families: (i) ZntR from the MerR family, (ii) Zur and FurB from the Fur family, and (iii) SmtB and CzrA from the ArsR family. They all belong to the winged helix (helix-turn-helix) class of proteins with zinc-binding sites at the interface of the subunits. The ligand sets used by zinc sensors are similar to those of other zinc proteins. There are multiple zinc-binding sites in zinc sensors.

Metal sensors are metal-dependent transcriptional regulators. They are allosteric molecules and work as conformational switches, in which metal binding to the protein is linked to the protein/DNA interaction.[181] Treating the biophysical properties of the sensors in the framework of thermodynamic linkage theory allows investigation of how the coordination chemistry of the metal-sensing site is coupled to conformational changes of the sensor protein and interaction with DNA. The structural basis of this coupling is emerging, and it involves transmitting the energy from the binding event from the first coordination sphere to a network of secondary interactions. The molecular mechanisms will be discussed for the individual sensor molecules of three protein families (Figure 10). The coordination environments of these sensors are summarized in Table 4.

5.3.1. MerR Family: ZntR

As the extracellular zinc ion concentrations rise, ZntR from *E. coli* binds zinc and turns on the production of a zinc ion efflux pump, ZntA, which removes any excess zinc ions from the cell. In an N-terminally truncated fragment of ZntR, both metal-binding domains of the ZntR dimer are well-ordered with two zinc ions bound in a binuclear site and with each zinc in a tetrahedral geometry and a bridging sulfur (Table 4).[182] The use of the ligands is nonsequential, and the site is at the interface. One zinc is bound to Cys-114 and Cys-124 of the metal-binding loop, and to Cys-79 from the other monomer, whereas the second zinc is bound to Cys-115 and His-119 of the metal-binding loop and also to Cys-79 from the other monomer. The fourth ligand of each zinc is an oxygen atom of a bridging phosphate or sulfate. ZntR exhibits half-maximal induction at 1.15×10^{-15} M free zinc ion concentrations. ZntR regulates transcription by a metal-induced DNA-distortion mechanism,[183] as inferred from Hg-MerR-induced DNA distortion. Apo-MerR binds to DNA, but when Hg^{2+} is bound, Hg-MerR bends the DNA. MerR relaxes these bends and unwinds the center of the operator; the unkinking and untwisting of the bending introduced by Hg-MerR remodels the promoter, making it a better substrate for RNA polymerase, thus dramatically increasing the transcription rate.[184]

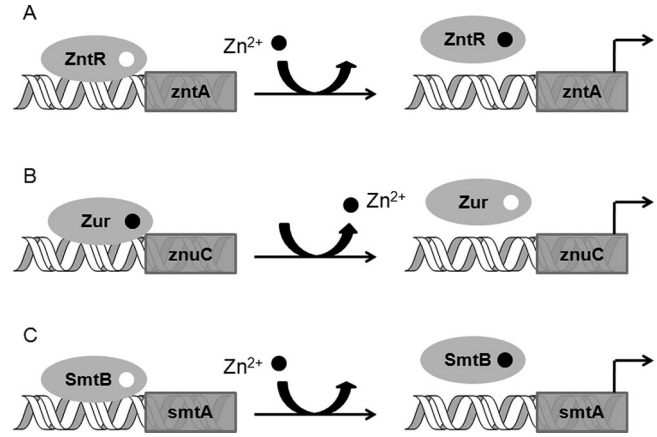

Figure 10. Bacterial zinc sensors from three families of winged helix proteins: (A) ZntR from the Mer family; (B) Zur from the Fur family; and (C) SmtB from the ArsR family. The sensors are allosteric proteins. Upon binding/releasing Zn^{2+}, they change their conformation and make the promoter accessible for transcription. The figure does not imply that the sensors dissociate from the DNA after the conformational change occurred.

5.3.2. Fur Family: Zur and FurB

The *znuABC* operon encodes a triplet of proteins, ZnuA (a periplasmic protein), ZnuB (a membrane protein), and ZnuC (an ATPase), and it is regulated by Zur.[185] Subfemtomolar ($<10^{-15}$) concentrations of free zinc ions trigger Zur repression of the *znuC* gene.[186] The purified *E. coli* Zur has two distinct metal-binding sites: one is functionally analogous to the zinc site in Fur, presumably a structural site with zinc in an $S_3(N/O)$ coordination environment; the second site binds zinc in an $S(N/O)_3$ coordination environment (Table 4).[186] In the presence of excess zinc ions, the Zur dimer binds to the regulatory sequence located within the central *znu* operon and prevents the binding of RNA polymerase.[185] When extracellular zinc ion concentrations become critically low, Zur repression of the *znu* operon is lifted and the proteins involved in zinc ion influx are expressed.

Another Fur-like zinc regulatory protein is FurB from *Mycobacterium tuberculosis*.[187] The crystal structure of FurB shows three distinct zinc binding sites (Table 4). The first zinc binding site is the regulatory site and is formed by two amino acids from the DNA binding-domain (Asp-62 and Cys-76) and two from the dimerization domain (His-81 and His-83). Thus, it is located at the hinge region between the two domains. The second zinc binding site is a structural site and coordinated by Cys-86 and Cys-89 from the dimerization domain and Cys-126 and Cys-129 from the C-terminus. It is not a protein-interface zinc site, but metal

Table 4. Coordination Environments of Bacterial Zinc Transporters and Zinc Sensors

			L_1		L_2		L_3		L_4	sensor/target
Sensing										
MerR family	ZntR[182]	Zn1	C79	34	C114	9	C124		O[a]	ZntA
		Zn2	C79	35	C115	3	H119		O[a]	
Fur family	Zur[186]	Zn1	S_3(N/O) (structural)							ZnuABC
		Zn2	$S(N/O)_3$ (regulatory)							
	FurB[187]	Zn1	D62	13	C76	4	H81	1	H83	
		Zn2	C86	2	C89	36	C126	2	C129	
		Zn3	H80	1	H82	18	E101	16	H118	
ArsR family	SmtB[b190−192]	Zn1	H_2O	−	C61	2	D64	32	H97	SmtA
		Zn2	D104	1	H106	10	H117′	2	E120′	
	CzrA[193]		D84	1	H86	10	H97′	2	H100′	CzrB
Transport										
Import										
ABC transporters	ZnuA-*Ec*[216]		E59	0	H60	82	H143	63	H207	Zur/FurB
	ZnuA-*Ec*[215]		H_2O	−	H60	82	H143	63	H207	
	ZnuA-*Syn*[217]		H_2O	−	H83	95	H179	63	H243	
Export										
P-type ATPases	ZntA[205]	Zn1	D58 (one or two O)			0	C59	2	C62	ZntR
		Zn2	C392, C394, D714							
CDF	YiiP[213]	Zn1	D45	3	D49	103	H153	3	D157	
		Zn2	D68, H75							
		Zn3 and Zn4	H232, H261, H283, D285							
	CzrB[214]	Zn1	H31, H47, E84							CzrA
		Zn2	H60, H82, E84							
		Zn3	D32, H47							
		Zn4	E57							

[a] The fourth ligand is an oxygen atom of a bridging phosphate or sulfate. [b] A mercuric acetate derivative of SmtB

binding at this site stabilizes the dimer significantly. A third zinc is tetrahedrally coordinated by His-80, His-82, Glu-101, and His-118 at the core of the dimerization domain. Both a structural role and a regulatory role have been suggested for this site in FurB, which has not been found in Zur.

5.3.3. ArsR Family: SmtB and CzrA

The *smt* locus of *Synechococcus*, a cyanobacterium, contains a metal-regulated gene, *smtA*, which encodes the prokaryotic MT SmtA, and a divergently transcribed gene, *smtB*, which encodes a *trans*-acting transcriptional repressor of *smtA*.[188] SmtA from *Synechococcus* PCC 7942 binds four zinc ions via nine cysteines and two histidines in a $Zn_4S_9N_2$ cluster resembling the Zn_4S_{11} cluster in mammalian MT. One of the four cysteine ligands of the two zinc centers with two terminal cysteine ligands is replaced by a histidine, yielding two ZnS_3N centers.[189] SmtB responds to potentially toxic concentrations of zinc ions and other heavy metal ions.[190] Analysis of a mercuric acetate derivative of SmtB crystals suggests a total of four zinc binding sites in the dimer: two are at the opposite ends of the dimer, while the other two are at the dimer interface with residues from each monomer (Table 4).[190] SmtB binds one metal ion per monomer in tetrahedral coordination geometry at pH 7.4. Zinc is bound in one of two mutually exclusive metal binding sites, termed α3N and α5. The zinc affinities differ at least 20-fold ($K_{Zn}^{\alpha 3N} \geq 10^{13}$ M^{-1}; $K_{Zn}^{\alpha 5} = 5 \times 10^{11}$ M^{-1}).[191] Cys-61, Asp-64, His-97, and a water molecule are the ligands of the α3N metal site. The α5 metal binding site provides Asp-104 and His-106 from one protomer and His-117' and Glu-120' from the other protomer as ligands.[191] It has a regulatory function in zinc sensing. The function of the high-affinity α3N site has yet to be defined.[192]

CzrA from *Staphylococcus aureus* is a homologue of SmtB.[193] It also binds zinc in a pair of tetrahedral, interhelical sites with two ligands derived from the α5 helix of one subunit, Asp-84 (Asp-104 in SmtB) and His-86 (His-106), and two from the α5 helix of the other, His-97' (His-117′) and His-100' (Glu-120′) (Table 4). The quaternary structural switch in this sensor is mediated by an intersubunit hydrogen-bond network that originates from the nonligating Nε2 atom of His-97' in CzrA to Arg-73 of loop A and then continues to Leu-69 of α-helix4, both located on the other protomer. This conformation has low affinity to DNA.

