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Coordination dynamics of biological zinc "clusters" in metallothioneins and in the DNA-binding domain of the transcription factor Gal4

(metalloproteins/isotope exchange/radiochromatography)

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ABSTRACT **The almost universal appreciation for the importance of zinc in metabolism has been offset by the considerable uncertainty regarding the proteins that store and distribute cellular zinc. We propose that some zinc proteins with so-called zinc cluster motifs have a central role in zinc distribution, since they exhibit the rather exquisite properties of binding zinc tightly while remaining remarkably reactive as zinc donors. We have used zinc isotope exchange both to probe the coordination dynamics of zinc clusters in metallothionein, the small protein that has the highest known zinc content, and to investigate the potential function of zinc clusters in cellular zinc distribution. When mixed and incubated, metallothionein isoproteins-1 and -2 rapidly exchange zinc, as demonstrated by fast chromatographic separation and radiometric analysis. Exchange kinetics exhibit two distinct phases ($k_{fast} \simeq 5000\ min^{-1} \cdot M^{-1}$; $k_{slow} \simeq 200\ min^{-1} \cdot M^{-1}$, pH 8.6, 25°C) that are thought to reflect exchange between the three-zinc clusters and between the four-zinc clusters, respectively. Moreover, we have observed and examined zinc exchange between metallothionein-2 and the Gal4 protein ($k \simeq 800\ min^{-1} \cdot M^{-1}$, pH 8.0, 25°C), which is a prototype of transcription factors with a two-zinc cluster. This reaction constitutes the first experimental example of intermolecular zinc exchange between heterologous proteins. Such kinetic reactivity distinguishes zinc in biological clusters from zinc in the coordination environment of zinc enzymes, where the metal does not exchange over several days with free zinc in solution. The molecular organization of these clusters allows zinc exchange to proceed through a ligand exchange mechanism, involving molecular contact between the reactants.**

The biological coordination chemistry of zinc is particularly rich in structural motifs (1), apparently reflecting the numerous functions and general importance of this element in biology (2). One particular motif is that found in biological zinc clusters (3), polynuclear complexes in which zinc is coordinated exclusively to thiolate sulfurs of cysteine residues. Their prominent structural feature includes both bridging and terminal cysteine ligands. Each zinc atom resides in a tetrahedral coordination environment and is apparently unable to accommodate additional ligands. How this coordination relates to biological function is unknown. We will address this question with reference to metallothionein (MT), a bilobal protein harboring two of these clusters.

Tetranuclear and trinuclear zinc clusters (Fig. 1 *A* and *B*) are located in two separate domains of mammalian MTs (4, 5). The three-metal cluster in the N-terminal β-domain has 6 terminal and 3 bridging cysteine ligands, whereas the four-metal cluster in the C-terminal α-domain has 11 cysteines, 6 of the terminal type and 5 that form bridges. As a consequence, all 20 cysteines of MT are involved in the binding of seven zinc atoms. Their unparalleled metal/ligand ratio makes these biological zinc clusters unique when compared with synthetic metal-thiolate compounds. For example, while the adamantane-like structure of cage molecules is related, their building principle differs slightly: Four of their zinc atoms are bound by 10 thiolate ligands—6 are bridging and 4 are terminal (6). In comparison, the four-zinc cluster in MT is stabilized by an additional ligand and has fewer bridging but more terminal ligands. The number of bridging ligands is a determinant of the reactivity of these clusters, since it has been observed that reaction rates generally decrease when the connectivity of the clusters increases (6).

MT was discovered as a cadmium-binding protein in horse kidneys (7) and identified later as a predominantly zinc-binding protein in liver. Indeed, under most physiological conditions zinc is the major metal associated with MT, with the exception of specific instances in which copper (whose significance, like that of zinc, remains to be determined) is present as well. The biological and chemical properties of mammalian MTs have suggested that they might play a role in cellular zinc homeostasis, a possible cornerstone of a general zinc buffer/distribution system (8–10). The molecular mechanism of action of MT in zinc metabolism, however, has remained uncertain, since biomolecules with which MT might interact *in vivo* have not been identified.

