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Oxidative metal release from metallothionein via zinc–thiol/disulfide interchange

(metalloproteins/glutathione/metal clusters/radiochromatography)

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ABSTRACT Mammalian metallothionein has been postulated to play a pivotal role in cellular zinc distribution. All seven of its metal atoms are bound with high thermodynamic stability in two clusters buried deeply in the molecule. If the protein is to function in metal delivery, there must be a biological mechanism to facilitate metal release. One means to achieve this would be a labilization of the clusters by interaction of metallothionein with an appropriate cellular ligand. To search for such a mediator, we have designed a rapid radiochromatographic method that can detect changes in the zinc content of ^{65}Zn-labeled metallothionein in response to other biomolecules. Using this methodology, we have established that rabbit liver metallothionein 2 interacts with glutathione disulfide with concomitant release of zinc. Under conditions of pseudo-first-order kinetics, the monophasic reaction depends linearly on the concentration of glutathione disulfide in the range from 5 to 30 mM with a second-order rate constant $k = 4.9 \times 10^{-3}$ s^{-1}·M^{-1} (pH 8.6; 25°C). Apparently, zinc release does not involve direct access of glutathione disulfide to the inner coordination sphere of the metals. Rather it appears that the solvent-accessible zinc-bound thiolates in two clefts of each domain of metallothionein [Robbins, A. H., McRee, D. E., Williamson, M., Collett, S. A., Xuong, N. H., Furey, W. F., Wang, B. C. & Stout, C. D. (1991) *J. Mol. Biol.* 221, 1269–1293] participate in a thiol/disulfide interchange with glutathione disulfide. This rate-limiting initial S-thiolation, which occurs with indistinguishable rates in both clusters, then causes the clusters to collapse and release their zinc. Such a mechanism of metal release would link the control of the metal content of metallothionein to the cellular glutathione redox status and raises important questions about the physiological implications of this observation with regard to a role of glutathione in zinc metabolism and in making zinc available for other biomolecules.

High-resolution structures of zinc proteins have provided detailed descriptions of the coordination environments of zinc and firmly established the role of zinc in a wide array of protein functions (1–3). While these structural aspects of zinc biochemistry are becoming clarified, knowledge of the dynamics of cellular zinc metabolism has lagged disproportionately. Thus far neither uptake, storage, nor transport systems for zinc are known, though metallothionein (MT) has long been implicated and suspected of participating in one or all of these stages of cellular zinc homeostasis (4–6).

Twenty of the approximately 60 amino acids of mammalian MTs are cysteines, whose side chains are the only ligands to the seven zinc atoms (for a review, see ref. 7). The metals are grouped in two unique metal clusters that are located in separate domains of the protein. One cluster binds three zincs with six terminal and three bridging cysteine sulfur atoms, while the other binds four zincs with the same number of terminal cysteine ligands, but five bridging cysteine sulfur atoms (8). Despite the high thermodynamic stability of the clusters [K_D (Zn) = 5×10^{-12} M; pH 7.4 (9)], they are remarkably reactive. *In vitro*, they undergo intramolecular metal exchange reactions within and between individual clusters (10). They also participate in intermolecular reactions—either metal exchange between MT isoforms (11) or metal transfer from MT to the apoforms of metalloproteins (12)—that are thought to proceed through ligand-exchange processes involving direct molecular contact between the reactants. Presumably, such reactivity would be limited to those few macromolecules with which MT could interact specifically and, therefore, should not be considered as evidence for a general mechanism for metal mobilization from MT. *In vivo*, zinc is readily available to other biomolecules. Thus, under conditions of zinc deficiency in cultured cells, zinc is released from MT with a rate constant of 0.6 h^{-1} (13). While the molecular mechanism for metal release is unknown, it does not appear to be controlled by proteolysis of MT, since this is at least one order of magnitude slower (13). These observations identified MT as a kinetically labile macromolecular pool of zinc (9) and led to the idea that MT might serve a role in the redistribution of zinc within the cell.

Knowledge about the chemical and biological mechanisms of metal release from MT are important missing links in the chain of events marking such a role of MT in zinc distribution. Aside from the acidic milieu in lysosomes, there are no physiological variations of pH sufficient to dissociate zinc from MT in cells. Also, since MT has very little secondary structure, it is difficult to invoke a conformational change as a means to alter the metal binding properties of the molecule. The highly compact structures of the protein and its clusters plus the absence of known physiological effector molecules further complicate the definition of a realistic mechanism of metal release from MT.

To study agents that would effect metal release from MT, we have developed a rapid, radiochromatographic technique that separates MT from potential reactants and allows examination of its metal content. In the course of these investigations we have found that disulfides release zinc from ^{65}Zn-labeled MT. Here, I describe the kinetics of metal release induced by glutathione disulfide (GSSG), the most abundant disulfide in the cell, and discuss the implications of an oxidative reaction to regulate MT.

