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Metals on the move: zinc ions in cellular regulation and in the coordination dynamics of zinc proteins

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Abstract Homeostatic control maintains essential transition metal ions at characteristic cellular concentrations to support their physiological functions and to avoid adverse effects. Zinc is especially widely used as a catalytic or structural cofactor in about 3000 human zinc proteins. In addition, the homeostatic control of zinc in eukaryotic cells permits functions of zinc(II) ions in regulation and in paracrine and intracrine signaling. Zinc ions are released from proteins through ligand-centered reactions in zinc/thiolate coordination environments, and from stores in cellular organelles, where zinc transporters participate in zinc loading and release. Muffling reactions allow zinc ions to serve as signaling ions (second messengers) in the cytosol that is buffered to picomolar zinc ion concentrations at steady-state. Muffling includes zinc ion binding to metallothioneins, cellular translocations of metallothioneins, delivery of zinc ions to transporter proteins, and zinc ion fluxes through cellular membranes with the result of removing the additional zinc ions from the cytosol and restoring the steady-state. Targets of regulatory zinc ions are proteins with sites for transient zinc binding, such as membrane receptors, enzymes, protein–protein interactions, and sensor proteins that control gene expression. The generation, transmission, targets, and termination of zinc ion signals involve proteins that use coordination dynamics in the inner and outer ligand spheres to control metal ion association and dissociation. These new findings establish critically important functions of zinc ions and zinc metalloproteins in cellular control.

Keywords Zinc · Signaling · Metallothionein · Muffling · Buffering

Abbreviations

ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
IP_3	Inositol 1,4,5-trisphosphate
MT	Metallothionein
MTF-1	Metal-response element (MRE)-binding transcription factor-1
ZnT	Zinc transporter

Introduction

Specific proteins control the cellular availability and re-distribution of the essential transition metal ions. They supply metalloproteins with metals, scavenge an excess of metal ions to avoid deleterious chemical reactions of the free metal ions, safeguard metal ions during their transit trough membranes (metal transporters) and cellular compartments (metallochaperones), store

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and release the metal ions (metal-storage proteins), or sense whether or not metal concentrations are adequate (metal-sensor proteins). Overall they achieve homeostatic control of transition metals, such as manganese, iron, copper, and zinc, by limiting the concentration ranges of total metal and free metal ions. This control enables metal-specific reactions while preventing coordination of the wrong metal to a metalloprotein. Yet steady-state concentrations of metal ions and their affinities for proteins are not the only aspect of cellular control. If metal affinities were the only criterion, metal ions could move only in one direction determined by the thermodynamic gradient. By restricting availability through compartmentalization and "by controlling the relative availabilities of metals, cells overcome otherwise inadequate metal-protein affinities" and determine when and where proteins and metal ions meet (Waldron and Robinson 2009). During metal re-distribution, metal ions are transferred from one protein to another. The transfer requires transient binding in dynamic coordination environments, in which protein conformational changes modulate the kinetics of metal association and dissociation (Maret and Li 2009). The biological mechanisms of metal control and the availability of metals from the environment are often neglected when investigating metalloproteins in vitro. As a result, it is not infrequent that metalloproteins are investigated with the wrong metal ion(s) and metal-protein interactions are deemed physiologically significant when in fact they are not (Maret 2010).