The metal clusters are thermodynamically quite stable [$K_D = 5 \times 10^{-13}$ M for rabbit Zn-MT; $K_D = 5 \times 10^{-17}$ M for rabbit Cd-MT at pH 7 (8)]. However, in MT, cadmium is kinetically labile and it exchanges in intermolecular and intramolecular reactions (11). Thus, saturation-transfer NMR demonstrates on a time scale of seconds that cadmium moves among the sites in the β-domain (intramolecular self-exchange) and between different β-domains by intermolecular exchange. Metal exchange within and between the α-domains and between α- and β-domains is much slower (12). Accordingly, work has concentrated on metals such as cadmium, which can be studied readily by physical techniques, but has virtually ignored the dynamics of zinc, the biologically significant element. In the series Zn → Cd → Hg, ligand exchange rates increase for both water (13) and thiolate sulfur ligands (14), despite a concomitant increase in thermodynamic stability. Therefore, zinc would be expected to have a smaller ligand exchange rate than does cadmium, much as experimental evidence for MT is still lacking. In an effort to relate the chemistry of MT to its possible biological activities, we have addressed the chemical reactivity and dynamics of zinc directly. The use of radioactive zinc and rapid chromatography reveals that zinc exchanges between MT isoforms, but zinc cluster structures and interprotein zinc isotope exchange are not limited to MT. A

Abbreviation: MT, metallothionein.

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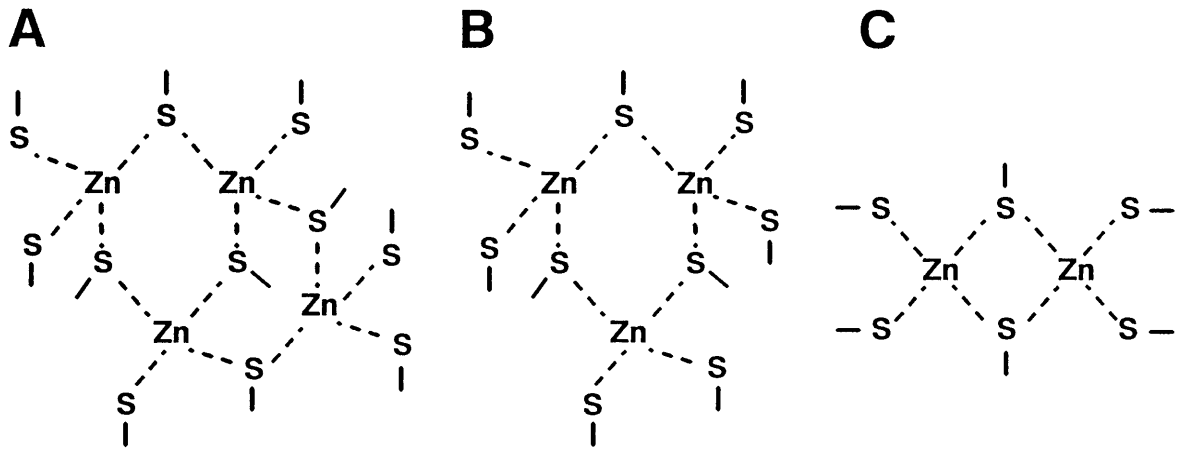

FIG. 1. Biological zinc clusters. (*A*) Metallothionein, four-zinc cluster in the α-domain; (*B*) metallothionein, three-zinc cluster in the β-domain; and (*C*) Gal4 protein, two-zinc cluster.

binuclear zinc cluster with six cysteine ligands, two of which act as bridging ligands, exists in the yeast transcription activator Gal4 (15–19) (Fig. 1*C*). This motif is conserved in at least 79 other fungal transcription factors, of which 56 exist in *Saccharomyces cerevisiae* (20), and organizes a relatively small protein domain for DNA recognition. Zinc exchange also takes place in a reaction between MT and the zinc cluster of Gal4. The results support a dynamic role of the clusters in MT in zinc transfer reactions and gain significance with regard to possible interactions of MT and zinc finger proteins, in which zinc/sulfur coordination plays a critical role. The unusual cluster structure involving thiolate sulfur ligands would seem to be a key feature in the function of MT as a cellular zinc reservoir.

