How to Hide Zinc in a Small Protein

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ABSTRACT
Small cysteine-rich proteins (metallothioneins) and related domains of some large proteins (e.g., lysine methyltransferases) bind tri- and tetranuclear zinc clusters with topologies resembling fragments of Zn^{II} sulfide minerals. These clusters are ubiquitous in animals, plants, and bacteria. Bacterial metallothioneins can also contain histidines as cluster ligands and embed Zn^{II} with a "treble-clef"-like finger fold. This unusual embedded Zn^{II} is "hidden" and surprisingly inert toward Zn or Cd exchange. Clearly, proteins can exert fine control over both the thermodynamics and kinetics of zinc binding in thiolate clusters. Genome sequences suggest that related zinc-finger sites are common in a variety of bacteria.

When Oxygen Came to Life, Zinc Came to Biology

Cyanobacteria are thought to have been the producers of the first oxygen, as much as 2.75–3.2 billion years ago. Oxygen evolution led to an increase in the concentration of available Zn^{II} by several orders of magnitude, because of the oxidation of sulfidic ZnS.[1,2] Sequenced genomes of many cyanobacteria thus, unsurprisingly, contain genes related to zinc homeostasis. Generally, these systems[3] consist of a zinc sensor[4,5] that regulates the expression of a protein that deals with elevated levels of zinc (Figure 1): either a membrane-bound zinc efflux pump (not shown) or a metallothionein (MT)-like intracellular zinc-binding protein (SmtA; Figures 2 and 3), probably involved in zinc sequestration.[6]

The occurrence of prokaryotic MT in cyanobacteria was reported in 1979.[7] Bacterial MT-like proteins from a *Pseudomonas putida* strain adapted to high Cd^{II} concentrations were subsequently characterized by ^{113}Cd NMR and shown to contain 4 distinct Cd sites.[8] The first amino

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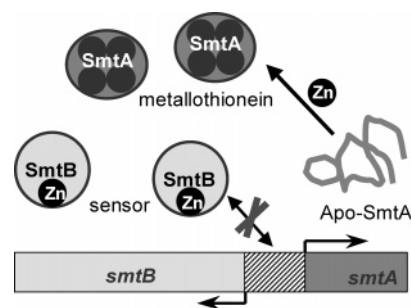
FIGURE 1. Induction of MT synthesis in the cyanobacterium *Synechococcus* PCC7942. The horizontal bar symbolizes genomic DNA. The sensor (*smtB*) and MT (*smtA*) genes are divergently transcribed (arrows) in the smt operon. Zinc-free SmtB binds to the operator–promoter region (hatched) and represses transcription of SmtB and SmtA. Zinc-loaded SmtB does not bind to DNA, and transcription can take place.

acid sequence of a prokaryotic protein with MT-like characteristics was reported for *Synechococcus* sp. in 1988.[9] Robinson et al.[10,11] characterized the metal-binding properties of the protein SmtA from *Synechococcus* PCC7942 and established its regulation on the transcriptional level by the zinc-dependent repressor SmtB (Figure 1).[4] Mutants lacking the gene for SmtA and SmtB are hypersensitive to zinc.[12] ^{111}Cd NMR spectroscopy established the involvement of histidine residues in metal binding,[13] and finally, the solution structure of SmtA was determined by NMR spectroscopy.[14]

Our NMR structure determination of SmtA (Figure 2) has provided the only structure of a hybrid MT/zinc finger and has also enabled the identification[15] of SmtA homologues (Figure 3).[16,17] It is now clear that MTs are more widespread in bacteria than previously thought.[6]

Intracellular Zinc Is Tightly Controlled

In cyanobacteria, the zinc-sensor protein is usually a repressor (e.g., SmtB) that binds to DNA in its zinc-free (apo) form and thus inhibits transcription of both itself and the zinc-handling protein (e.g., SmtA). The zinc-bound form of the sensor (e.g., Zn-SmtB) does not bind to DNA; thus, transcription and expression of the zinc-handling protein (and the sensor) can proceed, as long as the zinc concentration is high enough to produce the zinc-loaded sensor. The sensors have extraordinarily high stability constants ($>10^{14}$),[18,19] thus preventing DNA binding of the sensor, even at very low zinc concentrations (Figure 1). Only when no zinc is available for binding to SmtB does the transcription stop.

This model for zinc homeostasis can be compared to MT gene regulation in mammals (Figure 4).[20] It is now accepted that the concentration of zinc, like that of copper and iron, is indeed very tightly controlled and maintained at a very low level in cells.[18] Therefore, if there is no free zinc available, how do newly synthesized zinc-requiring

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(b)
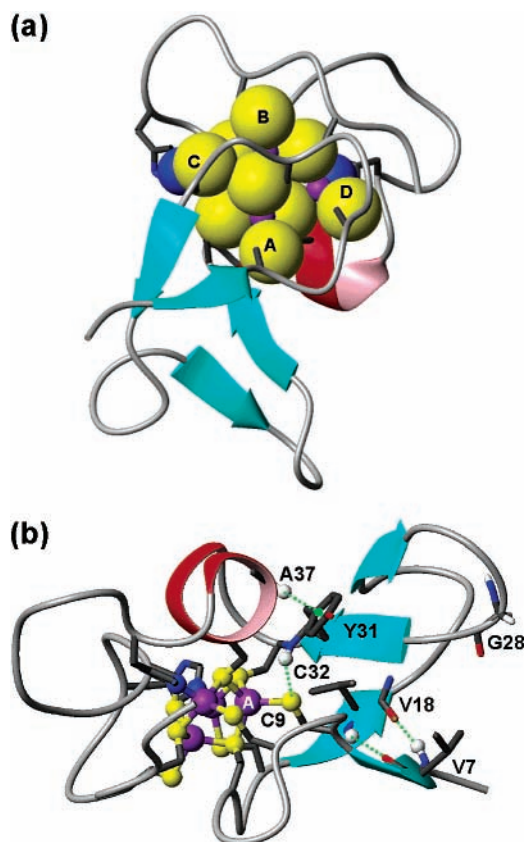
FIGURE 2. (a) Protein fold and mineral core of Zn_4SmtA from *Synechococcus* showing the close packing of thiolate sulfurs (yellow), Zn^{II} ions (purple), and coordinated His N (blue). The letters A−D refer to the four zinc sites (Figure 5) and for clarity are positioned on sulfurs coordinated to the respective zinc ions. (b) Same structure as in (a) but highlighting features around Zn(A): secondary structure elements, the CH/π interaction between the aromatic ring of Tyr31 and the CH(α) of Ala37, and a NH···S hydrogen bond between the backbone amide of Cys32 and the thiolate sulfur of Cys9.

apo proteins acquire their Zn^{II}? Various scenarios by which apo proteins might acquire zinc are possible: (i) from Zn complexes with low-molecular-weight ligands, such as histidine and glutathione.[21] This would imply a thermodynamic equilibrium distribution of zinc throughout the cell; (ii) from specific metallochaperones, a pathway that is now well-known for several Cu^I-requiring proteins.[22−24] Here, zinc distribution might be under kinetic control; and (iii) from another protein that essentially functions as a "zinc buffer". This protein might interact with a variety of other proteins and transfer zinc in ligand substitution reactions.

