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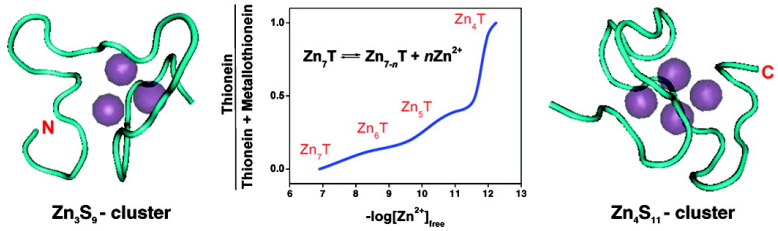

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Dual Nanomolar and Picomolar Zn(II) Binding Properties of Metallothionein

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Abstract: Each of the seven Zn(II) ions in the Zn_3S_9 and Zn_4S_{11} clusters of human metallothionein is in a tetrathiolate coordination environment. Yet analysis of Zn(II) association with thionein, the apoprotein, and analysis of Zn(II) dissociation from metallothionein using the fluorescent chelating agents FluoZin-3 and RhodZin-3 reveal at least three classes of sites with affinities that differ by 4 orders of magnitude. Four Zn(II) ions are bound with an apparent average log *K* of 11.8, and with the methods employed, their binding is indistinguishable. This binding property makes thionein a strong chelating agent. One Zn(II) ion is relatively weakly bound, with a log *K* of 7.7, making metallothionein a zinc donor in the absence of thionein. The binding data demonstrate that Zn(II) binds with at least four species: Zn_4T, Zn_5T, Zn_6T, and Zn_7T. Zn_5T and Zn_6T bind Zn(II) with a log *K* of ~10 and are the predominant species at micromolar concentrations of metallothionein in cells. Central to the function of the protein is the reactivity of its cysteine side chains in the absence and presence of Zn(II). Chelating agents, such as physiological ligands with moderate affinities for Zn(II), cause dissociation of Zn(II) ions from metallothionein at pH 7.4 ($Zn_7T \leftrightarrows Zn_{7-n}T + nZn^{2+}$), thereby affecting the reactivity of its thiols. Thus, the rate of thiol oxidation increases in the presence of Zn(II) acceptors but decreases if more free Zn(II) becomes available. Thionein is such an acceptor. It regulates the reactivity and availability of free Zn(II) from metallothionein. At thionein/metallothionein ratios > 0.75, free Zn(II) ions are below a pZn ($-\log[Zn^{2+}]_{free}$) of 11.8, and at ratios < 0.75, relatively large fluctuations of free Zn(II) ions are possible (pZn between 7 and 11). These chemical characteristics match cellular requirements for Zn(II) and suggest how the molecular structures and redox chemistries of metallothionein and thionein determine Zn(II) availability for biological processes.

Introduction

Metallothionein (MT) was discovered in 1957.[1] Mammalian MT is a dumbbell-shaped molecule with two domains. In one domain nine cysteinyl residues bind three Zn(II) ions, and in the other domain eleven cysteinyl residues bind four Zn(II) ions, forming two metal/thiolate clusters.[2,3] Each Zn(II) is in a tetrathiolate coordination environment, although the use of bridging thiolates causes an overall deficit of eight ligands in the two clusters when compared to mononuclear tetrathiolate coordination. A quest for a function of MT has been ongoing for 50 years. Studies of its remarkable coordination chemistry and structure have provided only limited insights into its biological function. Since bioinorganic chemistry is context-specific, insights from biology are required to direct the search for a function. In its cellular environment, MT is a redox-active protein, in which sulfur-ligand-centered chemistry confers redox activity on the clusters, a property that gains significance only in the context of biological redox partners.[4] Another example, where the biological context had been largely ignored, is the focus on only the structure of MT in attempts to explain its function, while in fact the apoprotein thionein (T) is present in tissues and cells at concentrations commensurate with those of MT.[5−7] Since T is a chelating agent, the fact that it is not saturated with Zn(II) *in vivo* demonstrates limited cellular availability and buffering of Zn(II) ions. In essence, the redox state and the metal load of the biologically active molecule must reflect the cellular milieu, which does not appear to be compatible with fully reduced and fully metal-loaded MT.[7] Additional knowledge from biological research is needed to interpret the results from chemical studies. For example, a purely chemical characterization of the Zn(II) binding properties of MT provides values of Zn(II) binding constants that become meaningful only in the context of the distinctive features of biological Zn(II) chemistry, such as the very low concentrations of cellular free Zn(II) ions ($[Zn^{2+}]_{free}$).[8−13]

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Zinc is a constituent of hundreds of enzymes and thousands of proteins with zinc finger domains, and it needs to be redistributed and made available to supply newly synthesized apo-metalloproteins with the Zn(II) ion. However, the availability of Zn(II) must be regulated tightly, because increases of cellular $[Zn^{2+}]_{free}$ are potent inhibitors of enzymes; are employed for biological control, such as gene expression; occur when cells proliferate, differentiate, or undergo programmed cell death (apoptosis); can cause protein misfolding; and, if sustained at higher levels for longer periods of time, are cytotoxic.[14−16] A complex network of proteins, including zinc transporters of the ZIP (SLC39) and ZnT (SLC30) families, zinc sensors such as metal response element-binding transcription factor-1 (MTF-1), and MTs, participate in cellular and subcellular zinc trafficking and homeostasis.[17−20] The present chemical studies seek an understanding of how MT and T control $[Zn^{2+}]_{free}$ availability. Sensitive fluorimetric detection methods based on chelating agents with high quantum yields upon the binding of Zn(II), the availability of relatively large amounts of expressed and highly purified MT, and redox control with the reducing agent tris(2-carboxyethyl)phosphine hydrochloride (TCEP), which has negligible affinity for Zn(II), make it feasible to examine the coordination dynamics of the zinc/thiolate clusters.[21−25] A remarkable heterogeneity in the affinity of MT for Zn(II) is found. It includes a weak Zn(II) binding site that makes significantly more Zn(II) available than when Zn(II) availability is based on average stability constants of 2×10^{12} M^{-1} (pH 7.0) for the seven Zn(II) ions.[26] In the presence of T, Zn(II) is not available at concentrations governed by the weak binding site in MT. The data demonstrate that MT and T can buffer Zn(II) in a much wider range of pZn ($-\log[Zn^{2+}]_{free}$) than previously postulated on the basis of average tight binding of Zn(II).[27] Competition of T or other Zn(II) acceptors with MT for Zn(II) modulates the thiol reactivity of MT. When the number of acceptor sites increases, Zn(II) dissociates from MT and the protein becomes more susceptible to oxidation, with concomitant dissociation of more Zn(II). The remarkably profound binding and redox properties of the zinc/thiolate clusters suggest ways of how the protein can participate in Zn(II) buffering in tissues that may require different Zn(II) availabilities.

(8) "Free" Zn(II) has been referred to as "freely available", "labile", or "rapidly exchangeable" Zn(II) that is readily bound to chelating agents. Either term is an operational definition and has its limitations. For the lack of a better term, we use the term free Zn(II), albeit with the understanding that the chemical nature of cellular ligands of Zn(II) is unknown.

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Experimental Section

Materials. Trizma base (Tris), Bis-Tris propane, glycine, bicine, L-alanine, 2,2′-dipyridyl, 2,2′-dithiodipyridine (DTDP), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), dithiotreitol (DTT), adenosine 5′-triphosphate disodium salt (ATP), nitrilotriacetic acid, trisodium salt monohydrate (NTA), ethylenediamine-*N*,*N*′-diacetic acid (EDDA), ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and ethylenediaminetetraacetate disodium salt dihydrate (EDTA) were purchased from Sigma-Aldrich. FluoZin-3 tetrapotassium salt, RhodZin-3 dipotassium salt, and TCEP·HCl were from Invitrogen. Perchloric acid double distilled (70%) was from GFC Chemicals Inc. *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) was OmniPure grade from EMD Chemicals Inc. Boric acid, zinc sulfate heptahydrate, hydrochloric acid (37%), formic acid (88%), sodium hydroxide, potassium hydroxide, sodium perchlorate monohydrate, potassium nitrate, sodium citrate dihydrate, potassium phosphate monobasic and dibasic, anhydrous were from Fisher. L-Histidine was from AppliChem GmbH, and Chelex 100 Resin (100−200 mesh, sodium form) was from BioRad.