Zinc(II) ions are bound as catalytic or structural cofactors in thousands of human zinc proteins (Andreini et al. 2006). This zinc proteome does not provide a sufficient dataset to account for all the functions of zinc in human biology. This article addresses this issue. It focuses on transient interactions of zinc ions with proteins and the functions of zinc ions in cellular regulation, signal transduction networks, and metabolic control. Cellular zinc ions that are not permanent cofactors of proteins have been referred to as labile, mobile, or rapidly exchanging zinc. All these terms specify characteristics that zinc ions may or may not have. Therefore, in lieu of a better term, I will refer to these zinc ions as free zinc ions to distinguish them from protein-bound zinc ions, albeit with the understanding that they are not devoid of any ligands and that the nature of the ligands is not known at present. Aside from compiling the proteins that regulate zinc and are regulated by zinc, thermodynamic and kinetic data about biological zinc coordination are required to define at which concentrations zinc ions have regulatory functions. As one aspect of such functions, zinc ions are thought to participate in information transfer as signaling ions and second messengers (Haase and Maret 2010). How zinc ions can serve such functions hinges on how fluctuations of zinc ions (zinc signals) are generated, controlled, transmitted to and terminated at their target sites. Thus, in addition to controlling and re-distributing zinc, the cell must control fluctuations of zinc ions. In part, this control is achieved by ZnT (SLC30A) and Zip (SLC39A) zinc transporters that coordinate subcellular, vesicular, and organellar translocations of zinc ions (Lichten and Cousins 2009). The three-dimensional structure of the *E. coli* Yiip Zn^{2+}/H^{+} antiporter, a member of the cation diffusion facilitator (CDF) family of proteins with significant sequence homology to human ZnTs, shows four zinc ions bound per monomer and suggests new principles for the molecular mechanisms of these proteins (Lu et al. 2009). A zinc-binding site between the transmembrane helices appears to be the primary transport site. A conformational change presumably induces dissociation of the one histidine and/or the three aspartate ligands and movement of the zinc ion from this site. The other zinc ions reside in the cytoplasmic domain and in the hinge region between the cytoplasmic domain and the transmembrane portion of the protein. One is a dinuclear site with a bridging aspartate and with each zinc ion bound by two histidine ligands. Each pair of histidine ligands stems from a different monomer of the homodimer. Outer shell chemical bonds constrain the histidine side chains and couple zinc ion binding to interactions of the cytoplasmic domains. Zinc binding at these sites leads to a scissor-like conformational change that affects the orientation of the transmembrane helices and the coordination of zinc at the primary transport site. Yiip thus senses zinc ion concentrations and functions as an allosteric protein in zinc-regulated zinc transport. The third site is located on the cytoplasmic site at a loop connecting two transmembrane helices. It has two histidines, one aspartate and a water molecule as ligands. Its function is unknown. The copper chaperone Hah1 superimposes on the cytoplasmic domain of Yiip. Based on molecular modeling, an Hah1 molecule or a related metallochaperone could dock to the cytoplasmic

domain after the zinc-dependent conformational change occurred and deliver a metal ion to the cavity between the cytoplasmic domain and the transmembrane portion of the transporter, perhaps aided by a histidine-rich region in the cytoplasmic domain (Fu 2011). Zinc metallochaperones, proteins that interact specifically with their targets and deliver zinc ions by an associate ligand-exchange mechanism, have not been identified.

Generating free zinc ions

At least three pathways increase zinc ion concentrations (Fig. 1). One is the release of zinc ions from vesicles into the extracellular space, where zinc ions can be involved in paracrine signaling. Best studied is the release of zinc ions from synaptic vesicles in a subset of glutamatergic neurons (Frederickson et al. 2005). The zinc transporter ZnT3 loads zinc ions into synaptic vesicles. In the vesicles, zinc ions are stored in a yet to be determined chemical form, and then secreted into the synapse in a calcium-dependent

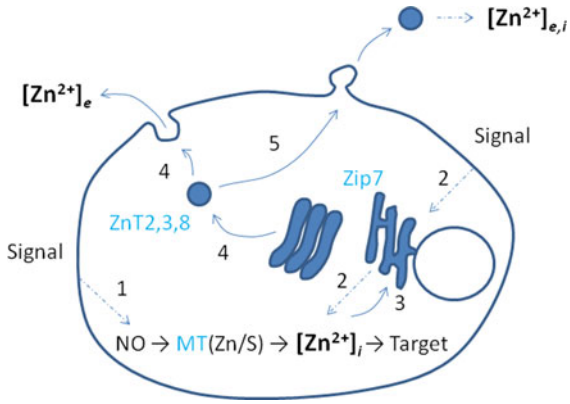

Fig. 1 Simplified pathways in cellular zinc(II) ion sorting, compartmentalization, and signaling. (1) Extracellular signals generate reactive species (NO, peroxide) that react with zinc/thiolate (Zn/S) coordination environments in metallothioneins (MT) and release zinc(II) ions as effectors of cellular targets. (2) Extracellular signals also stimulate the zinc transporter Zip7 on the ER membrane and increase the pool of cellular zinc(II) ions. Zinc(II) ions are loaded into the ER (3) or into the trans Golgi network that forms exocytotic vesicles (4) for extracellular targets or vesicles that shed from the plasma membrane to form extracellular matrix vesicles (5). The drawing does not imply that all three pathways operate in the same cell. Zinc signaling events among cytoplasm, nucleus, and mitochondria are not shown