In the latter mechanism, zinc distribution would not only be dependent on the kinetics of these reactions but also on the concentration of the "buffer" protein and its state of loading. MTs are thought to play such a zinc-buffering role,[25] and evidence that MTs are involved in interprotein metal exchange reactions,[26] including transfer to membrane proteins,[27] backs up this hypothesis. The physiological functions of MTs are still a matter of debate, which is widened by our studies of the structure and dynamics of SmtA.

Tetranuclear Cluster in a Bacterial MT.

The ^{111}Cd spectrum of Cd_4SmtA, reconstituted from apo-SmtA, contains 4 resonances above 550 ppm, typical of Cys_4 and Cys_3His environments. The replacement of Zn^{II} by Cd^{II} in SmtA is isostructural, as is the case for mammalian MT-2,[28] with only minor differences because of the cluster size. SmtA thus contains a $Zn_4Cys_9His_2$ cluster (Figures 2 and 5), in which each Zn^{II} ion is tetrahedral. Sites A and B have Cys_4 ligand sets, and sites C and D have Cys_3His ligand sets. There are 5 bridging and 4 terminal thiolates. Zn_2-, Zn_3-, and Zn_4-thiolate clusters have been structurally characterized in proteins (Figure 5). Although the protein sequences and metal−ligand connectivity patterns for SmtA and MT-2 are completely different (Figure 6), the topology of the four-metal cluster in SmtA closely resembles that of the α-domain cluster in mammalian MTs. There is an analogous situation for the C-terminal three-metal cluster found in the β domain of mammalian MTs and that in the pre-SET domains of histone-lysine-methyltransferases.[29] The latter proteins are involved in the regulation of transcriptional activity of genes in the chromatin fiber. The role of the pre-SET zinc cluster is not known.

Figure 7 compares the SmtA cluster with model cluster complexes and the mineral wurtzite (hexagonal ZnS). With the exception of the fungal Gal4 proteins (2 edge-sharing tetrahedra), the prevailing motifs are $M-S_4$ tetrahedra connected by their corners and six-membered M_3S_3 rings. In ZnS minerals and the model complexes, these rings can adopt either chair or boat conformations. The six-membered rings in wurtzite adopt both chair and boat conformations, whereas model complexes often contain adamantoid cages $[(\mu\text{-}SR)_6(Zn-SR)_4]^{2-}$, which are composed of four fused six-membered rings in chair conformations.[30] All of these complexes are essentially fragments of the cubic form of ZnS, sphalerite.[31]

In proteins, only distorted Zn_3S_3 boat conformations are commonly observed.[32] It is striking that the metal clusters from various proteins are very similar to one another, although the protein folds are not related. The four-metal cluster in MTs can be described as $[(\mu\text{-}SR)_5\text{-}(Zn-SR)_2(Zn(SR)_2)_2]^{3-}$. This unit has been observed in only one model compound, $[SZn_8(SBz)_{16}]$,[33] which can be described as two such units fused together in a perpendicular fashion. All of the rings in this compound adopt boat conformations. Although the M_4S_{11} unit found in the α domain of mammalian MTs has no strict parallel in minerals,[31] we consider that it is nevertheless appropriate to describe Zn−S clusters in proteins as small, distorted fragments of wurtzite (Figure 7). The cluster structure is dictated mainly by the close packing of sulfurs, with zinc in tetrahedral holes (see Figure 2), while the distortion of the six-membered rings might be attributable to the influence of the protein and might have functional significance.

Zinc Finger in a Zinc Cluster

Mammalian MTs make economic use of the protein synthesis machinery; the protein chain is just long enough
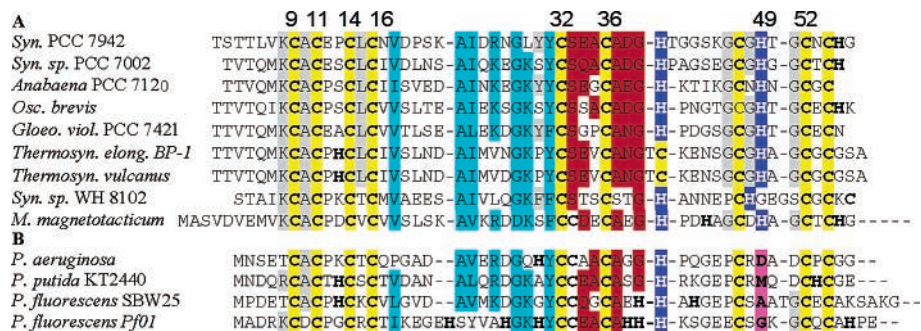
FIGURE 3. Predicted zinc clusters in bacterial proteins based on sequence homology with *Synechococcus* SmtA, as found by similarity searches in finished and unfinished genomes using BLAST[15] (www.ncbi.nlm.nih.gov/BLAST and http://www.kazusa.or.jp/cyano/blast.html). Group A sequences contain the full complement of 11 ligands, and the *Pseudomonas* sequences (group B) lack the equivalent of His49. Numbers refer to SmtA residues.
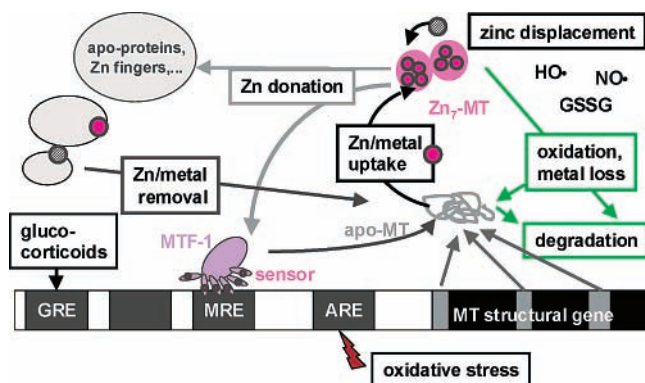

FIGURE 4. Intracellular handling of zinc and regulation of MT expression in mammals.[20] The horizontal bar symbolizes genomic DNA. MT sythesis is regulated by the metal-response element (MRE)-binding transcription factor (MTF-1), which contains six zinc fingers and activates MT transcription upon binding to the MRE region of DNA. Zinc binding to the fingers is necessary for activation.

to wrap around the Zn_4S_{11} and Zn_3S_9 mineral cores, and there is hardly any secondary structure. In contrast, the bacterial MT SmtA contains secondary structure elements, namely, two short antiparallel β sheets and an α helix (Figure 2). These secondary structure elements, together with zinc site A, constitute the zinc-finger portion of the protein.

The Protein Data Bank (PDB) currently contains over 400 structures of zinc fingers, and over 3% of the genes in the human genome contain at least one zinc-finger domain.[34] Although initially coined[35] for one motif, now called the "classical" zinc finger, in which a β hairpin and an α helix are connected via a Cys_2His_2 zinc site, there are now many classes of zinc fingers,[36] with Cys_2His_2, Cys_3His, and Cys_4 ligand sets. Zinc fingers typically bind to a wide variety of biomolecules, such as polynucleotides, proteins, and lipids.