Thionein Expression and Purification/Preparation of Metallothionein. Human T, isoform 2, (MDPNCSCAAGDSCTCAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKGASDKCSCCA) was expressed in *Escherichia coli* as an intein fusion protein without addition of metal ions to the growth medium, and the T was purified by affinity chromatography (IMPACT system, New England Biolabs Inc.).[24] T, obtained after intein cleavage induced by 0.1 M DTT, was acidified to pH 1 with 1.2 M HCl and spun at $14\,000 \times g$ for 10 min before loading onto a Sephadex G-25 (Amersham Biosciences) gel filtration column (1 cm × 120 cm). The column was eluted with 0.01 M HCl (degassed). Concentrations of T in the fractions were determined spectrophotometrically ($\epsilon_{220} = 4.8 \times 10^4$ M^{-1} cm^{-1}) and by thiol assays with DTNB and DTDP.[28] Thionein was stored at pH 2 at liquid nitrogen temperatures or used immediately for the experiments. The identity and purity of T were confirmed by mass spectrometry with a MALDI-TOF Voyager DE STR spectrometer (Applied Biosystems) (Biomolecular Resource Facility core at UTMB). The *m*/*z* values found/calculated were 6042.8/6043.2 $(M + H)^+$. Aliquots of purified T were mixed with zinc sulfate at a molar ratio of 1:10 under a nitrogen atmosphere, and the pH adjusted to 8.6 with a 1 M solution of Tris base. The sample was concentrated with Millipore Centricon centrifugal microconcentrators (MWCO 3,000), loaded onto a Sephadex G-50 (Amersham Biosciences) column (1 cm × 120 cm), and eluted with 20 mM Tris·HCl, pH 8.6. The concentration of MT was determined spectrophotometrically ($\epsilon_{220} = 1.59 \times 10^5$ M^{-1} cm^{-1}).[28] Zn(II) and thiols were determined by flame atomic absorption spectrophotometry and a thiol assay with DTDP, respectively.[29] Accordingly, MT contained 6.9 ± 0.2 Zn(II) and 20.3 ± 0.7 sulfhydryls per molecule.

Reduction of Thionein/Metallothionein. The use of a reducing agent is critical for the following experiments because T and MT are sensitive to oxidation. Since commonly employed reductants are thiols that can interfere with Zn(II) binding, the use of TCEP turns out to be critical. Zn(II) binding to TCEP is weak, with an apparent stability constant of 3×10^2 M^{-1} (pH 7.4) for the Zn(II)−TCEP complex. Therefore, submillimolar concentrations of TCEP do not compete with MT for Zn(II).[25] In contrast, DTT, a thiol-based reductant, binds Zn(II) with an apparent stability constant of 8×10^7 M^{-1}.[30]

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General Spectroscopic Methods. HEPES and borate buffers containing KNO_3 and $NaClO_4$, respectively, and TCEP were prepared fresh, the pH value adjusted to 7.4 with pure concentrated solutions of HCl, $HClO_4$, or NaOH, and stored with 2% (w/v) Chelex 100 overnight. Zn(II) stock solutions (50 mM, 1 mM, and 100 μM) were prepared from 99.5% $ZnSO_4 \times 7H_2O$, and their titers were determined by atomic absorption spectrophotometry. FluoZin-3 and RhodZin-3 potassium salts were dissolved in Milli-Q water to concentrations of about 1 mM and treated overnight with Chelex 100 resin. The purity of both dyes was greater than 97% by HPLC (Beckman Coulter, System Gold). Fluorimetric titrations with Zn(II) stock solutions were performed in triplicate to determine titers of 0.81 and 0.99 mM for FluoZin-3 and RhodZin-3, respectively.[13] TCEP and DTNB stock solutions were prepared daily in HEPES or borate buffers and filtered through Millipore 0.22 μm filters. A 500 μM DTDP stock solution for determination of thiols was prepared in 0.1 M formic acid and adjusted to pH 3.5 with NaOH.

UV Spectrophotometry. Electronic absorption spectra of T and MT were recorded at 25 °C with a Beckman Coulter DU 800 spectrophotometer in 1 cm quartz cuvettes. Stock solutions of T in 0.01 M HCl were diluted to final concentrations of 1 μM in degassed and Chelex-treated 50 mM borate, pH 7.4 ($I = 0.1$ from $NaClO_4$), 50 μM TCEP.

Fluorimetry. Fluorescence of FluoZin-3, RhodZin-3, and their Zn(II) complexes were measured at 25 °C in the ranges 480−560 nm and 530−670 nm with 492 and 550 nm excitation, respectively, with an SLM 8000 spectrofluorimeter with ISS data acquisition and Vinci Multidimensional Fluorescence Spectroscopy software. Measurements were performed in 1 cm × 1 cm fluorimetric PMMA (poly(methyl metacrylate)) cuvettes (Fisher). To avoid any carryover of fluorescent material, cuvettes were used only once.

MT was incubated with 0−5 μM FluoZin-3 or RhodZin-3 in 50 mM HEPES, pH 7.4 ($I = 0.1$ from KNO_3), 50 μM TCEP for 2 h. Maximum (F_{max}) and minimum (F_{min}) dye fluorescence were determined after adding zinc sulfate or EDTA to final concentrations of 100 and 500 μM, respectively. These values allow calibration of fluorescence response (F) and free Zn(II) concentration according to the formula $[Zn^{2+}]_{free} = K^{-1} \times (F - F_{min})/(F_{max} - F)$.

Reaction of Thionein and Metallothionein with DTNB in the Presence of Zn(II) Chelators and Zinc Sulfate. Oxidation of T or MT by DTNB was studied spectrophotometrically at equimolar concentrations of the reactants (1 μM) at 25 °C in buffered solutions, pH 7.4 ($I = 0.1$ from KNO_3) at 412 nm. Chelating agents (L-alanine, glycine, sodium citrate, ATP, L-histidine, 2,2′-dipyridyl, NTA, EDDA, EGTA, and EDTA) were added to final concentrations of 1 mM in 50 mM HEPES ($I = 0.1$ from KNO_3). The effects of 50 mM borate, phosphate, Tris, Bis-Tris propane, glycine, and bicine solutions at pH 7.4 were studied in the same way, while the effect of Zn(II) was studied in HEPES by adding 0−13 μM and 0−1000 μM of zinc sulfate to T and MT, respectively.

Unless noted otherwise, stability constants are given as apparent stability constants at pH 7.4. Because of different stoichiometries of some Zn(II)/ligand complexes (from 1:1 to 1:3 in terms of metal-to-ligand ratio), conditional stability constants, K', are expressed as the sum of every mononuclear Zn(II) complex over the product of $[Zn^{2+}]_{free}$ and free, unbound ligand (eq 1). According to such a definition, K' values vary at different pH values and at different reactant concentrations but are constant when the ligand is in large excess. This procedure allows comparison of the ligands on the basis of K' values under the same conditions (1 mM ligand over 1 μM Zn(II)) (Table S1), despite differences in coordination mode and acid−base properties of chelators. Stability and protonation constants were obtained from the Stability Constants Database − SCDbase.[31−45]

$$K' = \frac{\sum_{j,k} [ZnH_jL_k]}{[Zn^{2+}]_{free} \times \sum_j [H_jL]} \quad (1)$$

Competition of Metallothionein and NTA for Zn(II). The effect of NTA (10 μM − 20 mM) on Zn(II) release from MT (0.4 μM) was studied at 25 °C in 50 mM HEPES, pH 7.4, 50 μM TCEP ($I = 0.1$ from KNO_3). Samples incubated for 2 h were subjected to gel filtration on a G-25 Sephadex column (1 cm × 25 cm) using 50 mM HEPES, pH 7.4, containing TCEP as eluant at a flow rate of 10 mL/h. Fractions (0.5 mL) were diluted 3−5 times with 0.25 M HNO_3, and their zinc content was determined by graphite furnace atomic absorption spectrophotometry (Perkin-Elmer 5100 instrument). Complete Zn(II) transfer to EDTA was effected by incubating MT with EDTA (0.01 and 1 mM).

Results

Thionein as a Zn(II) Ligand. Zn(II) binding to a buffered solution of T can be studied by observing a ligand-to-metal charge transfer (LMCT) transition in the far UV region of the electronic absorption spectrum.[46] Spectral deconvolution analysis demonstrated at least three LMCT transitions centered at 231, 219, and 205 nm.[46] Analysis of the UV spectra (Figure 1A) by difference spectroscopy over the whole range of T titration with Zn(II) demonstrates a hypsochromic shift of the center of the bands from 222.4 to 217.8 nm (Figure 1B). After the addition of 5 equiv of Zn(II), the center remains at 217.8 nm (Figure S1) and the absorbance increases until 7 equiv of Zn(II) have been added (Figure 1C). UV spectral changes and titration breakpoints after addition of >3 equiv of Cd(II) have been detected in the formation of the cadmium/thiolate clusters in MT.[47] Furthermore, a hypsochromic shift of the absorption maximum occurs in the conversion of Cd_4T into Cd_7T as a result of significant decreases and increases of two bands at lower energy out of the three that constitute the absorption envelope.[48] Significantly, near the equivalence point (Figure 1C) the data points deviate considerably from a titration expected for tight Zn(II) binding with an average stability constant of 2×10^{12} M^{-1}, indicating that some Zn(II) is bound with weaker affinity.[26]