5.3.4. MTF-1

Zinc fingers of MTF-1 harbor the DNA-binding activity and may also be involved in zinc sensing. The six zinc fingers can be divided into two groups based on studies of a recombinant fragment of MTF-1 containing only the zinc finger domain (denoted MTF-zf).[194,195] One group of zinc fingers (F1−F4) is more important for the structure, while the second group (F5 and F6) contributes less to the affinity of MTF-1 for DNA and has a lower affinity for zinc, based on selective removal of zinc from these fingers with a chelating agent of modest zinc affinity (~10^7 M^{-1}). A possible mechanism for how zinc affinities are realized in coordination environments with identical donor sets is related to conformational changes associated with zinc binding, as discussed in section 4.1.

One model of MTF-1 metalloregulation involves intramolecular allosteric activation by zinc binding to F5 and F6.[195]

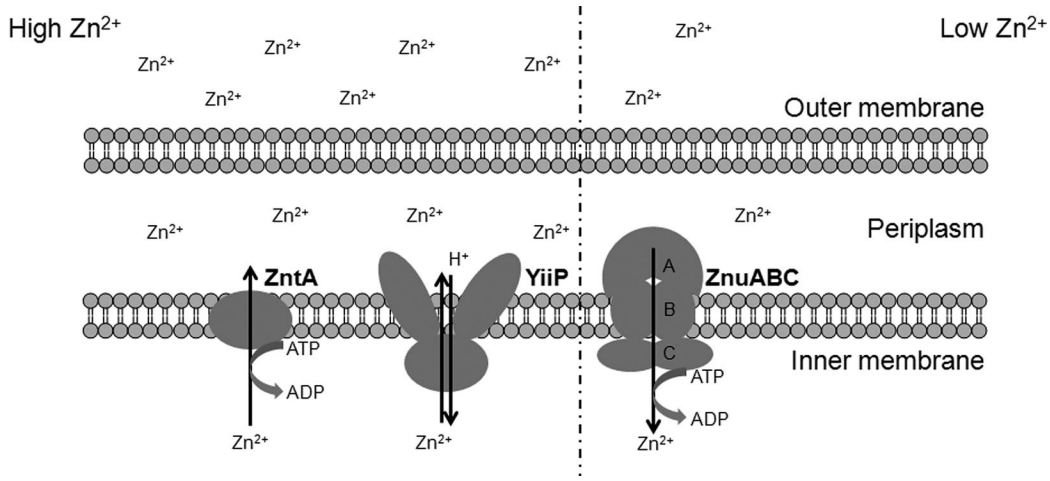

Figure 11. Zinc transport through the bacterial membrane. In a gram-negative bacterium, such as *E. coli*, the outer membrane is permeable to zinc ions. Three types of transporters are discussed in the text: an ATP-dependent exporter (ZntA), a cation diffusion facilitator (CDF), which is a Zn^{2+}/H^{+} antiporter (Yiip), and an ATP-dependent importer (ZnuABC) that is responsible for zinc uptake if zinc becomes limited. There is a proton gradient at the inner (cytoplasmic) membrane. The pathway of zinc transport through the proteins has not been established.

According to this model, F1−F4 are constitutively loaded with zinc and bind to the MRE. Binding of zinc to F5 and F6 then induces a conformational change of the protein to allow its C-terminal domain to interact with the polymerase complex and initiate RNA synthesis. In this model, only the two C-terminal zinc fingers with lower zinc affinities participate in zinc sensing, and this allows MTF-1 to sense elevated zinc ion concentrations. Another model suggests a role of F1 in metal sensing because F1 deletion mutants bind DNA constitutively but do not respond to induction by zinc.[196] According to this model, the reversible binding of zinc to F1 removes an allosteric block that prevents the strong DNA-binding fingers of MTF-1 (F2−F4) from interacting with the MRE.

A cluster of cysteine residues (-^{632}Cys-Gln-Cys-Gln-Cys-Ala-Cys^{638}-) that are outside the MRE-binding zinc finger domain may also participate in metal-dependent transcriptional activation.[197] The domain of human MTF-1, consisting of amino acids 567−753, has low affinity ($\sim 1 \times 10^6$ M^{-1}) for zinc ions, cobalt(II) ions, and cadmium(II) ions. Zinc binding to the cysteine-rich domain could alter the way in which MTF-1 interacts with other regions of the protein, with coactivators, or with other components of the transcription apparatus. Alternatively, this domain could be involved in zinc recruitment, exchange, or insertion into zinc finger domains.

The exact intracellular zinc ion concentrations sensed by MTF-1 are not known. However, it has been estimated that the cellular free zinc ion concentrations are in the picomolar range and underlie only relatively small fluctuations.[113,170,198] Therefore, sensing is likely to occur in the picomolar to low nanomolar range of zinc ion concentrations. The characteristics of zinc binding to MT also indicate zinc sensing in this range.[64] Binding of cadmium or copper to MT or the oxidation of MT with subsequent zinc dissociation activates MTF-1,[199] indicating a role for MT in controlling the availability of zinc ions for MTF-1-induced gene expression. In this way, MT not only controls its own induction, i.e., producing thionein (T) for lowering intracellular free zinc ion concentrations ($[Zn^{2+}]_i$), but also controls the expression of other MTF-1-dependent genes.

5.3.5. Zap1

In the yeast transcriptional activator Zap1, zinc sensing activities of the two domains, AD1 and AD2, were mapped to residues 332−402 and 552−705, with the latter region containing two special S_2N_2 zinc fingers, F1 and F2.[200] F1 and F2 form a single globular domain with a hydrophobic interface, resembling the structure of the Gli tumor suppressor.[124,201] The affinities of F1 and F2 for zinc are 5.3 ± 2.2 and 0.3 ± 0.1 nM, respectively, at pH 7.5. When compared to values of 3.0 ± 1.7 and 0.2 ± 0.0 nM for F3 and F4, respectively, from the DNA-binding domain, F1 and F2 have virtually identical zinc affinities.[200] However, when the rate of zinc dissociation in the presence of the chelating agent 4-(2-pyridylazo) resorcinol was examined, the dissociation rates differed: F1/F2 had a $t_{1/2}$ of 1.4 days while zinc dissociation from F3/F4 was much slower ($t_{1/2} > 14$ days).[200] It was suggested that zinc kinetics have a role in the zinc-sensing mechanism of the fingers. The difference in dissociation rates was mapped to the α-helix of F2.[202] The zinc affinities of the individual fingers (160 ± 20 nM and 250 ± 40 nM) are considerably different from the zinc affinity of the domain that contains both fingers (4.6 ± 1.2 nM).[124] Cooperative zinc binding is a distinctive feature of these interacting zinc fingers. The characteristics suggest sensing at a concentration of 5 nM zinc ions.

5.4. Zinc Transport

There are no 3D structures of eukaryotic membrane zinc transporters. Structural information is available for prokaryotic zinc transporters, however. These proteins also seem to use the typical ligands and ligand sets in their coordination environments. Similar to zinc sensors, multiple zinc binding sites have been identified in the crystal structures of zinc transporters. The activity of zinc transporters depends on the availability of zinc in the microenvironment, the level of zinc inside the cell, and the amount of zinc required for cellular functions at particular stages in the life cycle of a cell. Prokaryotic cells have both zinc export and zinc uptake systems (Figure 11). Bacteria have one or more low-affinity uptake systems for each metal ion, usually with relatively broad metal ion specificity. High-affinity systems are specific

and highly regulated by metal sensors, and they control uptake when the metal ion is limiting.[203] The significance of transporters for prokaryotic zinc homeostasis is that prokarya do not have control over zinc availability in the microenvironment and that subcellular structures sequestering zinc ions are unknown. In bacteria, export and uptake systems belong to the protein families of RND (resistance-nodulation-cell division) multidrug efflux transporters, P-type ATPases, and cation diffusion facilitators (CDF) for export of high concentrations of potentially toxic zinc ions, binding-protein-dependent ABC transporters, and phosphate or citrate cotransporters for the uptake of zinc that is necessary for growth.[204] Depending on the zinc ion concentration in the medium, different types of zinc transporters are synthesized. At limiting zinc ion concentrations, binding-protein-dependent ABC transporters, e.g. the ZnuABC complex, are induced to take up zinc. The Pit-like proteins may serve as cotransporters to ensure that the demands of the cell for zinc under zinc-depleted conditions are met. Exporters of the CzcABC-like RND transporters seem to be very efficient in protecting the cell against toxic zinc ion concentrations. Also, Czc-like CDFs and P-type ATPases, such as ZntA, protect the cell against high zinc ion concentrations.[204] Three examples of bacterial zinc transporters, ZntA, YiiP/CzrB, and ZnuABC, will be discussed in detail, because 3D structures are available. The coordination environments of these zinc transporters are summarized in Table 4.

5.4.1. P-type ATPase: ZntA

E. coli ZntA, a member of the P1B-type ATPase transporter family, exports zinc across the inner membrane against a concentration gradient by utilizing the energy derived from ATP hydrolysis. The N-terminal fragment of ZntA (residues 46−118) from the cytoplasmic region contains a solvent-exposed zinc coordination site with sulfur donors of Cys-59 and Cys-62 and one or both carboxylate oxygens of Asp-58[205] (Table 4). Metal ion selectivity and specificity remains unchanged when this N-terminal metal site is absent.[206] Another zinc binding site, involving Cys-392 and Cys-394 and located in the transmembrane domain, is essential for transport because mutations of the ligands result in a protein that cannot catalyze metal-ion-dependent ATP hydrolysis. Namely, the selectivity for metal ions is not due to different binding affinities, but rather due to binding of other metal ions not being able to activate ATPase activity.[207] In addition, Asp-714 in the transmembrane domain was identified as a metal ligand of this site.[208] Thus, the N-terminal and the transmembrane metal-binding sites appear to have similar coordination environments and similar zinc affinities ($K_a \approx 10^8$ M^{-1}). Therefore, metal transfer between the two sites could be facile. In fact, intermolecular zinc transfer between the two sites was observed in the homologous zinc efflux pump AztA from *Anabaena*.[209] It was suggested that metal binding to the N-terminal domain induces a strong negative cooperative effect on the transmembrane site, thereby accelerating release of metal ions on the other side of the membrane.[207]