process when the neuron is stimulated. One target is the postsynaptic *N*-methyl-D-aspartate (NMDA) receptor, which is inhibited by low nanomolar concentrations of zinc ions (Paoletti et al. 2009). Zinc ions diffuse in the synapse and affect intersynapses (Ueno et al. 2002). The activity of other receptors is also modulated by zinc ions in various experimental systems but the physiological effects of zinc ions will depend on their effective concentrations in the synapse (Sensi et al. 2009). Secretion of zinc ions from vesicles is not restricted to the nervous system and has been observed in a number of exocrine and endocrine glands. Somatotrophic cells in the pituitary gland, pancreatic acinar cells, β-cells of the islets of Langerhans, Paneth cells in the crypts of Lieberkuehn, cells of the tubuloacinar glands of the prostate, epithelial cells of the epididymal ducts and osteoblasts all secrete zinc ions (Danscher and Stoltenberg 2005). In pancreatic β-cells, zinc ions are imported into insulin granules via the zinc transporter ZnT8 and used to form and store hexameric insulin (Dunn 2005; Chimienti et al. 2005). Zinc ions are secreted together with insulin and thought to affect glucagon secretion from pancreatic α-cells (Gyulkhandanyan et al. 2008). Thyroid cubical epithelium and cortex of the adrenal gland also express ZnT8 and may secrete zinc ions (Murgia et al. 2009). In mammary epithelial cells, the zinc transporter ZnT2 loads exocytotic vesicles with zinc ions (Lopez and Kelleher 2009). These vesicles supply the milk with zinc and do not appear to be involved in any type of signaling. Another variation on the theme is the loading of vesicles with zinc and their shedding as zinc-rich extracellular matrix vesicles from chondrocytes, where the zinc is critically important for mineralization of cartilage, dentine, and bone (Sauer et al. 2003). The other two pathways involve intracellular release of zinc and suggest roles of zinc ions as a second messenger in intracrine signaling. First, zinc release from a store in the endoplasmic reticulum (ER) is mediated by the zinc transporter Zip7 (Taylor et al. 2008; Hogstrand et al. 2009). It has been observed in macrophages and is triggered by stimulation of the immunoglobulin E FcεRI receptor and ERK/IP_3 signaling and is preceded by Ca^{2+} release (Yamasaki et al. 2007), and from lysosomes of interleukin 2-stimulated T-cells as a required signal for proliferation of these cells (Kaltenberg et al. 2010). In cultured cortical neurons, zinc is

released from an ER store that is thapsigargin/IP_3-sensitive as is typical for calcium release from this store (Stork and Li 2010). The IP_3-pathway is also triggered by zinc ion binding to GPR39, an orphan G-protein-coupled receptor (Sharir et al. 2010). Thus, certain cells, such as nerve cells, may use both intracrine and paracrine zinc ion signals. Zinc release from organelles is an effective mechanism of signal amplification. Second, oxidation of sulfur donors in zinc/thiolate coordination environments of proteins leads to dissociation of zinc ions (Maret and Vallee 1998; Maret 2006). In this way, a redox signal is converted into a zinc signal with different and higher specificity, but without signal amplification. Since zinc itself is redox-inert in biology, the reactivity of the ligand donor instead of the central atom is used to change the stability of the complex. It is one of biology's solutions to the problem of how to mobilize zinc ions from their high affinity sites in proteins and how to use changes in the cellular redox poise to modulate zinc ion concentrations (zinc potentials, $pZn = -\log[Zn^{2+}]_i$) (Maret 2009).