MATERIALS AND METHODS

Materials. Buffers were prepared with water from a Milli-Q water system (Millipore), treated with Chelex-100 (Bio-Rad; ref. 21), and degassed. For storage longer than a few weeks, stock samples of proteins were kept under nitrogen at 4°C and handled with sterile pipet tips. $^{65}ZnCl_2$ (77.7–103.6 GBq/g) was obtained from DuPont/NEN.

Preparation of Zn_7-MT and of $^{65}Zn_7$-MT. Cd,Zn-MT-1 and -MT-2 isoforms from rabbit liver were converted to the apoform and reconstituted with $^{65}Zn^{2+}$ or nonradioactive Zn^{2+} to form $^{65}Zn_7$-MT and Zn_7-MT, respectively (22, 23).

Preparation of Gal4. The storage buffer (10 mM Tris·Cl/150 mM sodium chloride/1 mM 2-mercaptoethanol/10 μM Zn^{2+}, pH 8) of the bacterially expressed Gal4(149*) fragment (24) was exchanged against 10 mM Tris·Cl/15 mM sodium chloride, pH 8.6, using Centricon-10 centrifugal concentrators (Amicon). At least 15 mM sodium chloride is necessary to solubilize the protein fragment at pH 8.6. The metal content of the protein was 2.3 mol of zinc per monomer (M_r = 17,880) at a protein concentration of 20 μM. Stock solutions of the protein were prepared at this concentration, because zinc analyses of <2 mol of zinc per monomer were measured at lower protein concentrations due to weaker binding and loss of one of the zinc atoms during equilibrium dialysis (24). Protein concentration was determined spectrophotometrically (ε_{280} = 7300 $M^{-1}\cdot cm^{-1}$) and confirmed by amino acid analysis.

Zinc Isotope Exchange and Radiochromatography. Aliquots of reactants were incubated in a water bath at 25°C. MT isoforms were separated on a DEAE MemSep-1000 chromatography cartridge (Millipore) operated with an HPLC instrument. Chromatography was performed using a linear, 10-min gradient from 0 to 10 mM sodium chloride in 10 mM Tris·Cl (pH 8.6) at a flow rate of 9.6 ml/min. This system gives retention times of 2.5 and 5.2 min for MT-1 and MT-2, respectively. Rapid chromatographic separation of MT and Gal4 was achieved with a carboxymethyl MemSep-1000 chromatography cartridge (Millipore). Gal4 elutes at 2.4 min from this cartridge in a linear, 10-min gradient from 5 to 250 mM sodium chloride in 10 mM Tris·Cl (pH 8.0) at a flow rate of 9.6 ml/min. Under these conditions, MT does not bind to the cartridge and it elutes in the breakthrough fractions. Radioactivity was measured by γ-emission spectroscopy with a Searle model no. 1185 Automatic Gamma System operating at an energy range between 0.12 and 1.2 MeV. For the conversion of radioactivity into zinc concentration, a zinc standard from the particular batch of radioactive zinc was used. This eliminates variations in counter efficiency and automatically corrects for the radioactive decay of zinc ($t_{1/2}$ = 244 days).