5.4.2. Cation Diffusion Facilitators: YiiP and CzrB

E. coli YiiP, a member of the CDF zinc transporter family, is a homodimer of two integral membrane proteins,[210] each composed of six transmembrane segments (TMs) and a hydrophilic C-terminal domain located in the cytoplasm.[211]

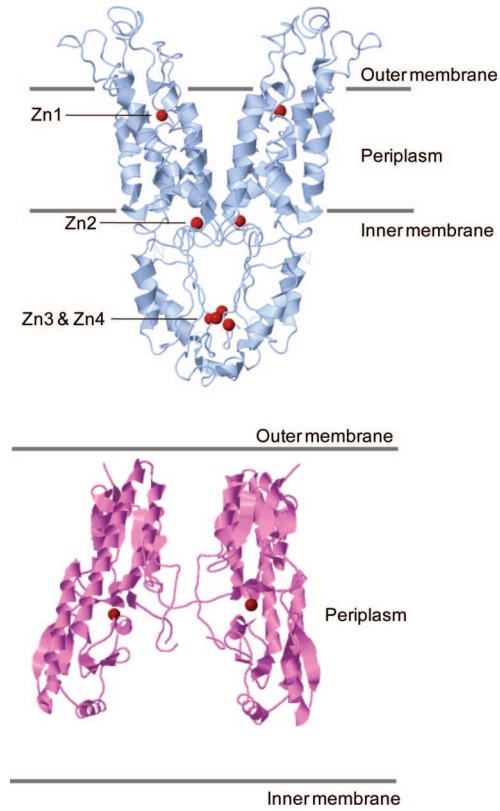

Figure 12. Structure of the dimeric Yiip (PDB 2QFI) (Top) and ZnuA (PDB 1PQ4) (Bottom) proteins. (Top) The position of four zinc ions per monomer was reported in this zinc transporter from the CDF family. Some of these zinc ions are either intrinsic elements of the protein structure or one is simply observing a snapshot of the zinc ions during their transit. (Bottom) The periplasmic ZnuA protein has one bound zinc ion per monomer.

It is a Zn^{2+}/H^+ antiporter. Affinities for zinc and cadmium are in the submicromolar range.[212] The crystal structure[213] revealed four zinc per monomer (Zn1−Zn4) (Figure 12). Zinc in site Zn1 is tetracoordinated (Table 4) with Asp-45 and Asp-49 from TM2 and Asp-157 and His-153 from TM5; all these residues are essential for zinc transport activity. Zinc in site Zn2 binds to the intracellular loop that connects TM2 and TM3, and this loop harbors many potential zinc-ligating residues. Zinc coordination appears to involve Asp-68 and His-75. This site is likely to be involved in subunit dimerization. Zn3 and Zn4 are located in a cleft between the two C-terminal domains and are bridged by the highly conserved Asp-285 in a bidentate coordination mode. This binuclear zinc center minimally involves His-232 from β-sheet 1, His-283 and Asp-285 from β-sheet 3, and His-261 from α-helix 2 of the neighboring subunit. A proposed mechanism of action for YiiP includes the binding of a putative zinc metallochaperone to the C-terminal domain and delivery of a cytoplasmic zinc to the intracellular cavity where the zinc is then translocated across the membrane to site Zn1 in the extracellular cavity.[213] It appears that, in the structure given (Figure 12), the metals are "frozen in time" during their transit through the membrane. The crystal structures of the cytoplasmic domain of the putative zinc transporter CzrB from *Thermus thermophilus* in its zinc-free and zinc-bound state at relatively high resolution (1.7−1.8 Å) provide further insights into zinc binding, protein dynamics, and function of this family of proteins (Table 4).[214] Binding of zinc to the cytoplasmic domain is described as "causing the dimer to snap shut" and generating pockets for

the binding of a putative metallochaperone and delivery of zinc. In the proposed model, the transporter senses cytoplasmic zinc ion concentrations because the transport-competent state of the protein is established only at high zinc ion concentrations. Each protomer binds four zinc ions. Zn4 is thought to have a function in crystallization. Its ligands are Oε1 from Glu-57 of each monomer. The remaining zinc ions form a trinuclear site with His-60 and His-82 binding Zn2 and Glu-84 bridging Zn1 and Zn2, which has His-31 and His-47 as additional ligands. While Zn1 and Zn2 have three ligands, Zn3 has only two: Asp-32 and His-47, the same ligand that also binds Zn1. Remarkably, aside from copper,zinc superoxide dismutase, it is the only other case where an imidazole of histidine is employed as a bridging ligand. All six side chains change their positions when their donor atoms bcome ligands for the zinc ions.

5.4.3. Binding-Protein-Dependent ABC Transporters: The ZnuABC Complex

Another *E. coli* zinc transporter for which relatively detailed structural information is available is ZnuA, the periplasmic component of the ZnuABC complex (Figure 12). *E. coli* ZnuA (ZnuA-*Ec*) belongs to the ABC-type periplasmic ligand binding proteins (PLBPs). The crystal structure of ZnuA shows at least two zinc binding sites: the primary binding site (K_d < 20 nM) is tetrahedrally coordinated by His-60, His-143, His-207, and one water molecule.[215] All three histidines are hydrogen-bonded to a second shell of amino acids, and yet, the affinity of this site is not as strong as the one for zinc in human carbonic anhydrase II. Glu-59 also interacts with zinc (Table 4); the second metal-binding site involves His-224 and several unidentified residues from the His-rich loop.[216] A homologue is ZnuA from *Synechocystis* strain 6803 (ZnuA-*Syn*). The metal-binding site of ZnuA-*Syn* ($K_d \approx 10$ nM)[217] is also formed by three histidines (His-83, His-179, and His-243) and one water molecule, but an interaction with a glutamate was not reported[218] (Table 4).

A Venus fly trap mechanism has been proposed for capture and release of large nonmetal ligands by PLBPs, which is, however, believed not to be the mechanism for zinc transport by ZnuA. Instead, a partial domain slippage mechanism has been proposed.[215] ZnuA-*Ec* has a conserved hydrogen-bonding network and salt bridges in the N-terminal domain, which restrict movement of the metal-binding arm. Upon metal release, a water molecule and Glu-59 occupy the vacant metal-binding site. The movement of the N- and C-terminal domains is also restricted by a conserved hydrogen-bonding network and salt bridges. The slippage of the bottom part of the C-terminal domain can be triggered by an alternative conformation of Arg-152 and a seesaw mechanism of the C-terminal α-helix. In ZnuA-*Syn*, flipping of the imidazole ring of His-243 (His-207 in ZnuA-*Ec*) was observed. It may be the first step toward release of the bound metal. Also, a conserved water molecule was detected in the C-terminal domain of ZnuA-*Syn*. The entry of this water molecule likely breaks a series of peptide backbone hydrogen bonds, which in turn would facilitate slippage of the top portion of the C-domain.

The His-rich loop of ZnuA-*Ec* likely harbors multiple zinc binding sites with at least 100-fold weaker binding constants than the primary metal-binding site.[217] The loop might chaperone zinc to the high-affinity site, perhaps by increasing the pool of zinc around the high-affinity site. At concentrations 100-fold higher than those needed for binding to the high-affinity site, zinc associated with the flexible loop may prevent association of ZnuA and ZnuB and thereby block zinc influx.

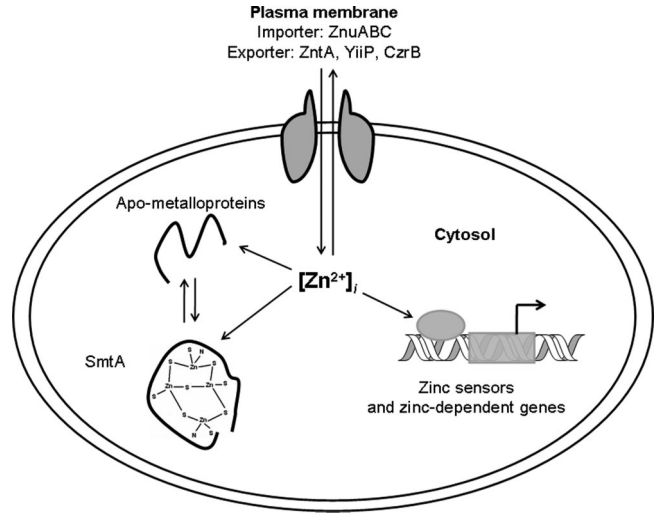

Figure 13. Zinc homeostasis and trafficking in a prokaryotic cell. Zinc import and export are controlled by plasma membrane transporters (for more details, see Figure 11). Intracellular distribution and control of zinc include bacterial metallothionein, such as SmtA in cyanobacteria, and zinc sensors for zinc-dependent gene transcription.

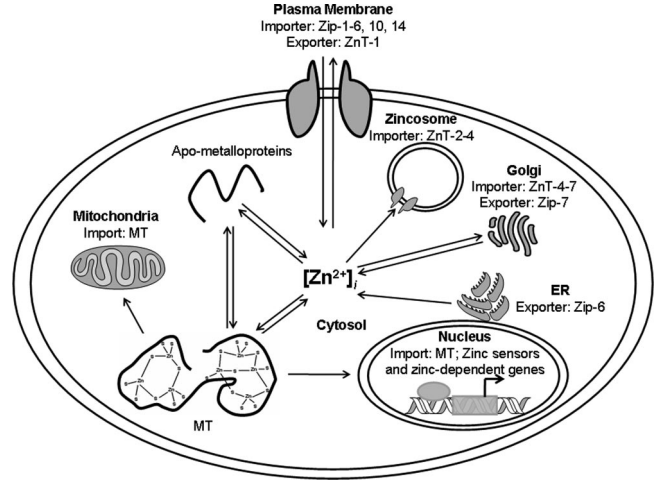

Figure 14. Zinc homeostasis and trafficking in a eukaryotic cell. Zinc import and export are controlled by plasma membrane transporters. In contrast to prokaryotic cells, a significant amount of zinc transport takes place between the cytosol and subcellular compartments. Metallothionein is the only molecule known to transport zinc into the mitochondrial intermembrane space and into the nucleus.