Maintaining transient increases of free zinc ions in a buffered environment

Different experimental strategies using fluorescent zinc-chelating probes resulted in estimates that put the resting cellular free zinc ion concentrations in multicellular eukarya at picomolar (Bozym et al. 2006; Krezel and Maret 2006; Vinkenborg et al. 2009). They confirm earlier estimates (Peck and Ray 1971). The overall cellular zinc concentration, however, is a few hundred micromolar and thus at least six orders of magnitude higher. The large ratio between bound and free zinc ions is a consequence of the generally tight binding of zinc to proteins with picomolar affinities. How is it then possible to express a zinc ion signal in a relatively strongly zinc-buffered cytosol? One possibility is that zinc ions are never free and information transfer occurs by relaying zinc ions via ligand exchange in protein–protein interactions. While such transfer occurs, there is ample evidence that cellular free zinc ion concentrations increase transiently and are potent biological effectors (Maret et al. 1997; Maret 2006; Krezel et al. 2007). We have measured zinc ion fluctuations in cultured cells under three different conditions: serum starvation, during the cell cycle, and when cells are subjected to mechanical forces such as stretching (Li and Maret 2009; Li et al. 2010). Under these conditions, cellular zinc ion concentrations increase above one nanomolar and within minutes return to their resting concentrations, or below, if no zinc is available from the environment. Under serum starvation and mechanical stress, zinc release depends on the formation of nitric monoxide, indicating conversion of a redox signal into a zinc signal. The characteristics of such a zinc signal depend on how strongly the cytosol is buffered at steady-state and on the activities of transporters that remove the surplus of zinc ions from the cytosol at pre-steady state. The combination of the two processes is called muffling (Thomas et al. 1991). Muffling describes the time-dependent changes that contribute to "buffering" in cells. The zinc-buffering capacity of intestinal epithelial cells at physiological pZn is about 65 μM (Krezel and Maret 2006). It depends on whether the cell is at rest, proliferates, differentiates, or undergoes apoptosis. Buffering metals is a thermodynamic process, in which transiently increased metal ion concentrations return fast to those at rest if the buffering capacity is strong, or reach a new steady-state if the buffering capacity is weak. Analyses of how cells handle zinc loads demonstrated that cells can tolerate much higher loads than determined by the zinc-buffering capacity at steady-state and suggest that zinc ion fluctuations are muffled (Colvin et al. 2008). Muffling reactions involve the binding of the surplus of zinc ions and moving them from the cytosol to an organelle or to the outside of the cell in order to restore the steady-state (Colvin et al. 2010). The muffling capacity augments the buffering capacity. Sequestration of zinc ions in a cellular compartment is one way of storing zinc ions in the cell and then releasing them again. This compartmentalization is a central aspect of the cellular homeostatic control of zinc and requires at least two dozen transporters. So far, no protein other than metallothioneins (MTs) has been identified in cellular zinc storage. Its micromolar concentrations in most cells make sufficient zinc available in a deliverable form to meet the demand of many cellular functions for zinc. The zinc-binding characteristics of human MT-2 are in agreement with its role in muffling reactions, namely binding zinc ions, translocating with bound zinc in the cell, and delivering zinc to a cellular compartment

(Colvin et al. 2008). Mammalian MTs contain 60+ amino acids and bind zinc exclusively to the sulfur donors of cysteines in two zinc/thiolate clusters. In humans, they are a family of at least ten different proteins (Li and Maret 2008). Despite the binding of up to seven zinc ions in tetrathiolate coordination environments, the binding sites in MT-2 have different affinities for zinc, ranging from nanomolar to picomolar (Krezel and Maret 2007). With these characteristics, MTs can serve as both zinc acceptor and zinc donor, and control zinc availability over a range of zinc ion concentrations commensurate with their affinities for zinc ions (Krezel and Maret 2008). Since thiolate ligands confer redox activity on the zinc/thiolate clusters of MT, the zinc donor potential of MT increases under more oxidizing conditions while its zinc acceptor potential increases under more reducing conditions and by de novo synthesis of the apoprotein thionein (Maret and Vallee 1998).