RESULTS AND DISCUSSION

Reaction Between $^{65}Zn_7$-MT-1 and Zn_7-MT-2. Zinc exchange between MT molecules can be studied by using two charge-separable isoforms of MT, which have essentially the same zinc cluster structures and differ only in a few amino acids that are not involved directly in metal binding. As a means of following this reaction, radioactive zinc is used as a label and isoproteins are separated by fast ion exchange chromatography. Incubation of equimolar amounts of labeled MT-1 and unlabeled MT-2 results in exchange of radioactive zinc (Fig. 2) until an equilibrium with a 50/50 distribution is reached. The progress curve describing the appearance of the label in the

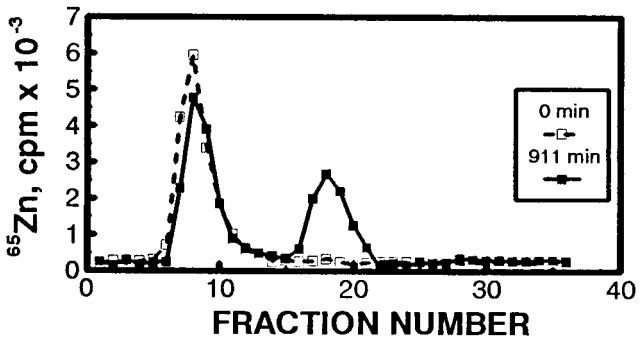

FIG. 2. Radiochromatograms of zinc exchange between $^{65}Zn_7$-MT-1 and Zn_7-MT-2. Proteins (10.3 μM total MT) were incubated, an aliquot was loaded at the times indicated on a DEAE MemSep-1000 cartridge at a flow rate of 9.6 ml/min, the cartridge was eluted in 10 min with a linear gradient from 0 to 10 mM sodium chloride in 10 mM Tris·Cl (pH 8.6), and the radioactivity in the fractions was measured. Total counts injected were 16,409 cpm for MT-1 and 21,364 cpm for the reaction mixture.

MT-2 fraction with time displays at least two kinetic phases (Fig. 3). Based on the observations that both domains of MT are structurally independent and that metal binding and release are independent and cooperative events in each domain (8, 25), the data were analyzed with a model that assigns the fast phase to the metals in the three-zinc cluster in the β-domain as a "kinetic class of amplitude 3" and the slow phase to the ones in the four-zinc cluster in the α-domain as a "kinetic class of amplitude 4." The data fit extremely well to such a two-parameter model of the form $Y_t = Y_\infty[7 - 3\exp(-k_3t) - 4\exp(-k_4t)]$ with $k_3 = 0.05\ \text{min}^{-1}$, $k_4 = 0.002\ \text{min}^{-1}$, and $Y_\infty = 0.5/7$ (Fig. 3). The bimolecular rate constants calculated from these data, $k_3 = 5048\ \text{min}^{-1}\cdot\text{M}^{-1}$ and $k_4 = 223\ \text{min}^{-1}\cdot\text{M}^{-1}$, allow comparison of data collected at any MT concentration and predict that these processes are relatively fast at the micromolar MT concentrations typically encountered in the cell (26) and even faster at higher concentrations. Thus, ^{111}Cd and ^{113}Cd NMR experiments performed at millimolar concentrations of Cd-MTs established a rate of cadmium exchange in the β-domain cluster in the order of 0.3–2.7 s^{-1} at 35°C (11), while rate constants for the much slower exchange between α-domains could not be measured. In another study, the interprotein cadmium exchange rate was determined with ^{109}Cd and separating and analyzing MT-1 and MT-2 isoforms by gel electrophoresis (8). A rate constant of $k = 7 \times 10^{-4}\ \text{s}^{-1}$ at 20°C was assigned to the exchange of cadmium atoms between the α-domains. By comparison, the present method is an alternative strategy that measures exchange rates of zinc, determines both processes simultaneously, and specifically establishes that the rate constants for the two processes differ by at least a factor of 20. Whether or not this difference suggests different roles of the metal atoms in the two clusters such as zinc storage in the α-domains and dynamic zinc transfer in the β-domain (27, 28) remains to be shown. The speculation that two zinc atoms in the β-domain are critical for the function of the protein can be attributed to the fact that they remain at specific positions in the β-domain of cadmium-induced Cd_5Zn_2-MT (4) and in metal exchange reactions between Zn-MT and Cd-MT *in vitro*, leading also to Cd_5Zn_2-MT (12). Notably, the two cadmium atoms that exhibit faster exchange rates in Cd_7-MT (11) occupy the two positions in the cluster in the β-domain, in which the two zinc atoms reside in the crystal structure of Cd_5Zn_2-MT (4). Kinetic data are not sufficient to trace individual zinc atoms of the clusters. The present model fitting the data does not rule out the possibility that more complex zinc exchange processes can be operative. Those could include zinc exchange between α- and β-domain clusters or preferential reactivity of specific zinc atoms in one of these.