Although a DALI (http://www.ebi.ac.uk/dali/) search of the PDB fails to detect any folds similar to SmtA, the zinc-finger fold in SmtA strikingly resembles those found in numerous other zinc-containing proteins (Figure 8). Examples include GATA, LIM domains, RING fingers, and the ribosomal protein L24. All of these have been classified as "treble-clef fingers".[37] The amino acid sequences (25–45 residues) differ considerably from that of
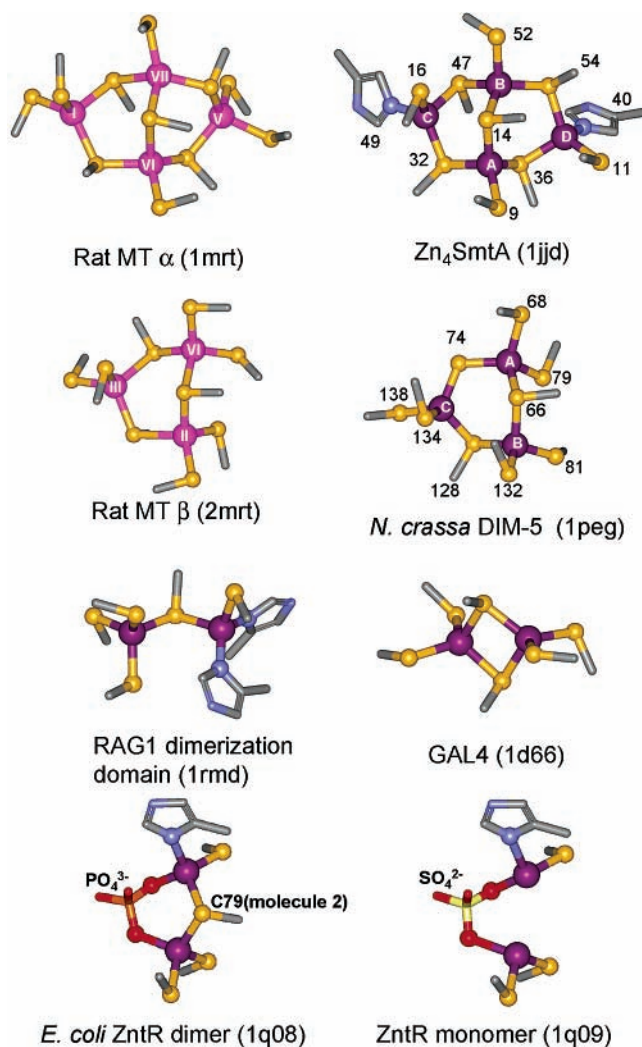

FIGURE 5. Multinuclear Zn–Cys sites in proteins. Zn (purple), S (gold), Cd (magenta), and N (blue). For coordinated Cys and His residues, only the side chains are shown.

SmtA, but the unique spatial arrangement of a "zinc knuckle", loop, β hairpin, and an α helix is the same as in SmtA (Figure 8).[38–40] Amazingly, the functional diversity of treble-clef proteins is greater than that of many larger domains, encompassing binding to other proteins, DNA, RNA, and small ligands and a role in enzymatic catalysis.[37]
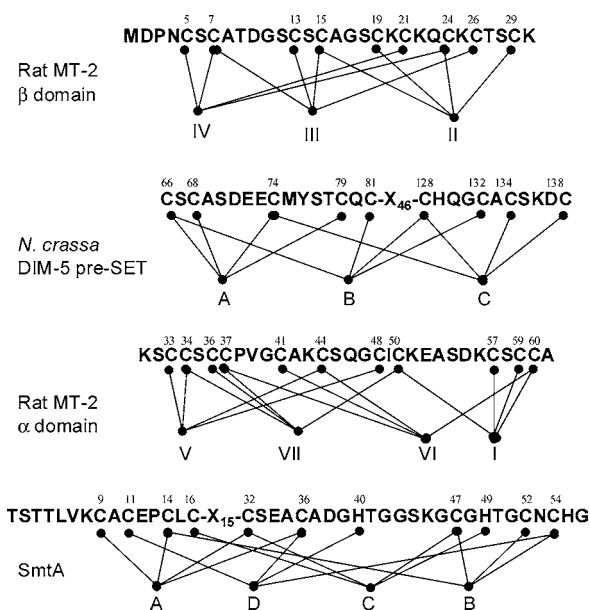
FIGURE 6. Ligand connectivity patterns for Zn or Cd in tri- and tetranuclear sites. Despite the differences in the location of the Cys residues in the sequences, the topologies of the metal clusters are very similar (see Figure 5). A common feature is that metals are coordinated by residues far apart in the sequence and that the connections for the individual sites cross each other; thus, the metal core efficiently "sews" together the peptide envelope.
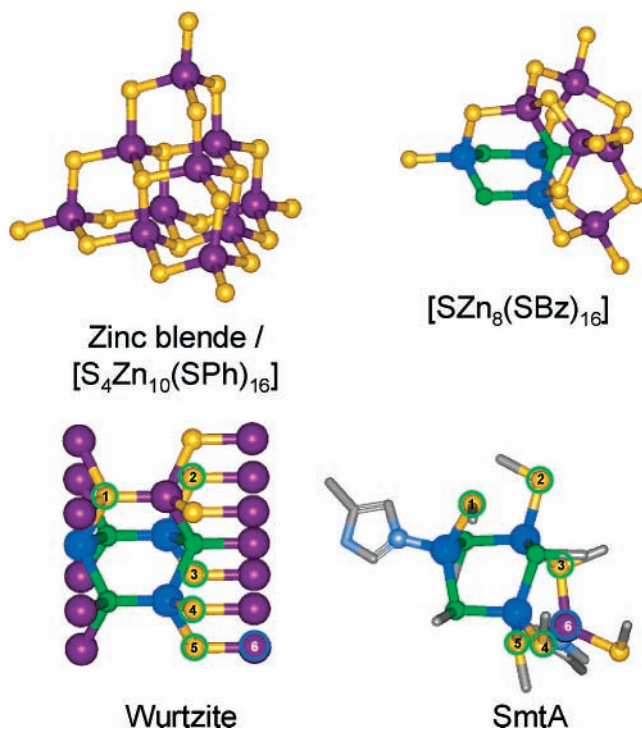
FIGURE 7. Topology of $Zn-S_4$ tetrahedra in the minerals zinc blende (sphalerite, cubic ZnS), wurtzite (hexagonal ZnS), and model complexes (charges not shown in formulas) in comparison with the $Zn_4S_9N_2$ cluster in SmtA. Zn_3S_3 rings with boat conformations are highlighted in green/blue, and sulfurs in equivalent positions are labeled with a green ring.