The thresholds for cellular zinc muffling separate physiology from pathophysiology. If the buffering/muffling capacity is exhausted, zinc ion concentrations will increase drastically and bind to additional proteins that usually do not bind zinc. It is worthwhile recapitulating that the theory of buffering predicts both increases and decreases of zinc availability outside the buffering range. Thus increasing the buffering capacity of a cell, e.g. by introducing certain antibiotics that bind zinc, decreases zinc availability and amounts to a state that can be described as a transient cellular zinc deficiency. The physiological consequences of such a state remain to be determined. Because of the remarkably low concentrations at which zinc ions are biological effectors, a significant number of compounds perturb zinc metabolism either directly or indirectly via redox changes. Such perturbation has significant implications for acute and chronic conditions caused by nutritional or environmental exposure, and thus for the balance between health and disease.

Targets of free zinc ions

Notwithstanding the many proteins that bind zinc with micromolar affinities in vitro, such relatively weak interactions between cytosolic proteins and zinc ions are not relevant physiologically, because commensurately high free zinc ion concentrations are cytotoxic and far outside the range of physiological zinc buffering in the cytosol. Four independent observations converge on defining a range of picomolar to low nanomolar concentrations at which zinc ions can serve regulatory functions: (i) the free zinc ion concentrations and their fluctuations, (ii) the zinc affinities of MT, (iii) the affinities of zinc proteins for zinc, and (iv) the affinities of proteins that are targets of zinc ion fluctuations. A description of these targets is the subject matter of this section. Picomolar to low nanomolar zinc ion concentrations inhibit some enzymes, indicating tonic inhibition of some of them and modulation of their activities by zinc ion fluctuations (Table 1).

Changing cellular zinc ion concentrations globally or locally provides ways of controlling many cellular processes. In fact, the control of zinc(II) ions at the remarkably low concentrations is the basis for achieving high specificity in biological control. The cellular zinc ion concentrations differ in growth-arrested, proliferating, differentiating or apoptotic cells, suggesting that global changes control the metabolic state and differentiation of a cell by affecting enzyme activities, protein–protein interactions, and gene expression patterns (Krezel et al. 2007). Cells have the capacity to adapt to different levels of zinc ions by regulating zinc transporters and MTs. Many zinc enzymes have binuclear metal-binding sites that bind either two zinc ions or a zinc ion and another metal ion. Investigations of isolated enzymes demonstrated that the occupancy of the second, so-called co-catalytic site with zinc can modulate enzymatic activity. Cellular zinc ion fluctuations suggest that such a modulation is physiologically significant. Nanomolar concentrations of zinc ions also inhibit the activities of enzymes (Maret et al. 1999). Zinc-inhibited proteins are generally not recognized as being zinc-dependent because they are isolated in the presence of a chelating agent to preserve their enzymatic activity. Zinc inhibits erythrocyte Ca^{2+}-ATPase with a K_i value of 80 pM (Hogstrand et al. 1999). Resting zinc ion concentrations in erythrocytes of 24 pM are expected to inhibit this enzyme partially (Simons 1991). The high affinity of some proteins for transient zinc binding reflects rather selective binding sites for zinc. *Locally* increased zinc ion concentrations can be higher than those globally increased and target yet other proteins. For example, zinc inhibits protein tyrosine phosphatase-1B (PTP-1B) with a K_d value of 16 nM (Haase

Table 1 Zinc inhibition of protein activities

Protein	Source	Zinc inhibition constant	pH	Reference
Ca^{2+}-ATPase	Human erythrocytes	80 pM (K_i)	7.4	Hogstrand et al. (1999)
Protein tyrosine phosphatase-1B (PTP-1B)	Human	15 nM (K_i)	7.4	Krezel and Maret (2008)
Caspase-3	Human	<10 nM (IC_{50})	7.5	Maret et al. (1999)
NMDA receptor, NR2a subunit	Human	<10 nM (IC_{50})	7.3	Paoletti et al. (1997)
Phosphoglucomutase	Rabbit muscle	4 pM (K_i)	8.5	Ray 1969
Dimethylarginine dimethylaminohydrolase	Bovine	4 nM (K_i)	7.4	Knipp et al. (2001)