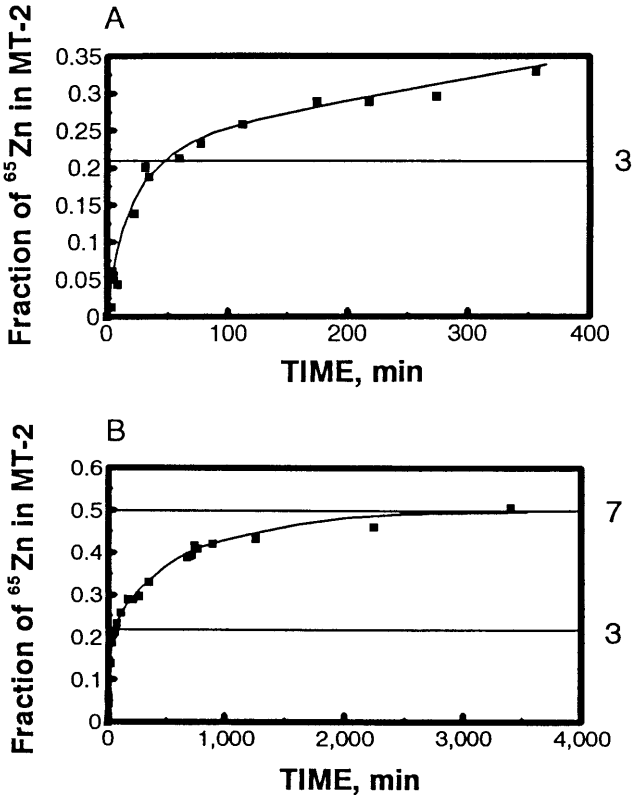

FIG. 3. (*A* and *B*) Time course of zinc exchange between $^{65}Zn_7$-MT-1 and Zn_7-MT-2. The horizontal lines represent amplitudes corresponding to the exchange of three and seven zinc atoms, respectively. Conditions are as described in the legend of Fig. 2.

Reaction Between Zn_2-Gal4 and $^{65}Zn_7$-MT-2. The reaction between MT and the zinc cluster of the transcription activator Gal4 was also examined to investigate whether or not such zinc exchange processes might also pertain to their interactions. Mixing aliquots of $^{65}Zn_7$-MT-2 and Zn_2-Gal4 followed by their separation at defined intervals of incubation demonstrates a time-dependent decrease of radioactivity in MT-2 (Fig. 4, two breakthrough fractions). The concomitant increase of radioactivity in the Gal4 fraction is a more direct means to follow the reaction, since it establishes that zinc derived from MT is indeed incorporated into Gal4 (Fig. 4). The assignment of the Gal4 fraction is based on the retention times of both native Gal4 and ^{65}Zn-labeled Gal4. A single exponential process describes the time-course of the reaction (Fig. 5). The apparent rate constant for this process is $k = 0.0120 \pm 0.0014\ \text{min}^{-1}$ ($n = 4$; $t_{1/2} = 59$ min).

To analyze this exchange process further, zinc exchange was followed at different ratios of the reacting partners and the bimolecular rate constants were calculated (Table 1). The exchange rate is quite constant over a wide range of ratios (0.1–0.7), but it decreases when the ratio approaches stoichiometric zinc values.