The protein sequences of SmtA and its homologues also show some similarity to "TRASH domains" (trafficking,
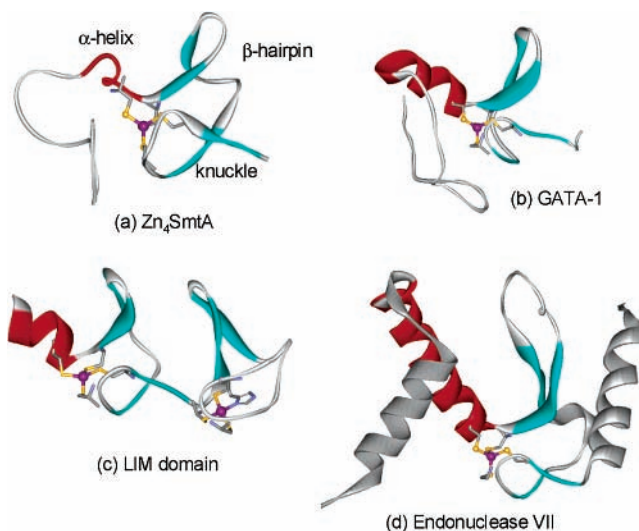
FIGURE 8. Proteins with treble-clef-like zinc-finger folds. (a) SmtA showing the characteristic arrangement of a zinc knuckle, β hairpin, and an α helix. For clarity, only Zn(A) is shown. (b) C-terminal zinc finger of chicken GATA-1 (PDB 1gat).[38] (c) Amino-terminal LIM domain from quail cysteine- and glycine-rich protein (PDB 1a7i).[39] (d) Phage T4 endonuclease VII (PDB 1en7).[40] Although the spacing between the two cysteine pairs in the $Zn(Cys)_4$ site is different in SmtA and typical treble-clef proteins, the geometry of the zinc site and the protein fold are very similar.

resistance, and sensing of heavy metals),[41] a newly defined protein family with predicted treble-clef folds. The members of this family either occur as stand-alone domains (i.e., presumably as individual, small proteins) or as part of larger proteins, especially putative cation-transporting ATPases. The SmtA-like zinc finger thus might have a role in mediating protein−protein contacts for metal-transfer reactions.

A New Protein Family: Bacterial MTs

As genome sequencing progresses, more candidate bacterial genes for MTs can be identified (Figure 3). All 11 zinc ligands are conserved in cyanobacterial sequences, but in the pseudomonad sequences, His49 is replaced by Asp, Met, Ala, or Gly. Crucially, the residues important for the zinc-finger fold are fully conserved: Gly28 is essential to form the tight turn at the tip of the β hairpin, and Tyr31 is involved in a CH/π interaction with the CH(α) atom of Ala37 from the α helix, thus stabilizing the mutual arrangement of the β hairpin and helix (Figure 2b). Residues in secondary structure elements also show a high level of conservation, e.g., Val7, Val18 (β bridge in the zinc knuckle; Figure 2b), Ala23−Tyr31 (β hairpin), and Ser33−Gly39 (α helix).

Together with our collaborators, we have cloned, overexpressed, purified, and characterized the MTs from *Anabaena*, *P. putida*, and *P. aeruginosa*.[16] Two-dimensional NMR experiments confirmed the preservation of all of the crucial elements for the treble-clef-like fold in these homologues of SmtA. Nevertheless, the metal-binding properties of the proteins from cyanobacteria and pseudomonads differ, as discussed below.

Metal Dynamics: Inert Zinc Finger

How does protein structure influence the dynamics of metal exchange and release? Extensive theoretical calculations[42] stress the involvement of second shell interactions in determining the structure and dynamics of Zn−Cys sites, with hydrogen bonds to the cysteine sulfurs playing a prominent role in stabilizing negatively charged thiolates. Apart from influencing the electrostatic environment, the close packing of a protein around a zinc site also restricts accessibility to the metal and/or the ligands. Finally, it is also important to take into consideration protein dynamics: a metal site in a highly fluxional, flexible protein will be more reactive than one in a rigid scaffold. These considerations are exemplified by the metal ion dynamics of MTs. The generation of native mammalian MT clusters is rapid (completed within a few milliseconds) and too fast to be observed by stopped-flow methods.[43] No intermediates in the loading process have been observed yet, and native clusters are formed. Metal release from MT clusters is also very fast.[44] Rapid metal uptake and release has been linked to the high flexibility of these proteins.[45] Mammalian MTs can be regarded essentially as large chelating ligands, which form long bridges and have little or no preorganization. This notion also explains why cluster assembly occurs cooperatively: the first metal encounters an unstructured random coil, whereas the second and subsequent metal ions benefit from the degree of organization achieved by the first metal. The result of cooperativity is a sigmoidal (as opposed to hyperbolical) response of saturation of binding sites to the zinc concentration. This means that within a certain concentration range, MT clusters can react sharply to small alterations in zinc concentrations. The tendency to transfer zinc from MT is highest at low zinc concentrations, whereas at high zinc concentrations, MT will be fully saturated with Zn^{II}. When the intracellular Zn^{II} concentration increases, apo MT will bind "free" zinc rapidly and with high stability. Interestingly, MTs prevent both zinc deficiency and toxicity.[46] The biological half-life of MT is short,[47] and the apo protein is rapidly degraded by proteases.[20,48] Thus, MTs are not long-term storage devices but highly dynamic systems for rapid response to alterations in zinc levels and, more importantly, to metabolic requirements.

How does the presence of a zinc finger affect cluster dynamics in bacterial MT? We have probed the reactivity of SmtA in two ways: by reacting the native protein with external Cd^{II} and Zn^{II} to study metal-exchange dynamics and with EDTA to investigate metal release, using NMR spectroscopy and electrospray mass spectrometry.

Metal Exchange and Substitution. There are only a few reported studies of zinc exchange in proteins, probably because of the inherent "silence" of Zn^{II} in most spectroscopic methods. The only method available until recently involved incubation of the native protein with radioactive[65] Zn^{II}.[49] We have developed a new approach using stable zinc isotopes and high-resolution mass spectrometry.[50]
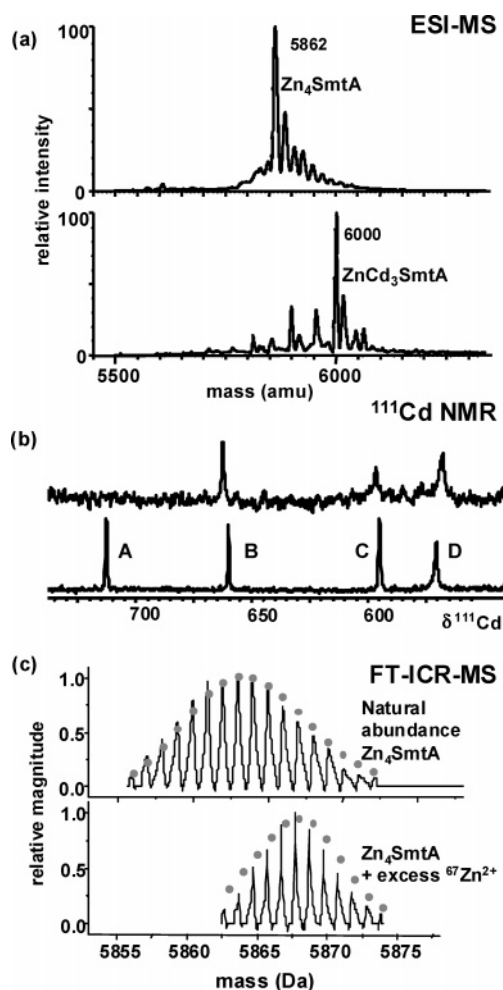