and Maret 2003; Krezel and Maret 2008). PTP-1B is tethered to the ER (Frangioni et al. 1992). Release of zinc ions from an ER store could provide sufficiently high local zinc ion concentrations for the inhibition of PTP-1B, which has a critical role in growth factor signaling. Cellular chelating agents abolish insulin or IGF-1 signal transduction, supporting the hypothesis that zinc inhibition of protein tyrosine phosphatases is indeed a physiological event (Haase and Maret 2003). One established target of zinc ions is metal-response element (MRE)-binding transcription factor-1 (MTF-1). MTF-1 controls zinc-dependent gene expression of proteins at increased zinc ion concentrations. Sensing is thought to occur through a pair of its six zinc fingers with a setpoint of a few nanomolar (Laity and Andrews 2007). But sensing is not necessarily signaling unless specific signals increase the zinc ion concentrations. There is evidence for this, too. Various conditions of stress increase zinc ion concentrations as a signal for MTF-1 to express genes that counteract the stress. Furthermore, MTF-1 is an essential transcription factor in development, suggesting that zinc signals are employed as developmental cues (Günes et al. 1998; Hogstrand et al. 2008). Zinc is known to bridge subunits of the same or different proteins in so-called zinc interface sites, where it can be a structural brace in organizing protein oligomerization (Maret 2004; Gundelfinger et al. 2006). Given the low availability of cytosolic zinc ions and their control, zinc presumably controls these interactions as well.

Modulating the chelating capacity of MTs through changes of either their total amount or their redox state affords a way of activating zinc-inhibited enzymes or reversing the regulatory effects of zinc ions (Maret et al. 1999). Elevated zinc ion concentrations induce MTF-1-controlled gene transcription of both thioneins, the apoproteins of MTs, and the zinc transporter ZnT1, resulting in the binding of the additional zinc to MTs and export from the cytosol. A host of other conditions also induces thioneins, indicating that thionein is produced for lowering the availability of cellular zinc, modulating zinc-dependent processes, and increasing the reducing power.

Outlook

Zinc ions serve as permanent cofactors of zinc metalloproteins. In addition, homeostatic control of cellular zinc and compartmentalization offers yet not completely charted possibilities for a role of zinc ions in cellular regulation. Particularly intriguing in this regard are the functions that zinc shares with calcium as a second messenger. The controlled release of zinc from the ER is more akin to calcium biology then to the biology of any of the other transition metal ions. However, calcium chemistry dictates different targets than zinc chemistry. Zinc has a preference for sulfur and nitrogen ligands, in addition to binding to oxygen donors, and engenders binding sites with affinities that are 2–3 orders of magnitude lower than those for calcium (from resting 100 nM to >1 μM fluctuations for calcium ions vs from resting 100 pM to >1 nM fluctuations for zinc ions). Employing redox-inert zinc ions as regulatory ions extends the control of proteins by the redox-inert magnesium and calcium ions to cover at least six orders of magnitude in metal ion concentrations (Maret 2001). While calcium activates kinases, zinc inhibits phosphatases. The overall effect is the same: Both metal ions activate phosphorylation signaling. Phosphatases are emblematic for control in phosphorylation signaling, and by choosing them as targets, zinc ions exert specific control. Separate targets for zinc and calcium do not exclude that the effects of zinc and calcium converge on some enzymes, such as protein kinase C.

In conclusion, the new findings establish a basis for regulatory functions of zinc ions in cellular signal transduction networks. Such regulation was postulated and discussed earlier. However, a lack of understanding at which concentration cellular zinc ions occur and are controlled precluded an explanation of how transition metal ions that bind to proteins so tightly function in biological control. Functions of zinc ions in inhibitory control, the importance of vesicular sequestration of zinc, and the complementarity of zinc and calcium ions in cellular regulation are entirely consistent with the answer given about 30 years ago to the question "Zinc: what is its role in biology?" (Williams 1984).

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