6. Chemical Biology of Zinc

In all of this work, zinc-dependent parameters were studied in isolated proteins. A cell, however, is a complex system with extensive spatial and temporal regulation and many interacting partners that affect the processes that are usually studied in isolation. Prokaryotic and eukaryotic cells differ primarily with regard to their subcellular organization of zinc metabolism (Figures 13 and 14). The properties of the isolated molecules may not always be sufficient to explain their behavior in the cell. Investigating cells with purely chemical approaches is an area that is now called chemical biology. In the remainder of this article, we will discuss this

approach because it provides critical information and the framework for function and purpose in the bioinorganic chemistry of zinc proteins.

To what extent thermodynamics and kinetics contribute to cellular zinc distribution is a central issue in zinc biology.[219] Differential stabilities of zinc complexes could provide a driving force for redistribution of zinc. Such a process would allow zinc transfer only in one direction, namely, from sites of lower to higher stability, unless, of course, there are significant changes in the concentrations of the binding partners. If an abundant zinc protein were to bind zinc as tightly as or even tighter than a less abundant one, the amount of protein available would dominate the hierarchy of distribution. Vice versa, if an abundant zinc protein were to bind zinc less tightly, zinc would dissociate. Metabolic energy would be wasted to assemble a protein that is not fully functional, because it relies on zinc as the limiting factor for its activity. Clearly, a distribution system based on thermodynamic gradients alone would be unsatisfactory and would challenge the capacity of cells to discriminate among competing metal ions.[15] As the selection of material in this article shows, chemical reactivity of sulfur donors and protein conformational changes that affect the properties of zinc sites provide pathways for kinetic control of zinc redistribution and regulation. A major factor in the interpretation of these processes is the availability of zinc ions in the cell.

The total cellular zinc concentration is in the range of a few hundred micromolar.[170] Most zinc is tightly bound, such that the steady-state concentration of free zinc ions is rather low. Estimates of free zinc ion concentrations in eukaryotic cells were made by various methods as early as 1971 and were in the range of hundreds of picomolar in rabbit skeletal muscle;[220] 24 pM in erythrocytes;[221] and 500 pM in neuroblastoma cells.[222,223] More recent estimates range from femtomolar in bacteria[224] to micromolar in eukaryotic cells.[225] The lowest estimates for eukaryotic cells are 5−10 pM for pheochromocytoma (PC12) cells.[198] In the presence of serum as an extracellular source of zinc ions, the free zinc ion concentrations of proliferating PC12 cells are between 0.7 and 1.4 nM.[226] A concentration of 1.07 nM was measured in primary cortical neurons.[227] Free zinc ion concentrations depend on the cellular zinc-buffering capacity, which affects their measurement with fluorescent chelating agents ("probes"). Introduction of a probe into a cell increases the buffering capacity and can result in underestimating free zinc ion concentrations. Measuring at different concentrations of the probe and then extrapolating to a zero concentration eliminates any contribution of the probe to zinc-buffering. Employing this procedure, human colon cancer (HT29) cells have a free zinc ion concentration of 614 pM (pZn = 9.2; pZn = −log [Zn^{2+}]).[170] This value is remarkably similar to the early estimates given above. Different slopes for the relationship between cellular zinc and probe concentrations in various states of the same cell, i.e., resting, proliferating, differentiated, and apoptotic, indicate different zinc-buffering capacities. The cellular cytosolic zinc-buffering capacity can be determined with chromophoric and fluorescent chelating agents that have relatively low affinity for zinc and, thus, little competition for cellular zinc-binding sites.[170] Zinc titrations in the presence of competing agents revealed 28 μM of tight zinc-binding ligands that are not saturated with zinc. Given a total cellular zinc concentration of 264 μM in HT-29 cells, the value of 28 μM corresponds to about 10% of unoccupied (surplus) sites. From the free zinc ion concentration of 614 pM, one calculates that these sites bind zinc with an average affinity of about 83 pM.[170] Thus, if one does not know the zinc-buffering capacity of a cell (defined as B_{Zn} = dcZn/dpZn) and it happens to be weak, free zinc ion concentrations determined at a single probe concentration can significantly differ from the true free zinc ion concentration. About 30% of the zinc-buffering capacity is due to thiols.[228]

Membrane transporters also participate in the availability of free zinc ions, controlling either zinc transport of zinc sequestered in subcellular vesicles or zinc transport through the cytoplasmic membrane. Such factors other than physicochemical buffering have been referred to as muffling.[229] Cation diffusion facilitators are critically involved in avoiding cytoplasmic zinc accumulation by either facilitating zinc efflux to the cell exterior or by sequestering zinc in intracellular organelles.[230] The mammalian forms of the CDF family are ZnTs. In humans, there are 14 ZIP (Zrt/Irt-like) proteins, designated SLC39A1-A14, which transport zinc into the cytosol, and 10 ZnT transporters, designated SLC30A1-A10, which generally transport zinc out of the cytosol.

How knowledge about cellular zinc metabolism and homeostasis bears on interpretations of the chemical properties of zinc proteins in terms of their functions will be discussed, using human MT as an example. Cellular free zinc ion concentrations are tightly regulated because free zinc ions are potent effectors of proteins. If cellular zinc ions increase above a certain threshold, zinc sensors, such as the metal response element (MRE)-binding transcription factor-1 (MTF-1), induce MT and other proteins as part of an adaptive response to zinc stress and its associated redox stress. MT can make zinc ions available to cytosolic zinc enzymes that typically have picomolar affinities for zinc ions.[30] Under more oxidizing conditions, MT can make zinc ions available to other sites with lower affinity for zinc.[30] Cellular zinc ions can fluctuate in a range between picomolar and low nanomolar concentrations,[231−233] exactly in the range where MT buffers chemically. These zinc ion fluctuations, so-called zinc potentials (pZn = −log[Zn^{2+}]), are thought to be utilized as zinc signals in cellular regulation. Zinc signals modulate the activity of zinc proteins and proteins that have not been recognized as zinc proteins,[168] such as zinc-inhibited enzymes and other processes. Effector roles of zinc in proteins other than zinc metalloproteins increase the functions of zinc beyond those in thousands of bona fide zinc proteins. Thus, using zinc as a third redox-inert metal ion allows the cell to regulate processes by extending the range, in which magnesium(II) (millimolar) and calcium(II) (micromolar) function, to nanomolar or even picomolar concentrations.[234]

7. Perspectives

In keeping with a classic citation from this journal,[235] "It is dangerous to speculate too far, but it is foolish not to speculate at all", we suppose some speculation should be at hand. We refer to the emerging field, in which association and dissociation of zinc is important for either modulation of protein function or redistribution of zinc, as **zinc kinesis** (the undirected movement of zinc) and **zinc taxis** (the directed movement of zinc). The chemistry of this field is in its infancy. For mobility of zinc in a protein structure, the structural elements that destabilize zinc sites are more important than the ones that stabilize them, which is, incidentally, the opposite way of how we think about the metal sites in metalloproteins. In the past, chemists searched

for ever stronger complexes, but with the knowledge presented in this article there is an opportunity to design complexes for the specific delivery of metal ions in many chemical and pharmaceutical applications.

Most of the terminology in the field of metal kinesis and taxis is borrowed from transportation and traffic. The terminology is often ill-defined. Terms, such as "metal receptor" or "metal transporter", need better definitions with regard to specific molecular functions. The term metallochaperone refers to the characteristics of proteins involved in copper metabolism.[236] Metallochaperones interact specifically with their targets, the apoproteins, and transfer metal ions by an associative mechanism. Following this definition, cytoplasmic zinc chaperones are unknown, though it is clear that cellular zinc concentrations are controlled tightly.[237] Zinc exchange between different MTs and between MT and other zinc finger proteins in an associative mechanism and ligand-substitution reactions has been observed, and it does not seem to involve recognition through complementary protein surfaces.[168,238] Thus, according to the above definition, MT is not a metallochaperone. Nevertheless it chaperones zinc in the cytosol and during the transport to mitochondria, to the nucleus, and into endosomal compartments when cells take up MT. For proteins, such as MT, some general terminology applies but it does not express the new functions that characterize the unique role of MT in zinc biology.

Specific protein dynamics and molecular mechanisms have evolved to transfer, redistribute, transport, and sense zinc ions. The conclusion from a review of the literature is that secondary interactions of amino acids with the metal ligands have a large influence on the functions and determine whether these functions require rigidity or flexibility of zinc coordination in the protein. The function of a zinc protein should not be inferred from the characteristics of inner coordination spheres alone, because zinc sites with identical donor sets have different functions. This property makes functional annotation of zinc proteins not as straightforward as it may seem.

In this article, we focused mainly on intracellular aspects of zinc/protein interactions. Future investigations will address intercellular zinc transfer and systemic control of zinc, which certainly demand additional and possibly different mechanisms of zinc proteins. The developmental biology of oogenesis and embryogenesis in the frog will serve as an example of how zinc is transported, stored, and redistributed.[239] During the maturation of the *Xenopus laevis* egg, the zinc content increases from 3 to 70 ng/oocyte. The protein vitellogenin transports zinc from the maternal liver through the blood to the oocyte. After uptake in the egg, vitellogenin is processed to lipovitellin, which is stored together with zinc in the yolk platelets. The fertilized egg is a closed system, in which 10% of the zinc is in the cytoplasm and 90% of the zinc is in the yolk sac, which is used for development only after the tadpole hatches.

The dynamic nature of developmental biology provides a fertile area for exploration of the inorganic biochemistry of zinc redistribution.

8. Abbreviations

MT	metallothionein
MTF-1	metal response element (MRE)-binding transcription factor-1
(HL)ADH	(horse liver) alcohol dehydrogenase
XPAzf	Xeroderma pigmentosa A zinc finger

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10. Note Added in Proof

The recent refinement of the structure of the E. coli YiiP transporter with a nominal resolution of 2.9 Å (Lu, M.; Chai, J.; Fu, D. *Nat. Struct. Mol. Biol.* **2009**, doi: 10.1038/nsmb.1662) illustrates some of the above principles that determine the dynamics of zinc sites in proteins. The investigation suggests a zinc-regulated zinc export mechanism for this dimeric protein from the cation diffusion facilitator (CDF) family.