The strength of Zn(II) binding to T was investigated in the presence of competing chelating agents that fluoresce more strongly when binding Zn(II). FluoZin-3 and RhodZin-3 were chosen as Zn(II)-sensitive fluorescent probes with apparent stability constants at pH 7.4 of 1.1×10^8 for FluoZin-3 and 7.1×10^7 M^{-1} for RhodZin-3 (Figure S2).[13] They emit with high quantum yields at 517 nm (Zn-FluoZin-3) and 575 nm (Zn-RhodZin-3) when excited at 492 and 550 nm, respectively.[49,50] During titrations of T with zinc sulfate in the presence of the

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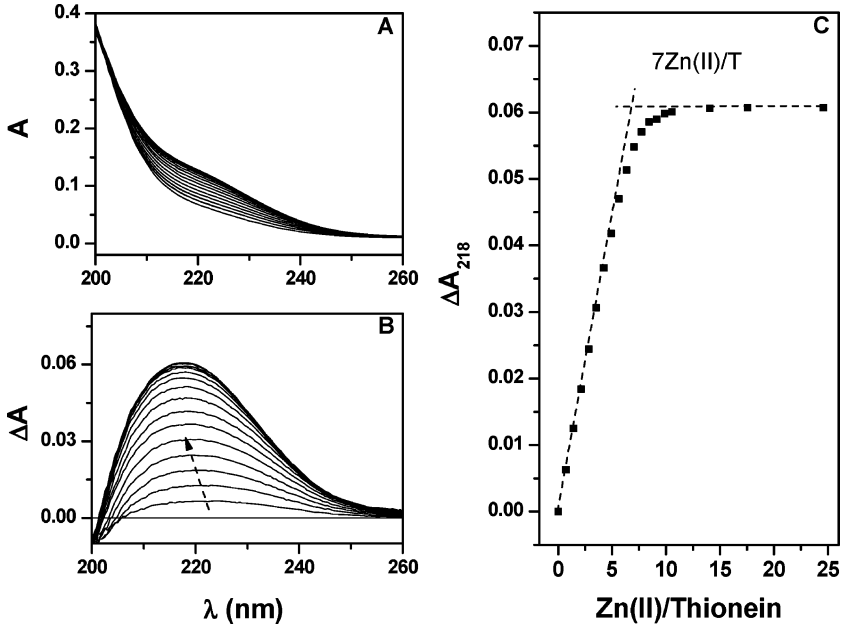

Figure 1. Spectrophotometric titration of thionein with Zn(II). T (1 μM) from a degassed stock solution in 0.01 M HCl was titrated with a 100 μM or 1 mM stock solution of Zn(II) to final concentrations of 0−25 μM in 50 mM borate, pH 7.4 (I = 0.1 from $NaClO_4$), 50 μM TCEP. (A) UV spectra. (B) UV-difference spectra obtained by subtracting the absorbance of the protein from each spectrum after adding Zn(II). The arrow indicates a hypsochromic shift of the center of the absorption envelope. (C) Absorbance changes at 218 nm as a function of the Zn(II)/T ratio.

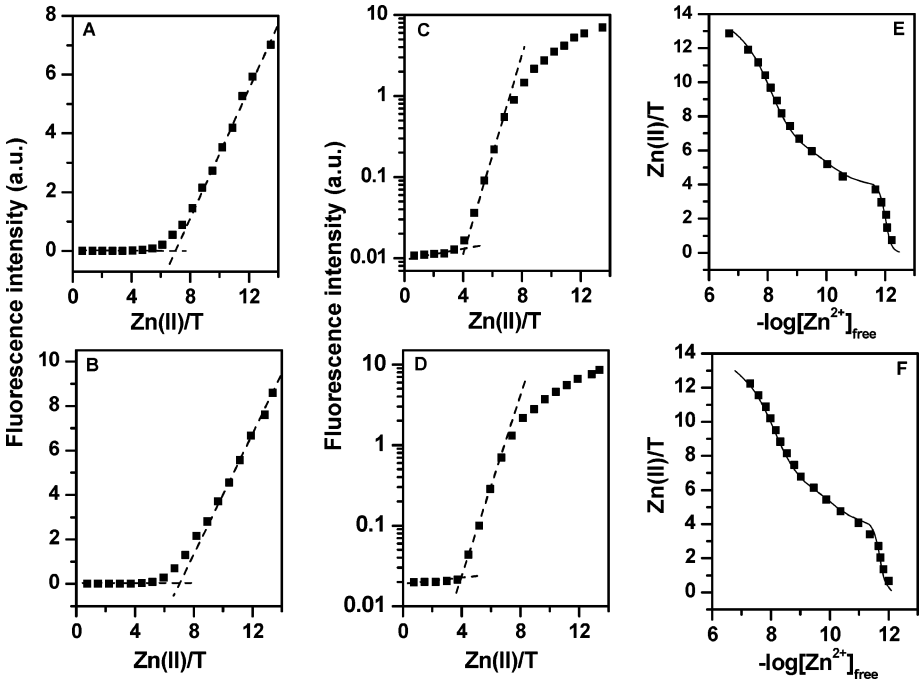

Figure 2. Titration of thionein with Zn(II) in the presence of FluoZin-3 (A) and RhodZin-3 (B). T (0.5 μM) in 50 mM HEPES, pH 7.4, 50 μM TCEP (I = 0.1), and 3.25 μM dye was titrated with Zn(II) to a final concentration of 13.5 μM. Fluorescence (au, arbitrary units) was measured at 517 and 575 nm with excitation at 492 and 550 nm for FluoZin-3 and RhodZin-3, respectively. Semilog plots of the same data are presented to visualize changes during titration with the first 7 equiv of Zn(II) (C and D). Fluorescence responses (A and B) were calibrated to obtain $[Zn^{2+}]_{free}$ and are shown as a function of Zn(II)/T (E and F). Data were fitted to a model with four independent species and their stability constants (Table 2), known concentrations of reagents, and stability constants of dyes, using species distribution software.[25,30] Stability constants for the two Zn(II) sites with intermediate affinity were chosen to obtain the best fit with the lowest χ^2.

fluorogenic dye, fluorescence increases significantly above Zn(II)/T ratios of 6 with a stoichiometry of seven Zn(II) ions (Figure 2A, B). A plot of fluorescence increase on a logarithmic scale reveals a titration breakpoint at four Zn(II) ions per protein (Figure 2C, D). Above this ratio, significant competition for Zn(II) occurs between partially Zn(II)-loaded T and FluoZin-3 or RhodZin-3. Thus, T binds four Zn(II) ions much more tightly than the fluorescent probes, while it binds an additional three Zn(II) ions more weakly. A two-step calibration of fluorescence allows determination of free Zn(II) ($-\log[Zn^{2+}]_{free}$) at various Zn(II)/T ratios (Figure 2E, F). Changes of $[Zn^{2+}]_{free}$ are small during the binding of the first four Zn(II) ions. Buffering of the additional three Zn(II) ions is weaker, resulting in significant changes of $[Zn^{2+}]_{free}$ over 3 orders of magnitude. Because of

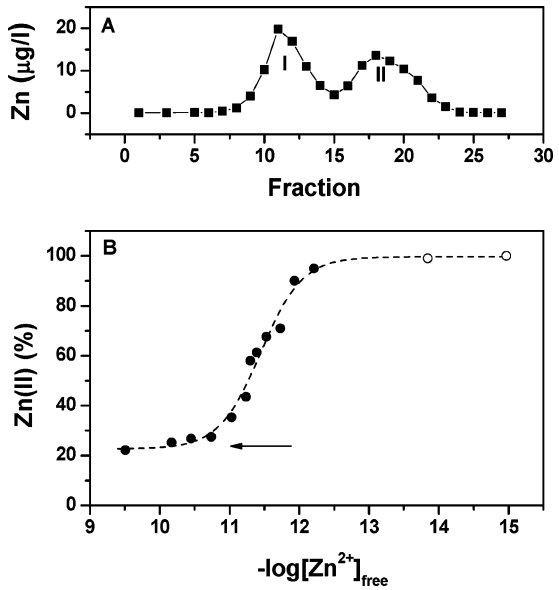

Figure 3. Zn(II) transfer from metallothionein to NTA. MT (0.4 μM) was incubated with NTA −●− (10 μM to 20 mM) or EDTA −○− (0.01 and 1 mM) in 50 mM HEPES, pH 7.4, 50 μM TCEP (I = 0.1). Samples equilibrated for 2 h were separated on Sephadex G-25 and fractions analyzed for Zn(II) with graphite furnace atomic absorption spectrophotometry. (A) Elution profile (gel filtration) of an MT sample treated with 1 mM NTA (−■−). Peaks I and II correspond to MT and the Zn(II)−NTA complex, respectively. (B) The percentage of Zn(II) transferred to NTA/EDTA was calculated by integrating the peak areas in the chromatograms and the data plotted as a function of free zinc, $-\log[Zn^{2+}]_{free}$. The arrow indicates a plateau that corresponds to ∼1.6 Zn(II) ions dissociated from MT at low concentrations of NTA.

this spread, the binding data reveal at least three additional sites with lower affinities than those of the four tightly bound Zn(II) ions. To further evaluate this finding, the Zn(II) binding characteristics of MT were investigated.