FIGURE 9. Metal-exchange reactions of Zn_4SmtA. Reaction of Cd^{II} with Zn_4SmtA. (a) Electrospray mass spectrometry shows that one Zn is not replaced by Cd^{II}. (b) One-dimensional ^{111}Cd NMR identifies Zn(A) as the inert site. Bottom spectrum, $^{111}Cd_4$-SmtA reconstituted from apo-SmtA; top spectrum, Zn_4SmtA incubated with excess $^{111}Cd^{II}$ at neutral pH. No ^{111}Cd enters site A. (c) One site (A) is also inert toward Zn self-exchange as observed by FT−ICR−MS.

Our findings were surprising in several ways. While three of the four zinc ions in SmtA exchanged rapidly with exogenous Zn^{II}, the fourth was inert (Figure 9). Exchange reactions of Zn_4SmtA with Cd^{II} followed by ^{111}Cd NMR and electrospray mass spectrometry revealed that the inert site is the Cys_4 zinc-finger site A.

This behavior was unexpected; typically, metals in MTs are very labile and exchange with external metal ions within seconds.[44] For mammalian MTs, stoichiometric amounts of Cd^{II} are sufficient to displace all Zn^{II} rapidly from native MT,[51] and thermodynamic considerations for both zinc fingers and MTs predict that Cys_4 sites should display a preference for Cd^{II} over Zn^{II}.[52,53]

An insight into why site A is inert toward metal exchange can be gained by consideration of the mechanism of exchange. A plausible scenario is that the terminal cysteine sulfurs of the cluster act as "bridging" ligands toward incoming metal ions. In SmtA, the terminal cysteines of sites B (Cys52), C (Cys16), and D (Cys11) are all accessible from the surface, whereas Cys9 of site A is

(a) MT-2 α: Zn$_4$Cys$_{11}$
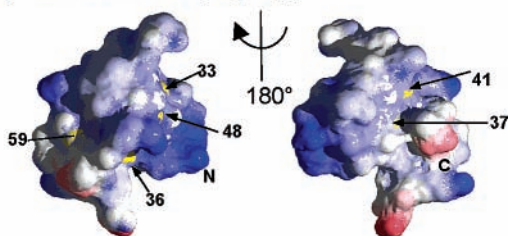
(b) SmtA: Zn$_4$Cys$_9$His$_2$
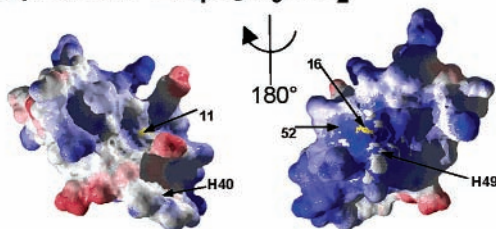
FIGURE 10. Molecular surfaces (red, negative surface charges; blue, positive surface charges; and yellow, Cys S) and space-filling models of (a) the α cluster of rat MT-2 (PDB 1mrt),[54] and (b) Zn$_4$SmtA (PDB 1jjd). All terminal cysteine sulfurs in rat MT-2 are accessible. In contrast, in SmtA, only the terminal Cys11 (left), Cys16, and Cys52 (right) from sites D, C, and B, respectively, are accessible via clefts from the surface. Cys9 from site A is completely buried.

protected by the secondary structure elements on this side of the protein (Figure 10). In contrast, the terminal cysteine sulfurs of all four metals in the MT-2 α cluster are accessible from the surface.

Studies of the metal-exchange dynamics of the *P. aeruginosa* protein (PmtA) by ESI−MS support this hypothesis. Replacement of a single cluster ligand (Asp for His) has a major effect not only on the stoichiometry but also on the metal-exchange behavior of the protein.

PmtA is isolated with 3 bound Zn^{II} ions; incubation of Zn$_3$PmtA at pH 6.0 with an excess of Cd^{II} leads to the complete replacement of all bound Zn^{II} and to the formation of Cd$_4$PmtA. Thus, PmtA does not have an inert metal site. Lowering the pH by one unit leads to Cd$_3$PmtA as the predominant species.

Asp44, the ligand in PmtA that replaces His49 of SmtA, is predicted to be part of site C, which is thus likely to be vacant in the three-metal species. An empty site C converts Cys28 of site A (the equivalent of Cys32 in SmtA) to a terminal, accessible Cys, thus rendering Zn(A) prone to exchange.

These observations help to explain why site A in SmtA is inert toward metal exchange: only the intact four-metal cluster provides an inert zinc-finger site, because three metal ions (B, C, and D) are required for the formation of three bridging thiolates (Cys14, Cys32, and Cys36; Figure 5). Thus, from one side, Zn(A) is protected by the metal cluster, irrespective of whether it is a Zn$_4$ or a Cd$_3$Zn cluster, with no detectable intramolecular metal transfer into site A. From the other side, the secondary structure, including an NH···S hydrogen bond from Cys32 to Cys9, shields the zinc from attack.
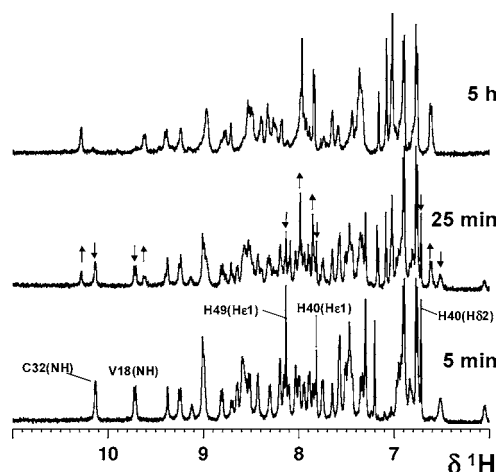
FIGURE 11. Low-field region of ^{1}H NMR spectrum of Zn$_4$SmtA at various times after addition of EDTA. A long-lived, folded intermediate is formed (top spectrum), as can be seen by the dispersion of NH and aromatic resonances. The arrows indicate disappearing and emerging resonances.