The coordination of three principal zinc binding sites is defined. Site A is tetrahedral with three aspartates and one histidine. Zinc in site B is bound to two histidines, one aspartate, and one water molecule. Site C is a binuclear zinc site with one aspartate bridging the two zinc ions. Additionally, each zinc ion in site C is bound to two histidines, while one of them binds a water molecule.

A significant structural feature of site A in the transmembrane domain, the "active site" for transport, is that there are no secondary interactions, thus allowing rapid on- and off-rates. The ligands of the zinc ions in sites B and C in the cytoplasmic domain have hydrogen bonding interactions with the outer shell of amino acids. The function of site B is unknown. The function of site C is believed to be in sensing the cytoplasmic zinc ion concentrations, inducing a movement of interdomain transmembrane helices and thereby modulating the coordination environment of zinc in site A for transport. The autoregulatory mechanism makes YiiP an allosteric protein.

11. References

(1) Frausto da Silva, J. J. R.; Williams, R. J. P. *The Biological Chemistry of the Elements*; Clarendon Press: Oxford, U.K., 1991; p 299.
(2) Maret, W.; Vallee, B. L. *Methods Enzymol.* **1993**, *226*, 52.
(3) Fragai, M.; Luchinat, C.; Parigi, G. *Acc. Chem. Res.* **2006**, *39*, 909.
(4) Vallee, B. L.; Auld, D. S. *Biochemistry* **1990**, *29*, 5647.
(5) Andreini, C.; Banci, L.; Bertini, I.; Rosato, A. *J. Proteome Res.* **2006**, *5*, 3173.
(6) Andreini, C.; Banci, L.; Bertini, I.; Rosato, A. *J. Proteome Res.* **2006**, *5*, 196.
(7) Maret, W. *J. Anal. At. Spectrom.* **2004**, *19*, 15.
(8) Maret, W. *J.Trace Elem. Med. Biol.* **2005**, *19*, 7.
(9) van Roon, A. M.; Loening, N. M.; Obayashi, E.; Yang, J. C.; Newman, A. J.; Hernandez, H.; Nagai, K.; Neuhaus, D. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 9621.
(10) Cavet, J. S.; Borrelly, G. P.; Robinson, N. J. *FEMS Microbiol. Rev.* **2003**, *27*, 165.
(11) Tottey, S.; Waldron, K. J.; Firbank, S. J.; Reale, B.; Bessant, C.; Sato, K.; Cheek, T. R.; Gray, J.; Banfield, M. J.; Dennison, C.; Robinson, N. J. *Nature* **2008**, *455*, 1138.
(12) Maret, W. *Pure Appl. Chem.* **2008**, *80*, 2679.
(13) Karlin, S.; Zhu, Z. Y. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 14231.
(14) Tamames, B.; Sousa, S. F.; Tamames, J.; Fernandes, P. A.; Ramos, M. J. *Proteins* **2007**, *69*, 466.
(15) Maret, W. *Biochemistry* **2004**, *43*, 3301.
(16) Myers, L. C.; Terranova, M. P.; Ferentz, A. E.; Wagner, G.; Verdine, G. L. *Science* **1993**, *261*, 1164.
(17) Springman, E. B.; Angleton, E. L.; Birkedal-Hansen, H.; Van Wart, H. E. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 364.
(18) Vallee, B. L.; Galdes, A. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1984**, *56*, 283.