Metallothionein as a Zn(II) Donor. EDTA or the apoforms of zinc metalloproteins compete with MT for Zn(II).[51−55] Several low molecular weight agents with intermediate Zn(II) affinity, such as NTA, 4-(2-pyridylazo)resorcinol (PAR), terpyridyl, and 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazone), also compete with MT for Zn(II).[56−58] Despite its relatively low affinity for Zn(II), remarkably, micromolar concentrations of NTA release Zn(II) from 0.4 μM MT (Figure 3A). In this experiment, MT was incubated with different concentrations of NTA for 2 h and the products were separated by gel filtration. Collected fractions were analyzed for Zn(II) by atomic absorption spectrophotometry. Analysis of the areas of zinc peaks I (MT) and II (NTA) (Figure 3A) allows determination of the percentage of Zn(II) transferred. The amount of Zn(II) removed from MT depends on the NTA concentration. Lower concentrations of NTA, 10−200 μM, bind 22−27% of Zn(II) in MT, whereas higher concentrations, such as 20 mM, bind 95%. $[Zn^{2+}]_{free}$ was calculated according to eq 2:

$$[Zn^{2+}]_{free} = \frac{1}{K} \times \frac{c_{Zn} \times \frac{II}{I+II}}{c_{NTA} - c_{Zn} \times \frac{II}{I+II}} \qquad (2)$$

where K and c_{Zn}, c_{NTA} are the apparent stability constant of the Zn(II)−NTA complex (2.1×10^8 M^{-1} at pH 7.4) and the total concentrations of Zn(II) and NTA, respectively. Fitting a plot of $-\log[Zn^{2+}]_{free}$ vs $Zn(II)_{transferred}$ with Hill's equation gives a Zn(II) stability constant (log K) of 11.8 ± 0.2 (Hill coefficient n = 1.5 ± 0.2 within a range of $-\log[Zn^{2+}]_{free}$ from 9.5 to 15) (Figure 3B). It is significant that even a slight molar excess of NTA competes with Zn(II) bound to MT because the function levels off at about 20% Zn(II) transferred (arrow). Such competition is possible only if one or more Zn(II) ions are bound with an affinity lower than 11.8.

FluoZin-3 and RhodZin-3, with apparent stability constants of $\sim 10^8$ M^{-1}, compete with T for Zn(II) (Figure 2) and, therefore, were employed to examine whether or not they compete with MT for Zn(II) and, if so, to determine the stability constants of the weakly bound Zn(II) ion(s). A time-dependent increase of fluorescence is observed immediately after mixing the dyes with MT. It reaches a maximum after 15−90 min. Hence, fluorescence was determined after a 2-h equilibration. Plotting the dye concentration vs its fluorescence demonstrates saturation above 2 μM dye with equivalence points at 0.65 and 0.55 μM for FluoZin-3 and RhodZin-3, respectively (Figure 4). The competition of the dyes with MT for Zn(II) is close to a dye-to-protein ratio of 1:1 based on equivalence points of 0.65/0.5 = 1.3 and 0.55/0.5 = 1.1 (Figure 4). The percentage of Zn(II) bound to the dye was calculated after complete oxidation of MT with DTDP to effect 100% dissociation of Zn(II). FluoZin-3 (3−5 μM) binds up to 20% (1.3 Zn(II) ions) of total Zn(II) from MT, while RhodZin-3 binds about 16% (1.1 Zn(II) ions) at the same concentration (Figure 4A, B). $[Zn^{2+}]_{free}$ at each concentration of dye was calculated based on F, F_{max}, F_{min}, and the apparent stability constant of the Zn(II)−dye complex. Plotting $-\log[Zn^{2+}]_{free}$ versus dye fluorescence and fitting the data with Hill's equation yield stability constants of 7.8 (±0.1) and 7.7 (±0.1) (log K) for a weakly bound Zn(II) ion in MT from either FluoZin-3 or RhodZin-3 titrations (Figure 4).

Reactivities of T and MT with 5,5′-Dithiobis(2-nitrobenzoic acid). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was employed to determine how zinc-binding agents affect the thiol reactivity of MT. Based on the stoichiometry of the reaction between thiols and disulfides, a maximum of 10 molecules of DTNB can react with the 20 cysteines in one molecule of T if only intramolecular disulfides are formed.[59] To decrease the possibility of intramolecular disulfide formation, a stoichiometry of 1:1 (1:20 in terms of thiols) between oxidant and apoprotein was chosen (Figure 5). The pseudo-first-order kinetic constant (k_{obsd}) obtained from monitoring the absorbance increase of TNB at 412 nm depends on the Zn(II)/T ratio (Figure 5A).[60] Up to 4 equiv of Zn(II) ions drastically diminish thiol reactivity from

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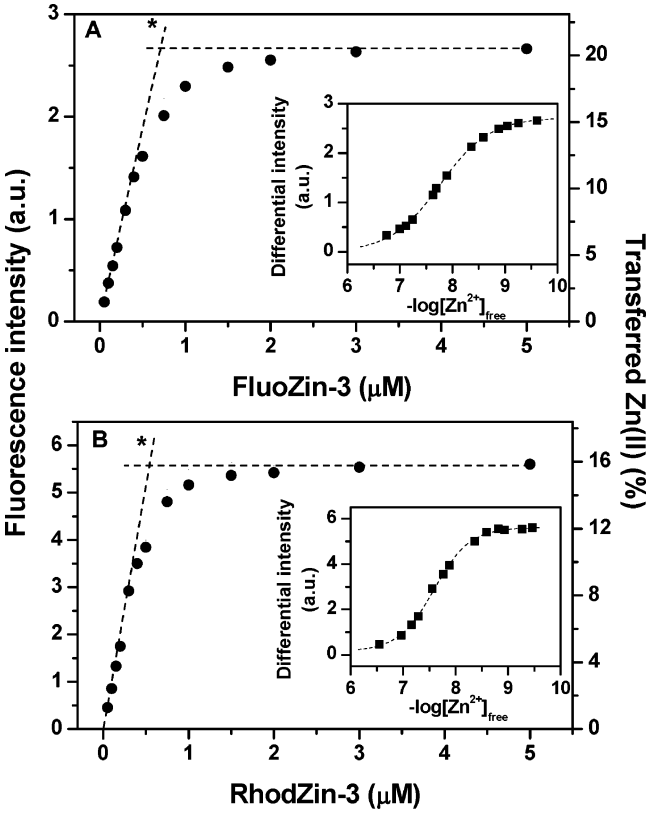

Figure 4. Zn(II) transfer from metallothionein to FluoZin-3 or RhodZin-3. MT (0.5 μM) was mixed with 0−5 μM FluoZin-3 (A) or RhodZin-3 (B) in 50 mM HEPES, pH 7.4, 50 μM TCEP ($I = 0.1$) and incubated for 2 h. Fluorescence was measured at 517 or 550 nm, and the background fluorescence of the dyes was subtracted and calibrated by adding Zn(II) sulfate or EDTA to final concentrations of 100 and 500 μM, respectively. Insets: $[Zn^{2+}]_{free}$ as a function of the dye concentrations (data fitted to Hill's equation). Asterisks indicate equivalence points of 0.65 and 0.55 μM for FluoZin-3 and RhodZin-3, respectively.

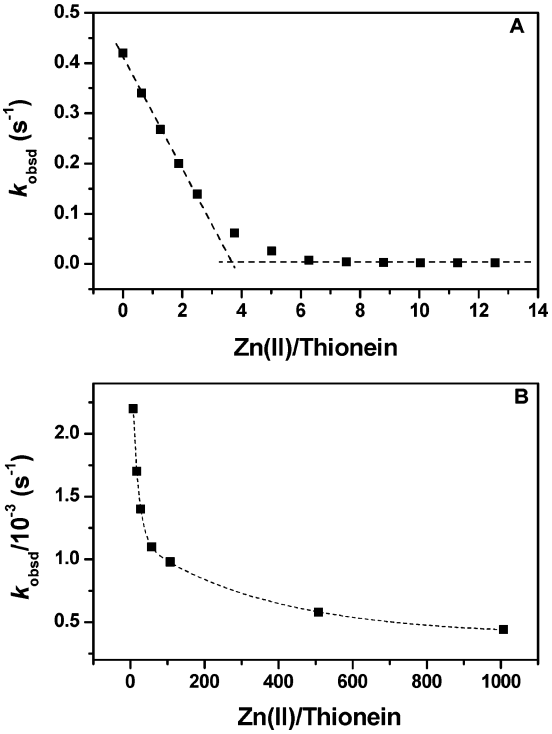

Figure 5. Oxidation of thionein with DTNB in the absence and presence of low (A) and high (B) concentrations of Zn(II). (A) Oxidation rates (k_{obsd}) of T with Zn(II). T (1 μM) was oxidized with 1 μM DTNB in 50 mM HEPES, pH 7.4 ($I = 0.1$) in the presence of 0−12.6 μM Zn(II). Different aliquots of T were mixed with a Zn(II) sulfate solution under a nitrogen stream and incubated at 25 °C for 30 s, and DTNB was added. (B) Oxidation rates (k_{obsd}) of MT as a function of an excess of Zn(II). Pseudo-first-order rate constants were determined from the linear rates of absorbance increase at 412 nm.