Why have the pseudomonad MTs "lost" a stable site C? Perhaps this labile site endows PmtAs with a higher zinc transfer potential. The two PmtA proteins studied are considerably less stable than SmtA. The presence of free thiols in the Zn$_3$ forms (e.g., Cys13 and Cys33 in *P. aeruginosa* PmtA) may render the proteins prone to oxidation and aggregation. Thus, these proteins are endowed with enhanced redox lability, which might play a role in their function. An intriguing parallel is found in mammalian MT-3, the isoform of MT in the brain, which has neuronal growth-inhibitory activity. MTs isolated from mammalian brains appear to be ($β$)Cu$_4$($α$)Zn$_3$−MT-3.[55] A Cu$_4$Zn$_4$ form can also be generated by reconstitution of the apo protein, but in contrast to other MTs, the fourth zinc in the α domain of MT-3 is redox-labile and air oxidation leads to the release of one Zn^{II}.[56]

Metal Release. Release of metal ions from MT clusters can be induced by lowering the pH, by chelating agents, or by modification of the thiolate sulfurs.[57] The pH for half-dissociation (pH at which the bound zinc/protein ratio is half that of fully loaded protein—this does not necessarily mean that each protein molecule contains half the amount of zinc: ESI−MS experiments suggest that for SmtA half the protein molecules are apo and that the remainder are fully loaded) of Zn^{II} from MT-2 is about 4.5 and for Cd^{II}, 3.5, compared to 4.1 for half-dissociation of Zn^{II} from SmtA.[11]

Polyaminocarboxylates such as EDTA and NTA can extract Zn^{II} and Cd^{II} efficiently from MTs.[58] The removal of Zn^{II} from MTs by these chelators proceeds much faster than the rate of dissociation of Zn^{II} from MT, suggesting direct attack by the chelator and emphasizing that access to the metal is an important factor in these reactions.

Reactions of MTs with EDTA are relatively slow and can be observed by ^{1}H NMR methods.[59] In general, metal depletion of both zinc fingers[60,61] and MTs[62] leads to the loss of an ordered protein structure. Interestingly, the release of Zn^{II} from SmtA leads to a long-lived metal-

depleted folded intermediate characterized by new amide NH peaks (Figure 11). The end product of the EDTA reaction is unfolded apo-SmtA, which suggests that once disassembly of the cluster has begun site A eventually also becomes accessible to metal chelators.

Conclusions

Mammalian MTs are well-known cysteine-rich proteins containing tetra- and trinuclear Zn_4Cys_{11} and Zn_3Cys_9 clusters and little or no secondary structure. Metal uptake, release, and exchange in the clusters is facile. We have used multinuclear NMR spectroscopy and electrospray mass spectrometry to elucidate the structure, composition, and dynamics of bacterial MTs. Our studies show that the cyanobacterial MT SmtA contains a $Zn_4Cys_9His_2$ cluster with a similar topology as mammalian Zn_4 clusters, despite the lack of protein sequence homology and the presence of two His N ligands. The presence of a β bridge, β hairpin, and an α helix, together with backbone NH to coordinated-Cys thiolate-S hydrogen bonding, render one zinc in the cyanobacterial Zn_4 cluster inert to metal exchange. This Zn^{II} ion together with the associated secondary structure is related to a functionally diverse set of so-called "treble-clef" zinc-finger proteins, which have specific interaction partners, including DNA, RNA, and other proteins. It may therefore be possible to identify specific recognition partners for SmtA, a task that has proven difficult for mammalian MTs.

Protein sequences homologous to SmtA can be identified in various bacterial genomes, including those of pseudomonads. These proteins adopt the same "treble-clef"-like fold as SmtA, but their metal-ion stoichiometries and exchange dynamics differ considerably. Alteration of a single cluster ligand results in the loss of the inertness of Zn(A), demonstrating that not only the protein structure but also the presence of a complete Zn_4 cluster is necessary for creating an inert site.

Our understanding of the roles of zinc thiolate clusters in biology is highly dependent on understanding not only the thermodynamics but also the kinetics of their assembly and disassembly. Intermediates in the loading/unloading pathways, even if short-lived, may have important biological roles and medical consequences and could include protonated and metal-deficient states, as well as ternary complexes. Further studies of the unusual coordination chemistry of metal thiolate clusters in proteins may therefore find important applications.

References

(1) Williams, R. J. P.; Da Silva, J. J. R. F. Evolution Was Chemically Constrained. *J. Theor. Biol.* **2003**, *220*, 323–343.

(2) Saito, M. A.; Sigman, D. M.; Morel, F. M. M. The Bioinorganic Chemistry of the Ancient Ocean: the Co-Evolution of Cyanobacterial Metal Requirements and Biogeochemical Cycles at the Archean-Proterozoic Boundary. *Inorg. Chim. Acta* **2003**, *356*, 308–318.

(3) Blencowe, D. K.; Morby, A. P. Zn^{II} Metabolism in Prokaryotes. *FEMS Mircobiol. Rev.* **2003**, *27*, 291–311.

(4) Morby, A. P.; Turner, J. S.; Huckle, J. W.; Robinson, N. J. SmtB is a Metal-Dependent Repressor of the Cyanobacterial Metallothionein Gene *smtA*: Identification of a Zinc Inhibited DNA–Protein Complex. *Nucleic Acids Res.* **1993**, *21*, 921–925.

(5) Busenlehner, L. S.; Pennella, M. A.; Giedroc, D. P. The SmtB/ArsR Family of Metalloregulatory Transcriptional Repressors: Structural Insights into Prokaryotic Metal Resistance. *FEMS Microbiol. Rev.* **2003**, *27*, 131–143.

(6) Robinson, N. J.; Whitehall, S. K.; Cavet, J. S. Microbial Metallothioneins. *Adv. Microb. Physiol.* **2001**, *44*, 183–213.

(7) Olafson, R. W.; Abel, K.; Sim, R. G. Prokaryotic Metallothionein—Preliminary Characterization of a Blue-Green-Alga Heavy Metal-Binding Protein. *Biochem. Biophys. Res. Commun.* **1979**, *89*, 36–43.

(8) Higham, D. P.; Sadler, P. J.; Scawen, M. D. Cadmium-Resistant *Pseudomonas putida* Synthesizes Novel Cadmium Proteins. *Science* **1984**, *225*, 1043–1046.

(9) Olafson, R. W.; McCubbin, W. D.; Kay, C. M. Primary- and Secondary-Structural Analysis of a Unique Prokaryotic Metallothionein from a *Synechococcus* sp. cyanobacterium. *Biochem. J.* **1988**, *251*, 691–699.

(10) Huckle, J. W.; Morby, A. P.; Turner, J. S.; Robinson, N. J. Isolation of a Prokaryotic Metallothionein Locus and Analysis of Transcriptional Control by Trace-Metal Ions. *Mol. Microbiol.* **1993**, *7*, 177–187.

(11) Shi, J.; Lindsay, W. P.; Huckle, J. W.; Morby, A. P.; Robinson, N. J. Cyanobacterial Metallothionein Gene Expressed in *Eschericia coli*. Metal-Binding Properties of the Expressed Protein. *FEBS Lett.* **1992**, *303*, 159–163.

(12) Turner, J. S.; Morby, A. P.; Whitton, B. A.; Gupta, A.; Robinson, N. J. Construction of Zn^{2+}/Cd^{2+} Hypersensitive Cyanobacterial Mutants Lacking a Functional Metallothionein Locus. *J. Biol. Chem.* **1993**, *268*, 4494–4498.