(19) Lipscomb, W. N.; Strater, N. *Chem. Rev.* **1996**, *96*, 2375.
(20) Parkin, G. *Chem. Rev.* **2004**, *104*, 699.
(21) Auld, D. S. *Encyclopedia of Inorganic Chemistry*; Wiley: Chichester, U.K., 2005; p 5884.
(22) Bertini, I.; Luchinat, C.; Maret, W.; Zeppezauer, M. *Zinc Enzymes*; Birkhauser: Boston, 1986.
(23) Miller, J.; McLachlan, A. D.; Klug, A. *EMBO J.* **1985**, *4*, 1609.
(24) Auld, D. S. *Handbook of Metalloproteins*; Wiley: Chichester, U.K., 2004; p 403.
(25) Kwon, K.; Cao, C.; Stivers, J. T. *J. Biol. Chem.* **2003**, *278*, 19442.
(26) Maret, W.; Jacob, C.; Vallee, B. L.; Fischer, E. H. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1936.
(27) Gazaryan, I. G.; Krasinskaya, I. P.; Kristal, B. S.; Brown, A. M. *J. Biol. Chem.* **2007**, *282*, 24373.
(28) Hogstrand, C.; Kille, P.; Nicholson, R. I.; Taylor, K. M. *Trends Mol. Med.* **2009**, *15*, 101.
(29) Paoletti, P.; Vergnano, A. M.; Barbour, B.; Casado, M. *Neuroscience* **2009**, *158*, 126.
(30) Krezel, A.; Maret, W. *J. Biol. Inorg. Chem.* **2008**, *13*, 401.
(31) Haase, H.; Maret, W. *Exp. Cell Res.* **2003**, *291*, 289.
(32) Costello, L. C.; Liu, Y.; Franklin, R. B.; Kennedy, M. C. *J. Biol. Chem.* **1997**, *272*, 28875.
(33) Frey, D.; Braun, O.; Briand, C.; Vasak, M.; Grutter, M. G. *Structure* **2006**, *14*, 901.
(34) Knipp, M.; Braun, O.; Gehrig, P. M.; Sack, R.; Vasak, M. *J. Biol. Chem.* **2003**, *278*, 3410.
(35) Larsen, K. S.; Auld, D. S. *Biochemistry* **1989**, *28*, 9620.
(36) Larsen, K. S.; Auld, D. S. *Biochemistry* **1991**, *30*, 2613.
(37) Gomez-Ortiz, M.; Gomis-Ruth, F. X.; Huber, R.; Aviles, F. X. *FEBS Lett.* **1997**, *400*, 336.
(38) Bukrinsky, J. T.; Bjerrum, M. J.; Kadziola, A. *Biochemistry* **1998**, *37*, 16555.
(39) Debela, M.; Magdolen, V.; Grimminger, V.; Sommerhoff, C.; Messerschmidt, A.; Huber, R.; Friedrich, R.; Bode, W.; Goettig, P. *J. Mol. Biol.* **2006**, *362*, 1094.
(40) Debela, M.; Goettig, P.; Magdolen, V.; Huber, R.; Schechter, N. M.; Bode, W. *J. Mol. Biol.* **2007**, *373*, 1017.
(41) Debela, M.; Hess, P.; Magdolen, V.; Schechter, N. M.; Steiner, T.; Huber, R.; Bode, W.; Goettig, P. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 16086.
(42) Ippolito, J. A.; Baird, T. T., Jr.; McGee, S. A.; Christianson, D. W.; Fierke, C. A. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 5017.
(43) Auld, D. S. *Biometals* **2001**, *14*, 271.
(44) Maret, W. *Handbook of Metalloproteins*; Wiley: Chichester, U.K., 2004; p 432.
(45) Kim, P. W.; Sun, Z. Y.; Blacklow, S. C.; Wagner, G.; Eck, M. J. *Science* **2003**, *301*, 1725.
(46) Romir, J.; Lilie, H.; Egerer-Sieber, C.; Bauer, F.; Sticht, H.; Muller, Y. A. *J. Mol. Biol.* **2007**, *365*, 1417.
(47) Solovyov, A.; Gilbert, H. F. *Protein Sci.* **2004**, *13*, 1902.
(48) Korndorfer, I. P.; Brueckner, F.; Skerra, A. *J. Mol. Biol.* **2007**, *370*, 887.
(49) Callaghan, A. J.; Redko, Y.; Murphy, L. M.; Grossmann, J. G.; Yates, D.; Garman, E.; Ilag, L. L.; Robinson, C. V.; Symmons, M. F.; McDowall, K. J.; Luisi, B. F. *Biochemistry* **2005**, *44*, 4667.
(50) Gundelfinger, E. D.; Boeckers, T. M.; Baron, M. K.; Bowie, J. U. *Trends Biochem. Sci.* **2006**, *31*, 366.
(51) Conrady, D. G.; Brescia, C. C.; Horii, K.; Weiss, A. A.; Hassett, D. J.; Herr, A. B. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 19456.
(52) Dudev, T.; Lin, Y. L.; Dudev, M.; Lim, C. *J. Am. Chem. Soc.* **2003**, *125*, 3168.
(53) Vallee, B. L.; Williams, R. J. *Proc. Natl. Acad. Sci. U.S.A.* **1968**, *59*, 498.
(54) Williams, R. J. *Eur. J. Biochem.* **1995**, *234*, 363.
(55) Reddi, A. R.; Gibney, B. R. *Biochemistry* **2007**, *46*, 3745.
(56) Petros, A. K.; Reddi, A. R.; Kennedy, M. L.; Hyslop, A. G.; Gibney, B. R. *Inorg. Chem.* **2006**, *45*, 9941.
(57) Bal, W.; Schwerdtle, T.; Hartwig, A. *Chem. Res. Toxicol.* **2003**, *16*, 242.
(58) Reddi, A. R.; Guzman, T. R.; Breece, R. M.; Tierney, D. L.; Gibney, B. R. *J. Am. Chem. Soc.* **2007**, *129*, 12815.
(59) Witkiewicz-Kucharczyk, A.; Bal, W. *Toxicol. Lett.* **2006**, *162*, 29.
(60) Arseniev, A.; Schultze, P.; Worgotter, E.; Braun, W.; Wagner, G.; Vasak, M.; Kagi, J. H.; Wuthrich, K. *J. Mol. Biol.* **1988**, *201*, 637.
(61) Robbins, A. H.; McRee, D. E.; Williamson, M.; Collett, S. A.; Xuong, N. H.; Furey, W. F.; Wang, B. C.; Stout, C. D. *J. Mol. Biol.* **1991**, *221*, 1269.
(62) Zhang, X.; Tamaru, H.; Khan, S. I.; Horton, J. R.; Keefe, L. J.; Selker, E. U.; Cheng, X. *Cell* **2002**, *111*, 117.
(63) Min, J.; Zhang, X.; Cheng, X.; Grewal, S. I.; Xu, R. M. *Nat. Struct. Biol.* **2002**, *9*, 828.
(64) Krezel, A.; Maret, W. *J. Am. Chem. Soc.* **2007**, *129*, 10911.
(65) Yang, Y.; Maret, W.; Vallee, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 5556.
(66) Krezel, A.; Maret, W. *Biochem. J.* **2007**, *402*, 551.
(67) Vazquez, F.; Vasak, M. *Biochem. J.* **1988**, *253*, 611.
(68) Vasak, M.; Kagi, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 6709.
(69) Cox, E. H.; McLendon, G. L. *Curr. Opin. Chem. Biol.* **2000**, *4*, 162.
(70) Wommer, S.; Rival, S.; Heinz, U.; Galleni, M.; Frere, J. M.; Franceschini, N.; Amicosante, G.; Rasmussen, B.; Bauer, R.; Adolph, H. W. *J. Biol. Chem.* **2002**, *277*, 24142.
(71) de Seny, D.; Heinz, U.; Wommer, S.; Kiefer, M.; Meyer-Klaucke, W.; Galleni, M.; Frere, J. M.; Bauer, R.; Adolph, H. W. *J. Biol. Chem.* **2001**, *276*, 45065.
(72) Heinz, U.; Kiefer, M.; Tholey, A.; Adolph, H. W. *J. Biol. Chem.* **2005**, *280*, 3197.
(73) Jacob, C.; Maret, W.; Vallee, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3489.
(74) Chong, C. R.; Auld, D. S. *Biochemistry* **2000**, *39*, 7580.
(75) Chong, C. R.; Auld, D. S. *J. Med. Chem.* **2007**, *50*, 5524.
(76) Lindskog, S.; Malmstrom, B. G. *J. Biol. Chem.* **1962**, *237*, 1129.
(77) Nelbach, M. E.; Pigiet, V. P., Jr.; Gerhart, J. C.; Schachman, H. K. *Biochemistry* **1972**, *11*, 315.
(78) Valentine, J. S.; Pantoliano, N. W. *Copper Proteins*; Wiley: New York, 1981; p 291.
(79) Maret, W. *Antioxid. Redox Signal.* **2006**, *8*, 1419.
(80) Maret, W.; Vallee, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3478.
(81) Giles, G. I.; Tasker, K. M.; Collins, C.; Giles, N. M.; O'Rourke, E.; Jacob, C. *Biochem. J.* **2002**, *364*, 579.
(82) Alves de Sousa, R.; Galardon, E.; Rat, M.; Giorgi, M.; Artaud, I. *J. Inorg. Biochem.* **2005**, *99*, 690.
(83) Garcia-Saez, I.; Docquier, J. D.; Rossolini, G. M.; Dideberg, O. *J. Mol. Biol.* **2008**, *375*, 604.
(84) Jacob, C.; Maret, W.; Vallee, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1910.
(85) Chen, Y.; Maret, W. *Eur. J. Biochem.* **2001**, *268*, 3346.
(86) Chen, Y.; Maret, W. *Antioxid. Redox Signal.* **2001**, *3*, 651.
(87) Kroncke, K. D.; Fehsel, K.; Schmidt, T.; Zenke, F. T.; Dasting, I.; Wesener, J. R.; Bettermann, H.; Breunig, K. D.; Kolb-Bachofen, V. *Biochem. Biophys. Res. Commun.* **1994**, *200*, 1105.
(88) Bernal, P. J.; Leelavanichkul, K.; Bauer, E.; Cao, R.; Wilson, A.; Wasserloos, K. J.; Watkins, S. C.; Pitt, B. R.; St Croix, C. M. *Circ. Res.* **2008**, *102*, 1575.
(89) Chen, Y.; Irie, Y.; Keung, W. M.; Maret, W. *Biochemistry* **2002**, *41*, 8360.
(90) Smirnova, J.; Zhukova, L.; Witkiewicz-Kucharczyk, A.; Kopera, E.; Oledzki, J.; Wyslouch-Cieszynska, A.; Palumaa, P.; Hartwig, A.; Bal, W. *Chem. Res. Toxicol.* **2008**, *21*, 386.
(91) Wang, G.; Strang, C.; Pfaffinger, P. J.; Covarrubias, M. *J. Biol. Chem.* **2007**, *282*, 13637.
(92) Levina, A.; Bailey, A. M.; Champion, G.; Lay, P. A. *J. Am. Chem. Soc.* **2000**, *122*, 6208.
(93) Schwerdtle, T.; Walter, I.; Hartwig, A. *DNA Repair (Amst)* **2003**, *2*, 1449.
(94) Piatek, K.; Schwerdtle, T.; Hartwig, A.; Bal, W. *Chem. Res. Toxicol.* **2008**, *21*, 600.
(95) Lee, S. H.; Maret, W. *Antioxid. Redox Signal.* **2001**, *3*, 531.
(96) Koehler, C. M. *Trends Biochem. Sci.* **2004**, *29*, 1.
(97) Ivanova, E.; Ball, M.; Lu, H. *Proteins* **2008**, *71*, 467.
(98) Mesecke, N.; Bihlmaier, K.; Grumbt, B.; Longen, S.; Terziyska, N.; Hell, K.; Herrmann, J. M. *EMBO Rep.* **2008**, *9*, 1107.
(99) Morgan, B.; Ang, S. K.; Yan, G.; Lu, H. *J. Biol. Chem.* **2009**, *284*, 6818.
(100) Daithankar, V. N.; Farrell, S.; Thorpe, C. *Biochemistry* **2009**, *48*, 4828.
(101) Hao, Q.; Maret, W. *FEBS J.* **2006**, *273*, 4300.
(102) Wilker, J. J.; Lippard, S. J. *J. Am. Chem. Soc.* **1995**, *117*, 8682.
(103) Bombarda, E.; Cherradi, H.; Morellet, N.; Roques, B. P.; Mely, Y. *Biochemistry* **2002**, *41*, 4312.
(104) Chiou, S. J.; Riordan, C. G.; Rheingold, A. L. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3695.
(105) Picot, D.; Ohanessian, G.; Frison, G. *Inorg. Chem.* **2008**, *47*, 8167.
(106) Smith, J. N.; Shirin, Z.; Carrano, C. J. *J. Am. Chem. Soc.* **2003**, *125*, 868.
(107) Hightower, K. E.; Fierke, C. A. *Curr. Opin. Chem. Biol.* **1999**, *3*, 176.
(108) Dauter, Z.; Wilson, K. S.; Sieker, L. C.; Moulis, J. M.; Meyer, J. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8836.
(109) Simonson, T.; Calimet, N. *Proteins* **2002**, *49*, 37.
(110) Fabris, D.; Zaia, J.; Hathout, Y.; Fenselau, C. *J. Am. Chem. Soc.* **1996**, *118*, 12242.
(111) Maynard, A. T.; Covell, D. G. *J. Am. Chem. Soc.* **2001**, *123*, 1047.
(112) Kuchar, J.; Hausinger, R. P. *Chem. Rev.* **2004**, *104*, 509.
(113) Thompson, R. B. *Curr. Opin. Chem. Biol.* **2005**, *9*, 526.