0.42 s^{-1} to 0.05 s^{-1}. After addition of 6 equiv, the rate is 0.008 s^{-1}. The rate decreases slightly upon further addition of zinc sulfate, but the reaction is not quenched completely. The results indicate that four to six Zn(II) ions engage virtually all of the cysteinyl residues of T in coordination. If the incomplete quenching is due to dissociation of weakly bound Zn(II) from MT, adding Zn(II) to MT is expected to shift the equilibrium between fully and partially Zn(II)-loaded MT to the former (eq 3), *thereby decreasing the reaction with DTNB* (emphasis added). Indeed, adding 10, 100, or 1000 μM Zn(II) to MT in HEPES reduces k_{obsd} to 1.4, 0.97, and 0.44 × 10^{-3} s^{-1} (Figure 5B), which is beyond the apparent limiting values in titrations of T with zinc sulfate (Figure 5A). Thus, the reaction rate is controlled by dissociation of weakly bound Zn(II) from MT, and even fully Zn(II)-loaded MT reacts with DTNB at pH 7.4.

$$Zn_7T \leftrightarrows Zn_{7-n}T + nZn^{2+} \quad (3)$$

These observations raise the possibility that relatively weak biological Zn(II)-binding agents compete with MT for Zn(II). If Zn(II) binding to MT is as strong as suggested by an average K of 10^{11}−10^{12} M^{-1}, the agents will bind virtually no Zn(II) from MT. If, on the other hand, the binding is as weak as suggested by the competition with FluoZin-3 and RhodZin-3, the agents will compete with MT for Zn(II) and their Zn(II)-binding properties may be important physiologically. The reactivity of MT with DTNB was employed to examine this question. Fully Zn(II)-loaded protein was mixed with DTNB in the presence of ligand, and absorbance readings at 412 nm were taken immediately. Pseudo-first-order rates were fitted to obtain k_{obsd}. In the absence of ligand, the value for MT in HEPES buffer is 2.2 × 10^{-3} s^{-1}. This value is identical to the one obtained from the reaction of DTNB with T when incubated with 7 equiv of Zn(II) (Figure 5A). Pseudo-first-order rates in the presence of weak physiological Zn(II) ligands are 4.3 (citrate), 4.9 (ATP), and 5.4 × 10^{-3} s^{-1} (L-histidine). Chelating agents with intermediate strength increase k_{obsd}: 1.2 (dipyridyl), 1.9 (NTA), 2.8 (EDDA), and 4.1 × 10^{-2} (EGTA) s^{-1} (Figure 6A, Table S1). The strongest chelating agent used, EDTA, increases k_{obsd} to 0.23 s^{-1}. At concentrations of 1 mM (vs 1 μM Zn(II)), glycine and L-alanine with conditional Zn(II) stability constants of 7.4 × 10^2 and 2.1 × 10^2 M^{-1} at pH 7.4, respectively, virtually do not affect the rate.[31,32] The ligands used bind Zn(II) with micromolar to femtomolar affinities at pH 7.4 (Table S1). Notably, the stronger the affinity of the chelating agent for Zn(II), the faster the oxidation of MT with DTNB (Figure 6A). A plot of the conditional stability constants of the ligands (log K') vs log k_{obsd} is linear over the entire range of chelating agents (Figure 6B, Table S1). The effects of chelating agents with relatively low Zn(II) affinity demonstrate that some Zn(II) in MT is bound with an affinity significantly lower than 11.8 (log K). Increased reactivity with higher affinities of the chelating agents indicates competition between MT and chelating agents for Zn(II) and an increase in the free thiol/thiolate ratio in partially Zn(II)-loaded MT.

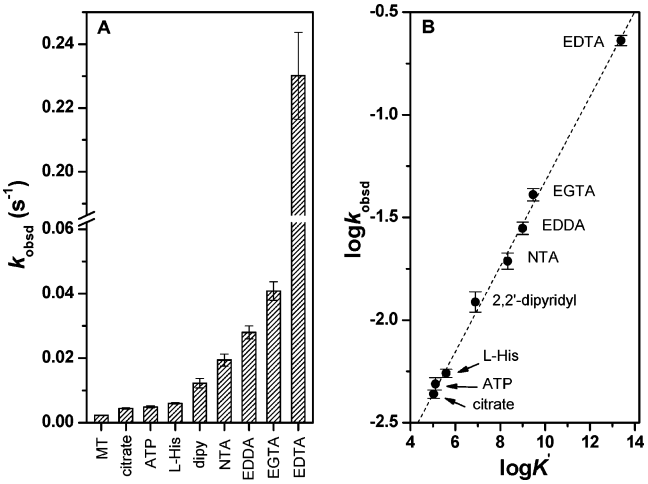

Figure 6. Effects of chelating agents and an excess of Zn(II) on the rate of metallothionein oxidation with DTNB. (A) MT (1 μM) was mixed with 1 mM chelator in 50 mM HEPES, pH 7.4 ($I = 0.1$) and 1 μM DTNB, and k_{obsd} was determined. (B) The affinity of each chelating agent was transformed to a conditional stability constant K' (eq 1, Table S1) and plotted against k_{obsd} ($R = 0.998$, $p < 0.0001$).

Table 1. Pseudo-first-order Rate Constants (k_{obsd}) of the Reaction between 1 μM Metallothionein and 1 μM DTNB in Different Buffers (50 mM, pH 7.4, $I = 0.1$)[a]

buffer (50 mM)	$k_{obsd} \times 10^3$ (s^{-1})
HEPES·Na^+	2.2 ± 0.1
borate·Na^+	2.1 ± 0.1
Tris·HCl	3.5 ± 0.1
Bis-Tris propane	5.6 ± 0.4
phosphate·K^+	5.7 ± 0.2
glycine	6.7 ± 0.3
bicine[a] (100 mM)	20.5 ± 0.3
(75 mM)	15.5 ± 0.5
(50 mM)	11.6 ± 0.8
(25 mM)	7.9 ± 0.4
(10 mM)	3.9 ± 0.1

[a] The dependence of k_{obsd} on the buffer concentration is given for bicine.

Importantly, rates of the reaction between MT and DTNB depend on the type of buffer, its Zn(II)-binding characteristics, and its concentration. The rate in borate is identical to that in HEPES (Table 1). In Tris·HCl or Bis-Tris propane, k_{obsd} is 2 and 3 times higher, respectively. Commonly used buffers (50 mM) increase the reactivity in the following order: HEPES $\approx$ borate < Tris·HCl < Bis-Tris propane $\approx$ phosphate < glycine < bicine (Table 1). This order correlates with the conditional stability constants of the Zn(II)/buffer complexes.[40−45] Glycine at a concentration of 50 mM affects MT/Zn(II) equilibria, while 1 mM has no measurable effect (see above). A similar concentration-dependent rate increase was observed with bicine, which binds Zn(II) tighter than glycine (Table 1).[45] In summary, chelating agents, Zn(II)-coordinating buffers, or Zn(II) modulate the reactivity of MT with an oxidant such as DTNB because these agents affect the equilibrium between fully and partially Zn(II)-loaded MT (eq 3). The thiol reactivity of MT with redox reagents depends on the availability of Zn(II) in solution. At low Zn(II) concentrations, the reactivity of MT is high, whereas at high Zn(II) concentrations its reactivity is correspondingly lower. Thus, in any study examining the thiol reactivity of MT, the choice of buffer is critical.

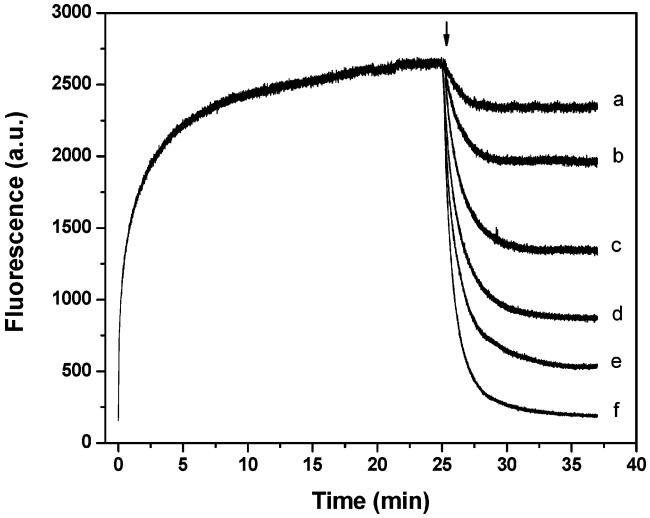

Figure 7. Competition between metallothionein and thionein for Zn(II) as monitored by FluoZin-3. MT (0.2 μM) was incubated with 2 μM FluoZin-3 for 25 min in 50 mM HEPES, pH 7.4, 50 μM TCEP ($I = 0.1$). At the end of the incubation (arrow), different amounts of T were added to final concentrations of 0.01 (a), 0.02 (b), 0.04 (c), 0.06 (d), 0.08 (e), and 0.14 μM (f). Only one trace for the initial increase of fluorescence is shown because each experiment gave identical results.