Zinc exchange rates observed here are between 1 and 2 orders of magnitude faster than those reported for the Gal4(62*) protein, a shorter fragment, with free zinc ions (24). In this study, an examination revealed biphasic kinetics, with $t_{1/2} = 24$ h and $t_{1/2} = 96$ h at 4°C and pH 7.0 for both processes. Unless there is an exceptionally high activation energy in this reaction, the lower temperature in this experiment accounts for only a 4-fold decrease in rates in comparison with the zinc exchange between MT-2 and Gal4(149*). The presence of only one process in the reaction between MT and Gal4 could mean that MT determines the rate-limiting step, that the larger

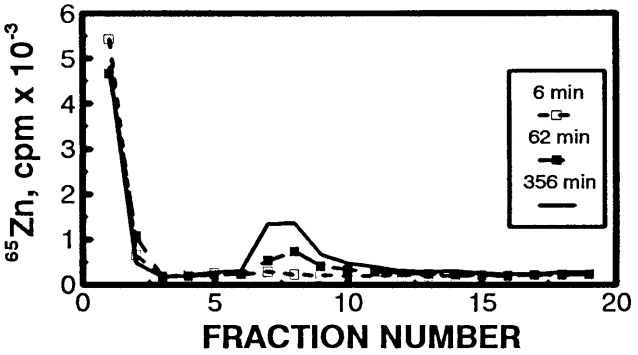

FIG. 4. Radiochromatograms of zinc exchange between $^{65}Zn_7$-MT-2 and Zn_2-Gal4. Proteins (1.5 μM MT-2 and 7 μM Gal4) were incubated, an aliquot was loaded at the times indicated on a carboxymethyl MemSep-1000 cartridge at a flow rate of 9.6 ml/min, the cartridge was eluted in 10 min with a linear gradient from 5 to 250 mM sodium chloride in 10 mM Tris·Cl (pH 8.0), and the radioactivity in the fractions was measured.

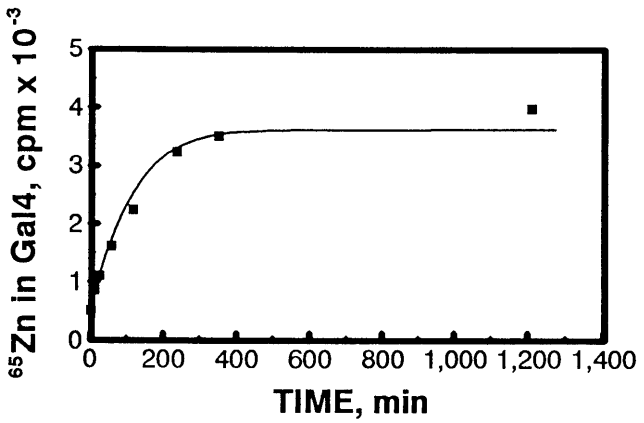

FIG. 5. Time course of zinc exchange between $^{65}Zn_7$-MT-2 and Zn_2-Gal4. The experimental data were fitted by a single exponential according to the equation $Y_t = Y_\infty[1 - \exp(-kt)]$, with $k = 0.0107$ min^{-1} and $Y_\infty = 3600$ cpm. Conditions as described in the legend of Fig. 4.

peptide confers the same reactivity on both zinc atoms, or that only one zinc exchanges. These alternatives cannot be differentiated at present.

There are few studies of ^{65}Zn exchange with zinc enzymes and apparently no studies of zinc exchange between proteins other than MTs. Zinc in carbonate hydratase (coordination: three histidines) exchanges extremely slowly; only a minuscule fraction of the radiolabel was incorporated after 50 days at equimolar concentrations of the enzyme and $^{65}Zn^{2+}$ (29). Similarly, zinc exchange in aspartate transcarbamoylase (coordination: four cysteines) could not be detected after 40 days (30), and zinc in superoxide dismutase did not exchange with $^{65}Zn^{2+}$ in solution (31). Zinc in liver alcohol dehydrogenase is also bound firmly. In this protein, zinc exchange was studied at pH 5.5, where the low pH value is expected to effect significant labilization of the metals. On the basis of exchange kinetics and effects of buffer ions, it was possible to distinguish between the two zinc atoms in each subunit of the dimeric enzyme—i.e., the so-called catalytic (coordination: one histidine and two cysteines) and noncatalytic (coordination: four cysteines) zinc atoms (32). Both zinc atoms exchange in sodium phosphate buffer. In sodium acetate buffer, however, only the noncatalytic zinc atoms exchange. These are the more rapidly exchanging ones, and they react with a bimolecular rate constant $k = 500$ $h^{-1}\cdot M^{-1}$ at 4°C. It was argued that this relatively rapid exchange rate is due to the formation of a binuclear complex, which can form between the readily accessible ligands of the noncatalytic zinc atom and free zinc in solution, but not between those of the buried catalytic zinc atom and free zinc (33). Collectively, these data demonstrate that zinc exchange mediated by MT is orders of magnitude faster than the exchange between free zinc ions and other zinc proteins in which zinc acts primarily either in catalysis or in stabilization of protein structure.