(114) Jiang, L. J.; Maret, W.; Vallee, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3483.
(115) Schneider, G.; Eklund, H.; Cedergren-Zeppezauer, E.; Zeppezauer, M. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5289.
(116) Rees, D. C.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 7151.
(117) Hakansson, K.; Carlsson, M.; Svensson, L. A.; Liljas, A. *J. Mol. Biol.* **1992**, *227*, 1192.
(118) Gomis-Ruth, F. X.; Grams, F.; Yiallouros, I.; Nar, H.; Kusthardt, U.; Zwilling, R.; Bode, W.; Stocker, W. *J. Biol. Chem.* **1994**, *269*, 17111.
(119) Whittaker, M. M.; Whittaker, J. W. *Biochemistry* **2008**, *47*, 11625.
(120) Selevsek, N.; Rival, S.; Tholey, A.; Heinzle, E.; Heinz, U.; Hemmingsen, L.; Adolph, H. W. *J. Biol. Chem.* **2009**, *284*, 16419.
(121) Heinz, U.; Hemmingsen, L.; Kiefer, M.; Adolph, H. W. *Chem.—Eur. J.* **2009**, *15*, 7350.
(122) Thickman, K. R.; Davis, A.; Berg, J. M. *Inorg. Chem.* **2004**, *43*, 7897.
(123) Roehm, P. C.; Berg, J. M. *Biochemistry* **1997**, *36*, 10240.
(124) Wang, Z.; Feng, L. S.; Matskevich, V.; Venkataraman, K.; Parasuram, P.; Laity, J. H. *J. Mol. Biol.* **2006**, *357*, 1167.
(125) Miseta, A.; Csutora, P. *Mol. Biol. Evol.* **2000**, *17*, 1232.
(126) Butler, J. S.; Loh, S. N. *Biochemistry* **2007**, *46*, 2630.
(127) Meplan, C.; Richard, M. J.; Hainaut, P. *Oncogene* **2000**, *19*, 5227.
(128) Ostrakhovitch, E. A.; Olsson, P. E.; Jiang, S.; Cherian, M. G. *FEBS Lett.* **2006**, *580*, 1235.
(129) Dobson, C. M. *Nat. Rev. Drug Discovery* **2003**, *2*, 154.
(130) Banci, L.; Bertini, I.; Durazo, A.; Girotto, S.; Gralla, E. B.; Martinelli, M.; Valentine, J. S.; Vieru, M.; Whitelegge, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 11263.
(131) Noy, D.; Solomonov, I.; Sinkevich, O.; Arad, T.; Kjaer, K.; Sagi, I. *J. Am. Chem. Soc.* **2008**, *130*, 1376.
(132) Wilkinson-White, L. E.; Easterbrook-Smith, S. B. *Biochemistry* **2007**, *46*, 9123.
(133) Brown, D. R. *Dalton Trans.* **2009**, 4069.
(134) Villanueva, J.; Hoshino, M.; Katou, H.; Kardos, J.; Hasegawa, K.; Naiki, H.; Goto, Y. *Protein Sci.* **2004**, *13*, 797.
(135) Eklund, H.; Samama, J. P.; Jones, T. A. *Biochemistry* **1984**, *23*, 5982.
(136) Maret, W.; Andersson, I.; Dietrich, H.; Schneider-Bernlohr, H.; Einarsson, R.; Zeppezauer, M. *Eur. J. Biochem.* **1979**, *98*, 501.
(137) Dietrich, H.; Maret, W.; Wallen, L.; Zeppezauer, M. *Eur. J. Biochem.* **1979**, *100*, 267.
(138) Maret, W.; Zeppezauer, M. *Biochemistry* **1986**, *25*, 1584.
(139) Maret, W.; Makinen, M. W. *J. Biol. Chem.* **1991**, *266*, 20636.
(140) Makinen, M. W.; Maret, W.; Yim, M. B. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 2584.
(141) Meijers, R.; Cedergren-Zeppezauer, E. S. *Handbook of Metalloproteins*; Wiley: Chichester, U.K., 2004; p 5.
(142) Meijers, R.; Morris, R. J.; Adolph, H. W.; Merli, A.; Lamzin, V. S.; Cedergren-Zeppezauer, E. S. *J. Biol. Chem.* **2001**, *276*, 9316.
(143) Sanghani, P. C.; Robinson, H.; Bosron, W. F.; Hurley, T. D. *Biochemistry* **2002**, *41*, 10778.
(144) Sanghani, P. C.; Bosron, W. F.; Hurley, T. D. *Biochemistry* **2002**, *41*, 15189.
(145) Ryde, U. *Protein Sci.* **1995**, *4*, 1124.
(146) Hemmingsen, L.; Bauer, R.; Bjerrum, M. J.; Zeppezauer, M.; Adolph, H. W.; Formicka, G.; Cedergren-Zeppezauer, E. *Biochemistry* **1995**, *34*, 7145.
(147) Eklund, H.; Horjales, E.; Jornvall, H.; Branden, C. I.; Jeffery, J. *Biochemistry* **1985**, *24*, 8005.
(148) Maret, W.; Auld, D. S. *Biochemistry* **1988**, *27*, 1622.
(149) Maret, W. *Biochemistry* **1989**, *28*, 9944.
(150) Pauly, T. A.; Ekstrom, J. L.; Beebe, D. A.; Chrunyk, B.; Cunningham, D.; Griffor, M.; Kamath, A.; Lee, S. E.; Madura, R.; Mcguire, D.; Subashi, T.; Wasilko, D.; Watts, P.; Mylari, B. L.; Oates, P. J.; Adams, P. D.; Rath, V. L. *Structure* **2003**, *11*, 1071.
(151) Baker, P. J.; Britton, K. L.; Fisher, M.; Esclapez, J.; Pire, C.; Bonete, M. J.; Ferrer, J.; Rice, D. W. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 779.
(152) Rosenblum, G.; Meroueh, S.; Toth, M.; Fisher, J. F.; Fridman, R.; Mobashery, S.; Sagi, I. *J. Am. Chem. Soc.* **2007**, *129*, 13566.
(153) Sousa, S. F.; Fernandes, P. A.; Ramos, M. J. *J. Am. Chem. Soc.* **2007**, *129*, 1378.
(154) Koutmos, M.; Pejchal, R.; Bomer, T. M.; Matthews, R. G.; Smith, J. L.; Ludwig, M. L. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 3286.
(155) Xiang, S.; Short, S. A.; Wolfenden, R.; Carter, C. W., Jr. *Biochemistry* **1996**, *35*, 1335.
(156) Akabayov, B.; Lee, S. J.; Akabayov, S. R.; Rekhi, S.; Zhu, B.; Richardson, C. C. *Biochemistry* **2009**, *48*, 1763.
(157) Andersson, I.; Maret, W.; Zeppezauer, M.; Brown, R. D., III; Koenig, S. H. *Biochemistry* **1981**, *20*, 3433.
(158) Schneider, G.; Zeppezauer, M. *J. Inorg. Biochem.* **1983**, *18*, 59.
(159) Andersson, I.; Bauer, R.; Demeter, I. *Inorg. Chim. Acta* **1982**, *67*, 53.
(160) Martin, R. B. *J. Chem. Educ.* **1987**, *64*, 402.
(161) Hemmingsen, L.; Damblon, C.; Antony, J.; Jensen, M.; Adolph, H. W.; Wommer, S.; Roberts, G. C.; Bauer, R. *J. Am. Chem. Soc.* **2001**, *123*, 10329.
(162) Sawada, N.; Nagahara, N.; Sakai, T.; Nakajima, Y.; Minami, M.; Kawada, T. *J. Biol. Inorg. Chem.* **2005**, *10*, 199.
(163) Leszczyszyn, O. I.; Schmid, R.; Blindauer, C. A. *Proteins* **2007**, *68*, 922.
(164) Margoshes, M.; Vallee, B. L. *J. Am. Chem. Soc.* **1957**, *79*, 4813.
(165) Kagi, J. H.; Kojima, Y. *Exp. Suppl.* **1987**, *52*, 25.
(166) Feng, W.; Benz, F. W.; Cai, J.; Pierce, W. M.; Kang, Y. J. *J. Biol. Chem.* **2006**, *281*, 681.
(167) Nettesheim, D. G.; Engeseth, H. R.; Otvos, J. D. *Biochemistry* **1985**, *24*, 6744.
(168) Maret, W.; Larsen, K. S.; Vallee, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2233.
(169) Li, T. Y.; Kraker, A. J.; Shaw, C. F., III; Petering, D. H. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 6334.
(170) Krezel, A.; Maret, W. *J. Biol. Inorg. Chem.* **2006**, *11*, 1049.
(171) Li, Y.; Maret, W. *J. Anal. At. Spectrom.* **2008**, *23*, 1055.
(172) Hao, Q.; Hong, S. H.; Maret, W. *J. Cell. Physiol.* **2007**, *210*, 428.
(173) Chung, R. S.; Penkowa, M.; Dittmann, J.; King, C. E.; Bartlett, C.; Asmussen, J. W.; Hidalgo, J.; Carrasco, J.; Leung, Y. K.; Walker, A. K.; Fung, S. J.; Dunlop, S. A.; Fitzgerald, M.; Beazley, L. D.; Chuah, M. I.; Vickers, J. C.; West, A. K. *J. Biol. Chem.* **2008**, *283*, 15349.
(174) Stewart, A. J.; Blindauer, C. A.; Berezenko, S.; Sleep, D.; Sadler, P. J. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3701.
(175) Churchich, J. E.; Scholz, G.; Kwok, F. *Biochim. Biophys. Acta* **1989**, *996*, 181.
(176) Radtke, F.; Heuchel, R.; Georgiev, O.; Hergersberg, M.; Gariglio, M.; Dembic, Z.; Schaffner, W. *EMBO J.* **1993**, *12*, 1355.
(177) Zhao, H.; Eide, D. J. *Mol. Cell. Biol.* **1997**, *17*, 5044.
(178) Zhao, H.; Butler, E.; Rodgers, J.; Spizzo, T.; Duesterhoeft, S.; Eide, D. *J. Biol. Chem.* **1998**, *273*, 28713.
(179) Bird, A. J.; Zhao, H.; Luo, H.; Jensen, L. T.; Srinivasan, C.; Evans-Galea, M.; Winge, D. R.; Eide, D. J. *EMBO J.* **2000**, *19*, 3704.
(180) Bird, A.; Evans-Galea, M. V.; Blankman, E.; Zhao, H.; Luo, H.; Winge, D. R.; Eide, D. J. *J. Biol. Chem.* **2000**, *275*, 16160.
(181) Giedroc, D. P.; Arunkumar, A. I. *Dalton Trans.* **2007**, 3107.
(182) Changela, A.; Chen, K.; Xue, Y.; Holschen, J.; Outten, C. E.; O'Halloran, T. V.; Mondragon, A. *Science* **2003**, *301*, 1383.
(183) Outten, C. E.; Outten, F. W.; O'Halloran, T. V. *J. Biol. Chem.* **1999**, *274*, 37517.
(184) Ansari, A. Z.; Bradner, J. E.; O'Halloran, T. V. *Nature* **1995**, *374*, 371.
(185) Patzer, S. I.; Hantke, K. *J. Biol. Chem.* **2000**, *275*, 24321.
(186) Outten, C. E.; Tobin, D. A.; Penner-Hahn, J. E.; O'Halloran, T. V. *Biochemistry* **2001**, *40*, 10417.
(187) Lucarelli, D.; Russo, S.; Garman, E.; Milano, A.; Meyer-Klaucke, W.; Pohl, E. *J. Biol. Chem.* **2007**, *282*, 9914.
(188) Robinson, N. J.; Gupta, A.; Fordham-Skelton, A. P.; Croy, R. R.; Whitton, B. A.; Huckle, J. W. *Proc. Biol. Sci.* **1990**, *242*, 241.
(189) Blindauer, C. A.; Harrison, M. D.; Parkinson, J. A.; Robinson, A. K.; Cavet, J. S.; Robinson, N. J.; Sadler, P. J. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 9593.
(190) Cook, W. J.; Kar, S. R.; Taylor, K. B.; Hall, L. M. *J. Mol. Biol.* **1998**, *275*, 337.
(191) VanZile, M. L.; Chen, X.; Giedroc, D. P. *Biochemistry* **2002**, *41*, 9765.
(192) VanZile, M. L.; Chen, X.; Giedroc, D. P. *Biochemistry* **2002**, *41*, 9776.
(193) Eicken, C.; Pennella, M. A.; Chen, X.; Koshlap, K. M.; VanZile, M. L.; Sacchettini, J. C.; Giedroc, D. P. *J. Mol. Biol.* **2003**, *333*, 683.
(194) Chen, X.; Agarwal, A.; Giedroc, D. P. *Biochemistry* **1998**, *37*, 11152.
(195) Chen, X.; Chu, M.; Giedroc, D. P. *Biochemistry* **1999**, *38*, 12915.
(196) Bittel, D. C.; Smirnova, I. V.; Andrews, G. K. *J. Biol. Chem.* **2000**, *275*, 37194.
(197) Chen, X.; Zhang, B.; Harmon, P. M.; Schaffner, W.; Peterson, D. O.; Giedroc, D. P. *J. Biol. Chem.* **2004**, *279*, 4515.
(198) Bozym, R. A.; Thompson, R. B.; Stoddard, A. K.; Fierke, C. A. *ACS Chem. Biol.* **2006**, *1*, 103.
(199) Zhang, B.; Georgiev, O.; Hagmann, M.; Gunes, C.; Cramer, M.; Faller, P.; Vasak, M.; Schaffner, W. *Mol. Cell. Biol.* **2003**, *23*, 8471.
(200) Bird, A. J.; McCall, K.; Kramer, M.; Blankman, E.; Winge, D. R.; Eide, D. J. *EMBO J.* **2003**, *22*, 5137.
(201) Pavletich, N. P.; Pabo, C. O. *Science* **1993**, *261*, 1701.
(202) Bird, A. J.; Swierczek, S.; Qiao, W.; Eide, D. J.; Winge, D. R. *J. Biol. Chem.* **2006**, *281*, 25326.