Effect of Thionein on Zn(II) Availability from Metallothionein. T is an excellent chelator that binds up to seven Zn(II) ions with different affinities. Further studies were designed to address the question of how MT and T jointly control the availability of free Zn(II). Two limiting conditions apply. If there were only Zn_4T, virtually no Zn(II) would be available above a pZn ($-\log[Zn^{2+}]_{free}$) of 11.8. If there were only Zn_7T (MT), too much Zn(II) would be available (pZn of about 7). Such pZn values are not compatible with estimates of picomolar concentrations of free Zn(II) in eukaryotic cells.[9,10,12,13] Since both MT and T are present in tissues and cells, it was investigated how the presence of T influences the availability of Zn(II) from MT. Upon addition of FluoZin-3 to MT, fluorescence increases immediately in a process with $t_{1/2} \approx 1$ min (Figure 7). However, the reaction is biphasic, and full equilibration is not reached until at least 1 h later. When T is added, Zn(II) is transferred from the dye almost instantaneously to form MT (Figure 7). Based on Zn(II) affinities, MT and T jointly should buffer Zn(II) in the pZn range from 7 to 11. To examine this buffering capacity, 5 μM FluoZin-3 was added to mixtures of MT and T that contain a final concentration of 1 μM total protein (MT + T). Fluorescence was measured after a 2-h equilibration. A plot of measured $-\log[Zn^{2+}]_{free}$ versus the fraction of T (T/(T + MT)) yields a buffering profile that clearly demarcates ranges of picomolar and nanomolar Zn(II) buffering (Figure 8). Importantly, buffering in the low picomolar range would require an excess of T over MT. Thus, from such a Zn(II) buffering system and in the presence of a competing dye as an acceptor, Zn(II) ions are available in the nanomolar to picomolar range, in which free Zn(II) ions have been observed by direct measurements in cells.[12,13]

Metallothionein Speciation. T has an apparent average Zn(II) affinity of log $K = 11.8$ for the four tightly bound Zn(II) ions. A value of 7.7 pertains to a relatively weakly bound Zn(II) ion in MT. The slope in the range of the intermediate buffering capacity (Figure 3E, F) indicates that the remaining

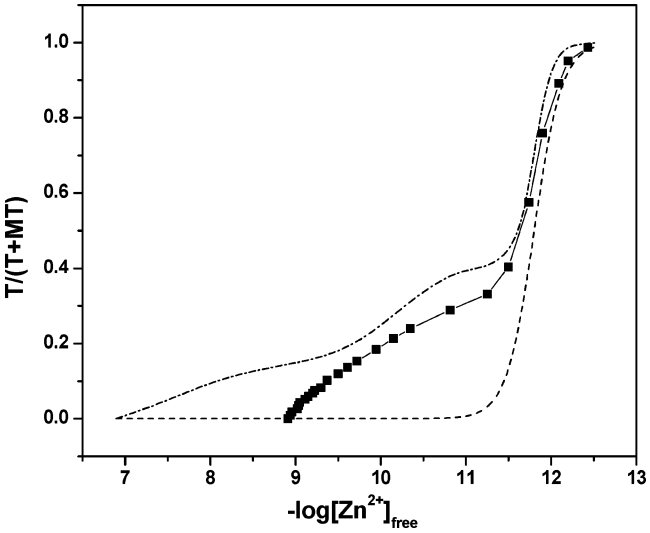

Figure 8. Effect of thionein on $[Zn^{2+}]_{free}$ controlled by metallothionein. MT + T (1 μM) was incubated with 5 μM FluoZin-3 in 50 mM HEPES, pH 7.4, 50 μM TCEP ($I = 0.1$) for 2 h. Reduced T was added to a solution of MT in such a way that the total protein concentration was 1 μM (T + MT). Fluorescence was measured at 517 nm with excitation at 492 nm. Free Zn(II) calculations are based on F_{min} and F_{max} values and the apparent stability constant of Zn-FluoZin-3. In the absence of FluoZin-3, Zn(II) buffering extends to pZn = 7, as shown by the curve calculated from the stability constants in Table 2 (-·-). The dashed line (- - -) corresponds to the theoretical behavior of MT and T if all seven Zn(II) ions had the same affinities as the four strongly bound Zn(II) ions.

Table 2. Apparent Stability Constants (log K) and Apparent Formation Constants (log β) of the Zn(II)−Thionein System at pH 7.4 ($I = 0.1$)

reaction	log K expt 1[a,f]	expt 2[b]	expt 3[c,f]	average[d]		log β
$4Zn^{2+} + T \leftrightarrows Zn_4T$	12.0[F] 11.7[R]	11.5(2)	11.8(1)[F]	K_{1-4}	11.8(2)	47.2[e]
$Zn^{2+} + Zn_4T \leftrightarrows Zn_5T$	10.5[F] 10.4[R]			K_5	10.45(7)	57.6[e]
$Zn^{2+} + Zn_5T \leftrightarrows Zn_6T$	9.9[F] 10.0[R]			K_6	9.95(7)	67.6[e]
$Zn^{2+} + Zn_6T \leftrightarrows Zn_7T$	7.8[F] 7.6[R]		7.7[F] 7.8[R]	K_7	7.7 (1)	75.3[e]

[a] Based on fitting binding isotherms (Figure 2). [b] Based on Zn(II) transfer to NTA (Figure 3). [c] Based on competition between either FluoZin-3 or RhodZin-3 and MT for Zn(II) (Figure 4). [d] Values of stability constants recalculated per one Zn(II). [e] $\log \beta\ Zn_4T = 4 \times \log K_1$; $\log \beta\ Zn_{4+n}T = \log \beta\ Zn_{(4+n)-1}T + \log K_{(4+n)}$, where $n = 1-3$. [f] F means experiment performed in the presence of FluoZin-3. R means experiment performed in the presence of RhodZin-3.

two Zn(II) ions in MT have binding constants in the range between 7.7 and 11.8. Stability constants for these Zn(II) ions could not be obtained with the titration methods employed. However, their affinities can be estimated by fitting the data to a model with four species: Zn_4T, Zn_5T, Zn_6T, and Zn_7T (Table 2). With the parameters listed in Table 2, an excellent fit of the experimental data is obtained (Figure 3E, F). For both dyes, a model with equal Zn(II) affinities of Zn_5T and Zn_6T gave a less satisfactory fit than a model with two different affinities. A species distribution can be calculated from these data (Figure 9). The NTA competition method employed detects neither differences in binding of the four tightly bound Zn(II) nor any significant degree or type of cooperativity. Given the complexity of the structure of MT, there is no *a priori* reason to assume that the four Zn(II) ions bind with equal affinity. Nevertheless, applying Ockham's razor to this issue and assuming four equal binding constants, a species distribution for $Zn_{1-4}T$ can

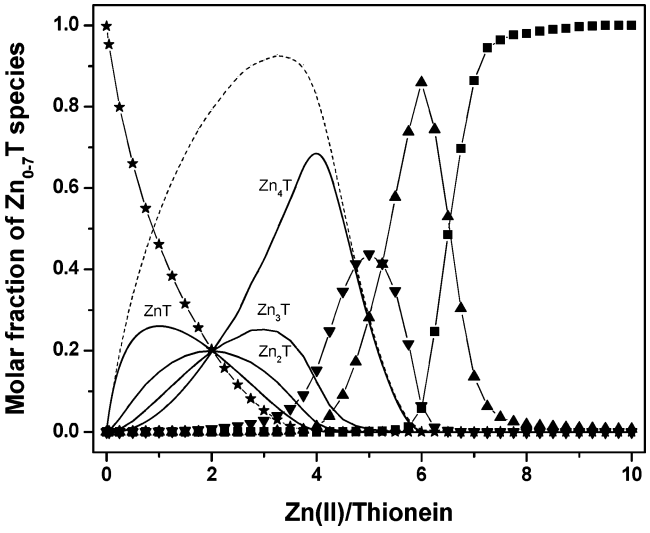

Figure 9. Distribution of $Zn_{0-7}T$ species at a 1 μM concentration of protein as a function of the Zn(II)/Thionein ratio; Zn_0T − free thionein (★), Zn_5T (▼), Zn_6T (▲), and Zn_7T (■). Speciation is based on the stability constants (Table 2). Distribution of $Zn_{1-4}T$ species is based on assuming identical apparent stability constants of $\log K = 11.8$ (solid lines). The sum of these four species is also given (dashed line) because these constants are indistinguishable by the methods employed.