(13) Daniels, M. J.; Turner-Cavet, J. S.; Selkirk, R.; Sun, H. Z.; Parkinson, J. A.; Sadler, P. J.; Robinson, N. J. Coordination of Zn^{2+} (and Cd^{2+}) by Prokaryotic Metallothionein—Involvement of His-Imidazole. *J. Biol. Chem.* **1998**, *273*, 22957–22961.

(14) Blindauer, C. A.; Harrison, M. D.; Parkinson, J. A.; Robinson, A. K.; Cavet, J. S.; Robinson, N. J.; Sadler, P. J. A Metallothionein Containing a Zinc Finger within a Four-Metal Cluster Protects a Bacterium from Zinc Toxicity. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 9593–9598.

(15) Altschul, S. F.; Madden, T. L.; Schaffer, A. A.; Zhang, J. H.; Zhang, Z.; Miller, W.; Lipman, D. J. Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs. *Nucleic Acids Res.* **1997**, *25*, 3389–3402.

(16) Blindauer, C. A.; Harrison, M. D.; Robinson, A. K.; Parkinson, J. A.; Bowness, P. W.; Sadler, P. J.; Robinson, N. J. Multiple Bacteria Encode Metallothioneins and SmtA-like Zinc Fingers. *Mol. Microbiol.* **2002**, *45*, 1421–1432.

(17) Liu, T.; Nakashima, S.; Hirose, K.; Shibasaka, M.; Katsuhara, M.; Ezaki, B.; Giedroc, D. P.; Kasamo, K. A. A Novel Cyanobacterial SmtB/ArsR Family Repressor Regulates the Expression of a CPx-ATPase and a Metallothionein in Response to Both Cu^I/Ag^I and Zn^{II}/Cd^{II}. *J. Biol. Chem.* **2004**, *279*, 17810–17818.

(18) Outten, C. E.; O'Halloran, T. V. Femtomolar Sensitivity of Metalloregulatory Proteins Controlling Zinc Homeostasis. *Science* **2001**, *292*, 2488–2492.

(19) VanZile, M. L.; Cosper, N. J.; Scott, R. A.; Giedroc, D. P. The Zinc Metalloregulatory Protein *Synechococcus* PCC7942 SmtB Binds a Single Zinc Ion per Monomer with High Affinity in a Tetrahedral Coordination Geometry. *Biochemistry* **2000**, *39*, 11818–11829.

(20) Davis, S. R.; Cousins, R. J. Metallothionein Expression in Animals: A Physiological Perspective on Function. *J. Nutr.* **2000**, *130*, 1085–1088.

(21) Krezel, A.; Wojcik, J.; Maciejczyk, M.; Bal, W. May GSH and L-His Contribute to Intracellular Binding of Zinc? Thermodynamic and Solution Structural Study of a Ternary Complex. *Chem. Commun.* **2003**, 704–705.

(22) Borrelly, G. P. M.; Blindauer, C. A.; Schmid, R.; Butler, C. S.; Cooper, C. E.; Harvey, I.; Sadler, P. J.; Robinson, N. J. A Novel Copper Site in a Cyanobacterial Metallochaperone. *Biochem. J.* **2004**, *378*, 293–297.

(23) Culotta, V. C.; Lin, S.-J.; Schmidt, P.; Klomp, L. W. J.; Casareno, R. L. B.; Gitlin, J. Intracellular Pathways of Copper Trafficking in Yeast and Humans. *Adv. Exp. Med. Biol.* **1999**, *448*, 247–254.

(24) Cobine, P.; Wickramasinghe, W. A.; Harrison, M. D.; Weber, T.; Solioz, M.; Dameron, C. T. The *Enterococcus hirae* Copper Chaperone CopZ Delivers Copper(I) to the CopY Repressor. *FEBS Lett.* **1999**, *445*, 27–30.

(25) Palmiter, R. D. The Elusive Function of Metallothioneins. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 8428–8430.

(26) Huang, M.; Shaw, C. F., III; Petering, D. H. Interprotein Metal Exchange Between Transcription Factor IIIa and Apo-Metallothionein. *J. Inorg. Biochem.* **2004**, *98*, 639−648.

(27) Costello, L. C.; Guan, Z.; Franklin, R. B.; Feng, P. Metallothionein Can Function as a Chaperone for Zinc Uptake Transport into Prostate and Liver Mitochondria. *J. Inorg. Biochem.* **2004**, *98*, 664−666.

(28) Messerle, B. A.; Schaeffer, A.; Vašák, M.; Kägi, J. H. R.; Wüthrich, K. Comparison of the Solution Conformations of Human [Zn$_7$]-Metallothionein-2 and [Cd$_7$]-Metallothionein-2 Using Nuclear Magnetic Resonance Spectroscopy. *J. Mol. Biol.* **1992**, *225*, 433−443.

(29) Zhang, X.; Tamaru, H.; Khan, S. I.; Horton, J. R.; Keefe, L. J.; Selker, E. U.; Cheng, X. D. Structure of the *Neurospora* SET Domain Protein DIM-5, a Histone H3 Lysine Methyltransferase. *Cell* **2002**, *111*, 117−127.

(30) Dance, I.; Fisher, K.; Lee, G. In *Metallothioneins: Synthesis, Structure, and Properties of Metallothioneins, Phytochelatins, and Metal−Thiolate Complexes*; Stillman, M. J., Shaw, C. F., III, Suzuki, K. T., Eds.; VCH: New York, 1992; pp 284−345.

(31) Henkel, G.; Krebs, B. Metallothioneins: Zinc, Cadmium, Mercury, and Copper Thiolates and Selenates Mimicking Protein Active Site Features—Structural Aspects and Biological Implications. *Chem. Rev.* **2004**, *104*, 801−824.

(32) González-Duarte, P. *Metallothioneins. Comprehensive Coordination Chemistry II*; Elsevier: Amsterdam, The Netherlands, 2003; Vol. 8., 213−228.

(33) Burth, R.; Gelinsky, M.; Vahrenkamp, H. Controlled Formation of Tri- and Octanuclear Benzylthiolate Complexes of Zinc. *Inorg. Chem.* **1998**, *37*, 2833−2836.

(34) Consortium, I. H. G. S. Initial Sequencing and Analysis of the Human Genome. *Nature* **2001**, *409*, 860−921.

(35) Klug, A.; Rhodes, D. "Zinc Fingers": A Novel Protein Motif for Nucleic Acid Recognition. *Trends Biochem. Sci.* **1987**, *12*, 464−469.

(36) Krishna, S. S.; Majumdar, I.; Grishin, N. V. Structural Classification of Zinc Fingers. *Nucleic Acids Res.* **2003**, *31*, 532−550.

(37) Grishin, N. V. Treble Clef Finger—A Functionally Diverse Zinc-Binding Structural Motif. *Nucleic Acids Res.* **2001**, *29*, 1703−1714.