Table 1. Effect of the variation of the ^{65}Zn donor/Zn acceptor ratio on zinc isotope exchange between MT-2 and Gal4

Protein concentrations, μM		Zinc ratio,	$k \times 10^{-2}$,
MT	Gal4	MT/Gal4	$min^{-1}\cdot M^{-1}$
1.5	7	0.83	1.62
2.2	11	0.77	8.48
1.5	15	0.36	8.42
0.6	15	0.14	7.69

A thorough assessment of the mechanism of cadmium exchange between MT isoforms by NMR techniques (11) has revealed that metals exchange through bimolecular association of MT molecules followed by cysteine ligand exchange in transiently interacting MT dimers that share metal-thiolate coordination bonds from both β-domains. Such a self-association with an association constant of 3×10^4 M^{-1} at pH 6.8 (11) is consistent with the dimerization of MT observed in the crystal structure (4) and with hydrodynamic properties of both Zn-MT and Cd-MT (11, 34). The relatively fast rate of zinc exchange between MT and Gal4 suggests that this process is also controlled by contact between the two molecules and ligand swapping.

Implications. The dissociation constant of zinc from rabbit liver Zn-MT is 5×10^{-13} M at pH 7.0 and $\approx 10^{-18}$ M in the higher pH range, in which the dissociation constant is independent of pH (8). The very existence of MT with such tight zinc binding lends considerable support to the emerging concept that very little free zinc exists in the cytoplasm and that zinc/protein complexes control cellular zinc traffic. Indeed, cytoplasmic free zinc concentrations have been estimated to be 2.4×10^{-11} M (24 pM) in human erythrocytes (35), ≈100 pM in rabbit skeletal muscle (36), and <100 pM in nonstimulated heart cells (37). Thus, the elucidation of the chemical principles adopted by biology to endow stable zinc complexes with dynamic functions becomes a major research objective. The zinc clusters found in biology exhibit requisite chemical characteristics to participate in cellular zinc distribution. They bind zinc tightly (thermodynamic stability) while featuring mechanisms that make zinc available (kinetic lability), as exemplified by zinc exchange rates that are orders of magnitude higher than those of zinc in other zinc proteins. In MT, stabilization of the zinc complex is achieved via a total of 28 intramolecular zinc/sulfur bonds (16 in the α-domain and 12 in the β-domain), even while high reactivity is provided by the 28 bonds in which only 20 cysteines participate. Thus, zinc/ligand ratios of <4 due to sulfur thiolate bridges poise the molecule for metal and ligand exchange.

The metabolism of zinc has become a major focus of nutritional research in humans and animals, and it seems timely to combine accomplishments in this area with known biochemistry. It was recognized about 40 years ago that a fraction of zinc in the liver freely exchanges with $^{65}Zn^{2+}$ injected into mice, while another fraction does not exchange at all (38). It is the exchangeable, labile zinc that has been thought to have "a vital role in zinc-dependent functions" (39). Its definition in chemical terms is perhaps one of the most important and challenging questions in zinc biochemistry. As our results on zinc exchange between cluster motifs demonstrate, zinc lability does not necessarily mean loosely bound. In fact, zinc can exchange fast by specific kinetic mechanisms and yet be bound in coordination environments of relatively high thermodynamic stability.

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