be calculated (Figure 9, dashed line). Below a Zn(II)/T ratio of 4, Zn(II) is available only to ligands that have affinities higher than 11.8 or are in significant excess over MT. At ratios above 6, the Zn(II) buffering capacity of MT is exhausted and a significant amount of Zn(II) becomes available to potential acceptors. What is particularly important is the intermediate range, where two species, Zn_5T and Zn_6T, make Zn(II) available. According to estimates of $-\log[Zn^{2+}]_{free}$ in the picomolar range, these species do seem to be important physiologically.[9,10,12,13]

Discussion

Cellular proteins bind Zn(II) with relatively high affinity ($\sim 10^{12}$ M^{-1}), and its availability is regulated tightly.[61] Accordingly, the concentrations of free Zn(II) ions are very low, posing a conundrum, namely how do thousands of zinc proteins obtain their zinc. Until recently, only total Zn(II) concentrations could be determined in biological samples. With the advent of fluorogenic chelating agents, it became possible to examine very low amounts of free Zn(II). Estimates are in the picomolar range.[9,10,12,13] To keep free Zn(II) concentrations so low, cells use a micromolar Zn(II) buffering capacity.[13] In contrast to most chemical metal buffering systems, biological systems use sulfur-donor-based chemistry to buffer Zn(II).[62] Based on previous estimates of the affinity of MT for Zn(II), MT should, essentially, be a thermodynamic sink for Zn(II) and should be saturated with Zn(II). The detection of binding sites with lower affinities, however, suggests a dynamic role of MT/T in cellular Zn(II) buffering. The chemical properties of MT need to be related to both the cellular requirements for Zn(II) and the speciation of MT in terms of metal load and redox state.[7] Thus, both chemical and biological approaches come to bear on the

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question of how the unique coordination chemistry of the zinc/thiolate clusters in MT controls cellular Zn(II).

In previous work, the Zn(II) affinity of MT was determined by competition with either protons or chelating agents and by observation of either LMCT (S → Zn(II)) transitions in MT or characteristic absorbance changes when the chelating agents bind Zn(II).[26,27,63] These approaches did not reveal differences in Zn(II) binding. The interpretation of pH titrations is based on a model of three cysteinyl protons competing with one metal ion in the clusters (a ratio of 20 thiols to 7 Zn(II) ions corresponds to a stoichiometry of 2.9). Equilibrium constants of $\beta_{Zn} = 2.1 \times 10^{-9}$ M^2 and β_{H_3}, based on an average thiol pK_a value of 8.9, were calculated according to eq 4:[26]

$$\beta_{Zn} = \frac{[(L^-)_3Zn(II)][H^+]^3}{[(LH)_3][Zn(II)]}$$
$$\beta_{H_3} = \frac{[(LH)_3]}{[(L^-)_3][H^+]^3} = \frac{1}{(K_a)^3} \quad (4)$$

The resulting cumulative stability constant $\beta_{MT} = 1 \times 10^{18}$ ($\beta_{Zn} \times \beta_{H_3}$) can be converted to any pH value. Thus, the apparent stability constants are 2×10^{12} and 3×10^{13} M^{-1} at pH 7.0 and 7.4, respectively. Calculated values of β_{Zn} and β_{H_3} are based on assuming equal contributions of 20 cysteines to either the LMCT transitions or the absorbance of thiol/thiolate, i.e., assuming that the absorbance at 220 nm is a linear function of metal or proton binding to T. The cysteinyl residues are grouped in seven motifs that are separated by stretches of at least three or more amino acids. Within these motifs, ligands are provided from Cys−X−Cys (7 times), Cys−Cys (3 times), and Cys−X−X−Cys (3 times) sequences.[64] Potential differences in pK_a values among the 20 thiols can significantly affect the value of the cumulative stability constant. Cysteine pK_a values differ even in apo-phytochelatins with the repetitive amino acid sequence $(\gamma$-Glu-Cys$)_n$.[65−66] Varying proximities of cysteinyl residues to Ser and Lys in MT-2 are expected to result in different pK_a values of thiol functions, and such differences are likely responsible for the fact that the Henderson−Hasselbalch slope ΔpH/Δlog([RS$^-$]/[RSH]) of pH titrations between 7 and 12 is <1.0.[26,67,68] In contrast to these methods that lead to a model of seven Zn(II) ions binding with equally strong affinity to MT, the results described in this manuscript demonstrate that fluorimetric measurements have the capacity to uncover at least three classes of Zn(II) binding sites in MT.

A Weak Zn(II) Binding Site in Metallothionein. Although a weak Zn(II) binding site in MT was implicated from the observation of Zn(II) transfer to apo-metalloenzymes, a method to quantitatively study this site and differentiate it from the others was not available.[52−54] Zn(II) competition with FluoZin-3 or RhodZin-3, which are dyes with very high quantum yields in response to Zn(II), demonstrates that one Zn(II) ion binds to the protein with an apparent stability constant of 5×10^7 M^{-1} (Figure 4, Table 2). However, since the dyes are in slight molar excess over the one Zn(II) ion in MT, more than one Zn(II) ion is released (20% and 16% for FluoZin-3 and RhodZin-3, respectively, with 14.3% corresponding to one Zn(II)). Also, more than one Zn(II) ion is available from MT when it is mixed with a 25-fold excess of NTA (Figure 3) with an apparent Zn(II) stability constant of 2.1×10^8 M^{-1} (pH 7.4), which is, thus, relatively close to those of the fluorogenic probes (Figure S2).[13,37] Higher concentrations of chelators compete with more than one Zn(II) ion, including Zn(II) from stronger binding sites. The contributions of these stronger binding sites are negligible in the presence of a 2−5-fold excess of FluoZin-3 or RhodZin-3 because of 2−4 orders of magnitude differences in affinities between strong and weaker binding sites (Table 2).

Previously, it was reported that 100 μM of 4-(2-pyridylazo)-resorcinol (PAR) with a conditional stability constant of 2×10^8 M^{-1} binds ≤14% of Zn(II) from 1 μM MT in 0.2 M TRIS-HCl, pH 7.4.[53,69] Using the stability data in Table 2, one can calculate that, under equilibrium and at this particular concentration, PAR binds 25% of Zn(II) from MT. 100 μM of the much weaker chelating agent 2-[2-[alpha(2-hydroxy-5-sulfophenylazo)benzylidene]hydrazino]benzoic acid (Zincon), with an apparent stability constant of 8×10^5 M^{-1} (25 °C, pH 7.4), binds only about 4% of total Zn(II) from MT, which is in agreement with a calculated value of 5% at equilibrium (Table 2).[70,71] Thus, Zincon competes with the weakly bound Zn(II) in MT while PAR can compete with the stronger bound Zn(II) ions. Based on an average overall tight binding of Zn(II) to MT, it was suggested that Zincon does not compete with MT for Zn(II), and accordingly, the detection of about 4% of Zn(II) in MT when Zincon was mixed with MT was attributed to spurious Zn(II) in the preparation.[53,58] Clearly, in studies of the reactivity of MT, conditions such as buffer, pH, and choice and concentration of chelating agent are critically important.

Strong Zn(II) Binding Sites in Metallothionein. T binds four Zn(II) ions with an apparent stability constant of 6×10^{11} M^{-1} per Zn(II) (Figure 3, Table 2), a value that is in good agreement with the previously determined average value of 2×10^{11} M^{-1} 25 °C, pH 7.4 obtained from competition with NTA or H_2KTSM_2.[63] An apparent stability constant of 7×10^{12} M^{-1} (pH 7.0) for MT was measured by competition with 8-hydroxyquinoline-5-sulfonic acid. This value seems to overestimate the affinity, because it would be even higher at pH 7.4.[27] Remarkably, aside from another value of 2×10^{12} M^{-1} in the literature, these are the only values available.[26] Experimental details about how these constants were obtained were not reported, and neither the source nor the isoform of MT was specified. Added to this scarcity of data is the fact that equal affinity of all binding sites was assumed. This assumption is chemically perhaps intuitive due to the similar coordination environments, but it turns out to be incorrect. The existence of one weakly bound Zn(II) in MT does not contradict the generally strong affinity of this protein for Zn(II), as determined in past studies. With apparent stability constants of four strongly, two moderately strongly, and one weakly bound Zn(II) (Table 2), one calculates an average binding constant ($^1/_7 \times (4K_{1-4} + K_5 + K_6 + K_7)$) of 4×10^{11} M^{-1}. This value is almost identical to the affinities of the strong binding sites and is not readily distinguishable from

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the average value 2×10^{11} M^{-1}.[63] Thus, the binding properties are dominated by the four strongly bound metal ions, and the contribution of one weakly bound metal ion is not readily detectable by less sensitive methods.