(203) Blencowe, D. K.; Morby, A. P. *FEMS Microbiol. Rev.* **2003**, *27*, 291.
(204) Hantke, K. *Biometals* **2001**, *14*, 239.
(205) Banci, L.; Bertini, I.; Ciofi-Baffoni, S.; Finney, L. A.; Outten, C. E.; O'Halloran, T. V. *J. Mol. Biol.* **2002**, *323*, 883.
(206) Mitra, B.; Sharma, R. *Biochemistry* **2001**, *40*, 7694.
(207) Liu, J.; Dutta, S. J.; Stemmler, A. J.; Mitra, B. *Biochemistry* **2006**, *45*, 763.
(208) Dutta, S. J.; Liu, J.; Hou, Z.; Mitra, B. *Biochemistry* **2006**, *45*, 5923.
(209) Liu, T.; Reyes-Caballero, H.; Li, C.; Scott, R. A.; Giedroc, D. P. *Biochemistry* **2007**, *46*, 11057.
(210) Wei, Y.; Li, H.; Fu, D. *J. Biol. Chem.* **2004**, *279*, 39251.
(211) Wei, Y.; Fu, D. *J. Biol. Chem.* **2005**, *280*, 33716.
(212) Wei, Y.; Fu, D. *J. Biol. Chem.* **2006**, *281*, 23492.
(213) Lu, M.; Fu, D. *Science* **2007**, *317*, 1746.
(214) Cherezov, V.; Hofer, N.; Szebenyi, D. M.; Kolaj, O.; Wall, J. G.; Gillilan, R.; Srinivasan, V.; Jaroniec, C. P.; Caffrey, M. *Structure* **2008**, *16*, 1378.
(215) Chandra, B. R.; Yogavel, M.; Sharma, A. *J. Mol. Biol.* **2007**, *367*, 970.
(216) Yatsunyk, L. A.; Easton, J. A.; Kim, L. R.; Sugarbaker, S. A.; Bennett, B.; Breece, R. M.; Vorontsov, I. I.; Tierney, D. L.; Crowder, M. W.; Rosenzweig, A. C. *J. Biol. Inorg. Chem.* **2008**, *13*, 271.
(217) Wei, B.; Randich, A. M.; Bhattacharyya-Pakrasi, M.; Pakrasi, H. B.; Smith, T. J. *Biochemistry* **2007**, *46*, 8734.
(218) Banerjee, S.; Wei, B.; Bhattacharyya-Pakrasi, M.; Pakrasi, H. B.; Smith, T. J. *J. Mol. Biol.* **2003**, *333*, 1061.
(219) O'Halloran, T. V. *Science* **1993**, *261*, 715.
(220) Peck, E. J., Jr.; Ray, W. J., Jr. *J. Biol. Chem.* **1971**, *246*, 1160.
(221) Simons, T. J. *J. Membr. Biol.* **1991**, *123*, 63.
(222) Adebodun, F.; Post, J. F. *J. Cell. Physiol.* **1995**, *163*, 80.
(223) Benters, J.; Flogel, U.; Schafer, T.; Leibfritz, D.; Hechtenberg, S.; Beyersmann, D. *Biochem. J.* **1997**, *322*, 793.
(224) Outten, C. E.; O'Halloran, T. V. *Science* **2001**, *292*, 2488.
(225) Brand, I. A.; Kleineke, J. *J. Biol. Chem.* **1996**, *271*, 1941.
(226) Li, Y.; Maret, W. *Exp. Cell Res.* **2009**, *315*, 2463.
(227) Colvin, R. A.; Bush, A. I.; Volitakis, I.; Fontaine, C. P.; Thomas, D.; Kikuchi, K.; Holmes, W. R. *Am. J. Physiol. Cell Physiol.* **2008**, *294*, C726.
(228) Krezel, A.; Hao, Q.; Maret, W. *Arch. Biochem. Biophys.* **2007**, *463*, 188.
(229) Thomas, R. C.; Coles, J. A.; Deitmer, J. W. *Nature* **1991**, *350*, 564.
(230) Palmiter, R. D.; Findley, S. D. *EMBO J.* **1995**, *14*, 639.
(231) Atar, D.; Backx, P. H.; Appel, M. M.; Gao, W. D.; Marban, E. *J. Biol. Chem.* **1995**, *270*, 2473.
(232) Smith, P. J.; Wiltshire, M.; Davies, S.; Chin, S. F.; Campbell, A. K.; Errington, R. J. *Am. J. Physiol. Cell Physiol.* **2002**, *283*, C609.
(233) Turan, B.; Fliss, H.; Desilets, M. *Am. J. Physiol.* **1997**, *272*, H2095.
(234) Maret, W. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 12325.
(235) Nord, F. F. *Chem. Rev.* **1940**, *26*, 423.
(236) O'Halloran, T. V.; Culotta, V. C. *J. Biol. Chem.* **2000**, *275*, 25057.
(237) Nies, D. H. *Science* **2007**, *317*, 1695.
(238) Otvos, J. D.; Liu, X.; Li, H.; Shen, G.; Basti, M. *Metallothionein III: Biological roles and medical implications*; Birkhauser: Basel, Switzerland, 2008; p 57.
(239) Falchuk, K. H.; Montorzi, M. *Biometals* **2001**, *14*, 385.
(240) Dunn, M. F. *Biometals* **2005**, *18*, 295.
(241) Pai, M. T.; Tzeng, S. R.; Kovacs, J. J.; Keaton, M. A.; Li, S. S.; Yao, T. P.; Zhou, P. *J. Mol. Biol.* **2007**, *370*, 290.
(242) Lee, B. M.; Buck-Koehntop, B. A.; Martinez-Yamout, M. A.; Dyson, H. J.; Wright, P. E. *J. Mol. Biol.* **2007**, *371*, 1274.
(243) Cohen, S. R.; Wilson, I. B. *Biochemistry* **1966**, *5*, 904.
(244) Baker, J. O.; Prescott, J. M. *Biochem. Biophys. Res. Commun.* **1985**, *130*, 1154.
(245) Kleemann, S. G.; Keung, W. M.; Riordan, J. F. *J. Inorg. Biochem.* **1986**, *26*, 93–106.
(246) Coleman, J. E.; Vallee, B. L. *J. Biol. Chem.* **1961**, *236*, 2244.
(247) Hirose, J.; Iwamoto, H.; Nagao, I.; Enmyo, K.; Sugao, H.; Kanemitu, N.; Ikeda, K.; Takeda, M.; Inoue, M.; Ikeda, T.; Matsuura, F.; Fukasawa, K. M.; Fukasawa, K. *Biochemistry* **2001**, *40*, 11860.
(248) Sellin, S.; Mannervik, B. *J. Biol. Chem.* **1984**, *259*, 11426.
(249) Lasch, J. *Ophthalmic Res.* **1979**, *11*, 373.
(250) Ray, W. J., Jr. *J. Biol. Chem.* **1967**, *242*, 3737.
(251) Simons, T. J. *Eur. J. Biochem.* **1995**, *234*, 178.
(252) Day, E. S.; Wen, D.; Garber, E. A.; Hong, J.; Avedissian, L. S.; Rayhorn, P.; Shen, W.; Zeng, C.; Bailey, V. R.; Reilly, J. O.; Roden, J. A.; Moore, C. B.; Williams, K. P.; Galdes, A.; Whitty, A.; Baker, D. P. *Biochemistry* **1999**, *38*, 14868.
(253) Voordouw, G.; Milo, C.; Roche, R. S. *Anal. Biochem.* **1976**, *70*, 313.
(254) Feder, J.; Garrett, L. R.; Kochavi, D. *Biochim. Biophys. Acta* **1971**, *235*, 370.
(255) Hunt, J. A.; Ahmed, M.; Fierke, C. A. *Biochemistry* **1999**, *38*, 9054.
(256) Hunt, J. A.; Fierke, C. A. *J. Biol. Chem.* **1997**, *272*, 20364.
(257) Kiefer, L. L.; Fierke, C. A. *Biochemistry* **1994**, *33*, 15233.
(258) Lesburg, C. A.; Huang, C.; Christianson, D. W.; Fierke, C. A. *Biochemistry* **1997**, *36*, 15780.
(259) Kiefer, L. L.; Krebs, J. F.; Paterno, S. A.; Fierke, C. A. *Biochemistry* **1993**, *32*, 9896.
(260) Kiefer, L. L.; Paterno, S. A.; Fierke, C. A. *J. Am. Chem. Soc.* **1995**, *117*, 6831.
(261) Huang, C. C.; Lesburg, C. A.; Kiefer, L. L.; Fierke, C. A.; Christianson, D. W. *Biochemistry* **1996**, *35*, 3439.

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