(38) Omichinski, J. G.; Clore, G. M.; Schaad, O.; Felsenfeld, G.; Trainor, C.; Appella, E.; Stahl, S. J.; Gronenborn, A. M. NMR Structure of a Specific DNA Complex of Zn-Containing DNA Binding Domain of GATA-1. *Science* **1993**, *261*, 438−446.

(39) Kontaxis, G.; Konrat, R.; Krautler, B.; Weiskirchen, R.; Bister, K. Structure and Intramodular Dynamics of the Amino-Terminal LIM Domain From Quail Cysteine- and Glycine-Rich Protein CRP2. *Biochemistry* **1998**, *37*, 7127−7134.

(40) Raaijmakers, H.; Vix, O.; Toro, I.; Golz, S.; Kemper, B.; Suck, D. X-ray Structure of T4 Endonuclease VII: A DNA Junction Resolvase with a Novel Fold and Unusual Domain-Swapped Dimer Architecture. *EMBO J.* **1999**, *18*, 1447−1458.

(41) Ettema, T. J. G.; Huynen, M. A.; de Vos, W. M.; van der Oost, J. TRASH: A Novel Metal-Binding Domain Predicted to be Involved in Heavy-Metal Sensing, Trafficking, and Resistance. *Trends Biochem. Sci.* **2003**, *28*, 170−173.

(42) Simonson, T.; Calimet, N. Cys(x)His(y)-Zn^{2+} Interactions: Thiol vs Thiolate Coordination. *Proteins* **2002**, *49*, 37−48.

(43) Ejnik, J.; Robinson, J.; Zhu, J. Y.; Försterling, H.; Shaw, C. F., III; Petering, D. H. Folding Pathway of Apo-Metallothionein Induced by Zn^{2+}, Cd^{2+}, and Co^{2+}. *J. Inorg. Biochem.* **2002**, *88*, 144−152.

(44) Otvos, J. D.; Liu, X.; Li, H.; Shen, G.; Basti, M. Dynamic Aspects of Metallothionein Structure. *Metallothionein III [Int. Conf. Metallothionein] 3rd* **1993**, 57−74.

(45) Romero-Isart, N.; Vašák, M. Advances in the Structure and Chemistry of Metallothioneins. *J. Inorg. Biochem.* **2002**, *88*, 388−396.

(46) Kelly, E. J.; Quaife, C. J.; Froelick, G. J.; Palmiter, R. D. Metallothionein I and II Protect Against Zinc Deficiency and Zinc Toxicity in Mice. *J. Nutr.* **1996**, *126*, 1782−1790.

(47) Krezoski, S. K.; Villalobos, J.; Shaw, C. F.; Petering, D. H. Kinetic Lability of Zinc Bound to Metallothionein in Ehrlich Cells. *Biochem. J.* **1988**, *255*, 483−491.

(48) Klaassen, C. D.; Choudhuri, S.; McKim, J. M.; Lehman-McKeeman, L. D.; Kershaw, W. C. In-Vitro and in-Vivo Studies on the Degradation of Metallothionein. *Environ. Health Perspect.* **1994**, *102*, 141−146.

(49) Meplan, C.; Richard, M. J.; Hainaut, P. Metalloregulation of the Tumor Suppressor Protein p53: Zinc Mediates the Renaturation of p53 After Exposure to Metal Chelators in Vitro and in Intact Cells. *Oncogene* **2000**, *19*, 5227−5236.

(50) Blindauer, C. A.; Polfer, N. C.; Keiper, S. E.; Harrison, M. D.; Robinson, N. J.; Langridge-Smith, P. R. R.; Sadler, P. J. Inert Site in a Protein Zinc Cluster: Isotope Exchange by High-Resolution Mass Spectrometry. *J. Am. Chem. Soc.* **2003**, *125*, 3226−3227.

(51) Nettesheim, D. G.; Engeseth, H. R.; Otvos, J. D. Products of Metal Exchange-Reactions of Metallothionein. *Biochemistry* **1985**, *24*, 6744−6751.

(52) Krizek, B. A.; Merkle, D. L.; Berg, J. M. Ligand Variation and Metal-Ion Binding-Specificity in Zinc Finger Peptides. *Inorg. Chem.* **1993**, *32*, 937−940.

(53) Vašák, M.; Kägi, J. H. R. Spectroscopic Properties of Metallothionein. *Met. Ions Biol. Syst.* **1983**, *15*, 213−273.

(54) Schultze, P.; Wörgötter, E.; Braun, W.; Wagner, G.; Vašák, M.; Kägi, J. H. R.; Wüthrich, K. Conformation of [Cd$_7$]-Metallothionein-2 from Rat Liver in Aqueous Solution Determined by Nuclear Magnetic Resonance Spectroscopy. *J. Mol. Biol.* **1988**, *203*, 351−268.

(55) Pountney, D. L.; Fundel, S. M.; Birchler, P. F. E.; Hunziker, P.; Vašák, M. Isolation, Primary Structures, and Metal Binding Properties of Neuronal Growth Inhibitory Factor (GIF) from Bovine and Equine Brain. *FEBS Lett.* **1994**, *345*, 193−197.

(56) Roschitzki, B.; Vašák, M. Redox Labile Site in a Zn$_4$ Cluster of Cu$_4$,Zn$_4$-Metallothionein-3. *Biochemistry* **2003**, *42*, 9822−9828.

(57) (a) Munoz, A.; Petering, D. H.; Shaw, C. F., III Reactions of Electrophilic Reagents that Target the Thiolate Groups of Metallothionein Clusters: Preferential Reaction of the α-Domain with 5,5′-Dithio-bis(2-nitrobenzoate) (DTNB) and Aurothiomalate (AuSTm). *Inorg. Chem.* **1999**, *38*, 5655−5659. (b) Jacob, C.; Maret, W.; Vallee, B. L. Control of zinc transfer between thionein, metallothionein, and zinc proteins. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3489−3494.

(58) Petering, D. H.; Krezoski, S.; Chen, P.; Pattanaik, A.; Shaw, C. F., III In *Metallothioneins: Synthesis, Structure, and Properties of Metallothioneins, Phytochelatins, and Metal−Thiolate Complexes*; Stillman, M. J., III, C. F. S., Suzuki, K. T., Eds.; VCH: New York, 1992.

(59) Nicholson, J. K.; Sadler, P. J.; Vašák, M. Probing the Reactivity of the Zinc and Cadmium Ions Bound to Rabbit Liver Metallothioneins with EDTA. *Experientia* **1987**, *52*, 191−201.

(60) Frankel, A. D.; Berg, J. M.; Pabo, C. O. Metal-Dependent Folding of a Single Zinc Finger from Transcription Factor IIIA. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 4841−4845.

(61) Berg, J. M.; Godwin, H. A. Lessons from Zinc-Binding Peptides. *Annu. Rev. Biophys. Biomol. Struct.* **1997**, *26*, 357−371.

(62) Galdes, A.; Vašák, M.; Hill, H. A. O.; Kägi, J. H. R. Proton NMR Spectra of Metallothioneins. *FEBS Lett.* **1978**, *92*, 17−21.