Medium Strength Zn(II) Binding Sites in Metallothionein. Transfer of more than one Zn(II) ion to an excess of chelators with medium strength, such as NTA, demonstrates removal of Zn(II) from stronger binding sites in MT. The binding constants of the weak and strong binding sites (Table 2) differ by 4 orders of magnitude. The properties of sites that bind Zn(II) with medium strength cannot be measured directly because of the overlapping affinities of weak and strong binding sites. Zn(II) titration of T in the presence of FluoZin-3 or RhodZin-3 demonstrates that two Zn(II) ions are bound with affinities that differ from the strong and weak binding sites (Figure 2). A fit of the binding isotherm with values of 9×10^9 and 3×10^{10} M^{-1} (K_6 and K_5) for the Zn(II) ions that bind with medium strength (Figure 2, Table 2) demonstrates excellent agreement between estimates of these parameters and experimental data.

Effect of Thionein on the Metal Load of Metallothionein. Different binding properties of MT are very well resolved in the case of its titrations with T. If MT were to bind seven Zn(II) ions with the same strong affinity, the Zn(II) buffering capacity would be limited to 11.8 ± 0.25 units on a $-\log[Zn^{2+}]_{free}$ scale, and Zn(II) would not be buffered in such a wide range as is experimentally observed (Figure 8). T is a strong acceptor of at least four Zn(II) ions, and MT is a donor of at least one weakly bound Zn(II). Therefore, MT containing Zn(II) bound with such a wide range of affinities is expected to transfer weakly bound Zn(II) as well as some of the Zn(II) ions bound with intermediate strength to T. The implications for biology are that any newly synthesized T re-equilibrates with any already existing MT, with the important consequences that MT and T do not coexist, as previously inferred from data that were interpreted as cooperative binding, and that metal clusters that are not fully loaded with Zn(II) are functionally relevant.[72] Using the affinities of MT for Zn(II) (Table 2), a species distribution as a function of Zn(II)/T ratios can be calculated (Figure 9). The strongly bound Zn(II) ions interact with T with little, if any, cooperativity ($n = 1.5 \pm 0.2$, Figure 3). Zn_6T and Zn_5T species exist when the T concentration relative to that of total protein (T/(MT + T)) is below 0.43 (corresponding to a T/MT ratio of 0.75). Above this value, only Zn_4T exists (Figures 8 and 9). It is now possible to compare this information with our knowledge about T/MT ratios in cells. Analysis of MT and T in human colon cancer (HT-29) cells by differential chemical modification with the fluorescent agent ABD-F (7-fluorobenz-2-oxa-1,3-diazole-4-sulfonamide) yields T/MT ratios in the range 0.28−0.43 for control cells and cells treated with zinc sulfate.[13] Thus, the cellular T/MT ratios are below the critical value 0.43, clearly supporting the physiological importance of the Zn_6T and Zn_5T species. The differential chemical modification assay does not have the capacity to distinguish between T that is completely devoid of Zn(II) and a mixture of species.[6] The finding of binding sites with different affinities for Zn(II) resolves this issue. Though MT and T can be prepared and studied in the test tube, the form of the protein in cells is a mixture of species with a complement of less than seven Zn(II) ions. Therefore, the nomenclature used for the isolated forms becomes fuzzy when applied to biological systems. Indeed, all four species (Zn_4T-Zn_7T) are observed by mass spectrometry when the apo-form of the zinc finger peptide Ncp7 (13−33) is mixed with rabbit MT-2.[73] In cells, "metallothionein" is a dynamic protein with species constantly changing due to Zn(II) transfer to apo-metalloproteins and re-equilibration when T expression is induced. Furthermore, species distribution depends on the total protein concentration, which is estimated to be micromolar but can vary 400-fold.[74,75] Changing MT concentrations in the range $10^{-4}-10^{-8}$ M modulates $[Zn^{2+}]_{free}$ from 0.2 to 10.6% of total Zn(II) as a result of Zn(II) dissociation from Zn_7T and formation of Zn_6T (Figure S3). Thus, at the millimolar concentrations of protein used for structure determination in solution, there is virtually no dissociation of zinc from Zn_7T. However, in the physiological range of MT concentrations, Zn_7T and Zn_6T are the predominant forms in the absence of T. Zn_6T predominates below a concentration of 30 nM of total MT.

Reactivity of Metallothionein as a Consequence of Different Zn(II) Affinities. Chelating and other Zn(II)-binding agents serve as surrogates for biological zinc acceptors, and DTNB serves as a surrogate for oxidants or reactive electrophiles. DTNB oxidizes thiols faster in partially Zn(II)-depleted MT than in fully Zn(II)-loaded MT because of the higher reactivity of free thiols compared with Zn(II)-bound thiolates (Figure 5A).[54] Even ligands with a relatively weak affinity for Zn(II) compete with MT for Zn(II) and increase the rate of thiol oxidation (Figure 6A, B). Different oxidation rates were observed when DTNB oxidizes MT in the presence of ATP, ADP, and AMP whose Zn(II) affinities decrease in this order.[76] Addition of Zn(II) to MT, however, decreases the oxidation rate because the equilibrium ($Zn_7T \leftrightarrows Zn_{7-n}T + nZn^{2+}$) shifts toward Zn_7T (Figure 5B). These observations uncover a property of MT that is important biologically, namely that the chemical activity of MT changes when biological ligands compete with MT for Zn(II) and affect its metal load. In other words, Zn(II) availability and reactivity of MT are linked in a way that reflects the demand for Zn(II). MT can switch between functions in either storage or delivery of Zn(II). At high availability of Zn(II) the molecule is least reactive. However, if the availability of Zn(II) is low, the molecule becomes increasingly more reactive to supply Zn(II) for cellular functions.

Speciation and Structure. Any discussion of MT function is based on the high resolution solution and crystal structures of the protein, which has 20 reduced cysteines and 7 Cd(II) ions. There are limitations with this model. First, Zn(II) is expected to behave differently from Cd(II) because it binds at least 4 orders of magnitude weaker and its exchange kinetics are slower. Second, both oxidized and partially metal-loaded species are present in tissues, supporting the notions that redox reactions are important in the regulation of Zn(II) availability and that the protein does not exist in the form known from its three-dimensional structure.[7,77] Thus, there are issues about

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where in the structure of MT the weak Zn(II) binding site is located and whether or not the $Zn_{4-6}T$ species have unique three-dimensional structures. One weakly bound Zn(II) originates from the N-terminal β-domain.[53] Similarly, a more weakly bound Cd(II) has been localized to the C-terminal portion of the β-domain.[78] From studies with the isolated domain peptides, which can be synthesized chemically, the β-domain has an average binding constant of 2×10^{11} M^{-1} at pH 7.0.[79,80] The crystal structure of MT was determined with a Cd_5Zn_2T species, and a remarkable feature of this structure is that the two Zn(II) ions occupy defined positions in the β-domain.[3] Presumably, Cd(II) is bound to the same sites that have a strong affinity for Zn(II), suggesting from which sites Zn(II) ions have dissociated in Zn_6T and Zn_5T and which cysteines would become exposed for reactions with oxidants and electrophiles. Zn(II) binding in the C-terminal α-domain is stronger with at least two different classes of affinities.[79] Likewise, differences in cadmium binding in the Cd_4-α-domain were observed by UV and CD spectroscopies.[81] No evidence for cooperativity of cadmium binding in the formation of the cadmium/thiolate cluster in the isolated C-terminal domain of human MT-1a was found.[82] Co(II) is a much better isostructural probe than Cd(II).[83] The four tightly bound Zn(II) ions are thought to interact with T without using bridging ligands, in the same way postulated for the pathway for forming Co_7T, where EPR spectroscopy demonstrated that four Co(II) ions bind to T without spin−spin interactions.[84] Above a Co(II)/T ratio of 4, the intensity of the EPR signal decreases progressively due to antiferromagnetic coupling of the electron spins of neighboring Co(II) ions that become connected *via* sulfur bridges, preferentially in the α- and later in the β-domain.[84,85] From this observation and the fact that four to six Zn(II) ions quench the reactivity of all the cysteines, it seems clear that the four tightly bound Zn(II) ions do not correspond to the four Zn(II) ions in the Zn_4S_{11} cluster. The above data do not provide information about whether the binding mechanism for seven Zn(II) ions to MT is sequential or concerted. Even in the RING finger domain of BRCA1 with a pattern of ligands interleaving the two zinc sites, metal binding is sequential with anticooperativity.[86] Regarding the two zinc/thiolate clusters in MT, it is remarkable how variation of binding constants over 4 orders of magnitude can be achieved given that all Zn(II) ions reside in a tetrathiolate coordination environment.

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Supporting Information Available: Hypsochromic shift of the center of the LMCT bands of metallothionein when thionein is titrated with Zn(II), determination of the apparent stability constant of Zn(II)-RhodZin-3, species distribution of metallothionein, pseudo-first-order rate constants of the reaction between metallothionein and DTNB, and conditional binding constants of zinc ligands and chelating agents. This material is available free of charge via the Internet at http://pubs.acs.org.

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