MATERIALS AND METHODS

Materials. Purified rabbit liver MT-1 and MT-2 isoforms were kindly provided by J. H. R. Kägi (Universität Zürich, Switzerland). Zinc-65 chloride with a specific radioactivity of 2.1–2.8 Ci/g (77.7–103.6 GBq/g) was obtained from DuPont/NEN. Baker's yeast glutathione reductase (type III), glutathione (GSH), GSSG (free acid and sodium salt), and

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Abbreviations: MT, metallothionein; GSH, glutathione; GSSG, glutathione disulfide.

other disulfides were from Sigma; NADPH was from Boehringer Mannheim; 2-vinylpyridine was from Aldrich; and dithiothreitol, ultrapure, was from United States Biochemical.

Preparation of Thionein and ^{65}Zn-Thionein. Lyophilized Cd,Zn-MT, 3 mg, was dissolved in 1 ml 10 mM Tris·HCl, pH 8.6, and incubated with 30 mg of dithiothreitol overnight at ambient temperature (14). Thionein was separated from dithiothreitol and metal ions by adjusting the reaction mixture to pH 1 with HCl and applying it to a Sephadex G-50 (fine) column (1 × 100 cm) equilibrated with 10 mM HCl at room temperature. The protein was eluted with 10 mM HCl at a flow rate of about 10 ml/h. Thionein was located in the fractions by measurement of the absorbance at 220 nm (ε_{220} = 48,200 $M^{-1} \cdot cm^{-1}$) and by assaying the protein sulfhydryls with 2,2'-dithiopyridine (ε_{343} = 7600 $M^{-1} \cdot cm^{-1}$). Thionein fractions that contained an average of at least 19 free thiolates per protein molecule (M_r = 6000) were pooled, and an 8-fold molar excess of ^{65}Zn was added under a nitrogen atmosphere at 4°C. The reaction mixture was adjusted to pH 8.6 with 2 M Tris base, added in small aliquots over a period of 30 min under nitrogen and with stirring. ^{65}Zn-MT was then lyophilized and stored at 4°C. Aliquots were dissolved in degassed water and exhaustively dialyzed (Spectra/Por no. 7 dialysis tubing, molecular weight cutoff 1000; Spectrum Medical Industries) at 4°C against degassed 10 mM Tris·HCl, pH 8.6, to remove excess metal. The metal-to-protein stoichiometry was calculated on the basis of the protein concentration determined from a linear plot of different aliquots measured spectrophotometrically (ε_{220} = 159,000 $M^{-1} \cdot cm^{-1}$) and on the specific radioactivity of zinc in the protein. The stoichiometries for two preparations were 6.8 and 7.2 zinc atoms per protein, respectively.

Glutathione Assays. Concentrations of GSH and GSSG were determined with the enzymatic assay of Griffith (15).

Radiochromatography. Separations were performed by HPLC on a DEAE MemSep-1000 chromatography cartridge (Millipore) at a flow rate of 9.6 ml/min. Radioactivity was measured with a Searle Analytic automatic γ counter system, 1185 series, with an energy range from 0.12 to 1.2 MeV and an efficiency of 15–20%.

Kinetic Experiments. Reactants were mixed and incubated in a water bath at 25°C. Since there is some accumulation of ^{65}Zn on the chromatography cartridge, care was taken to regenerate the cartridge as recommended by the manufacturer. Because zinc is not completely recovered in the GSSG complex (see below) at all concentrations of disulfides, reaction kinetics were evaluated by following the decrease of radioactivity of the ^{65}Zn-MT peak. This chromatographic peak was unchanged after incubating ^{65}Zn-MT under identical experimental conditions in the absence of GSSG for 24 h.

RESULTS AND DISCUSSION

Introduction of ^{65}Zn into MT allows the metal to be followed with high sensitivity and, when combined with a rapid chromatographic separation technique, provides a means to resolve and monitor the interacting molecular species and thereby define the processes of intermolecular metal transfer.

Reaction of MT with GSSG. Incubation of ^{65}Zn-MT-2 with a relatively large excess of GSSG followed by rapid separation of aliquots of the reaction mixture by anion-exchange chromatography leads to a time-dependent decrease of radioactivity in the MT-2 fractions (Fig. 1, first peak) and concomitant formation of a new radioactive species (Fig. 1, second peak). The new peak appears at the same position in the chromatogram where complexes of ^{65}Zn with glutathione elute under identical chromatographic conditions. A zinc/GSH complex elutes as a single peak (Fig. 2) whereas a mixture of zinc with GSSG at the same metal-to-ligand ratio elutes as a composite peak (Fig. 3), albeit with indications that a single species is formed when a high excess of ligand (>100-fold) is used.

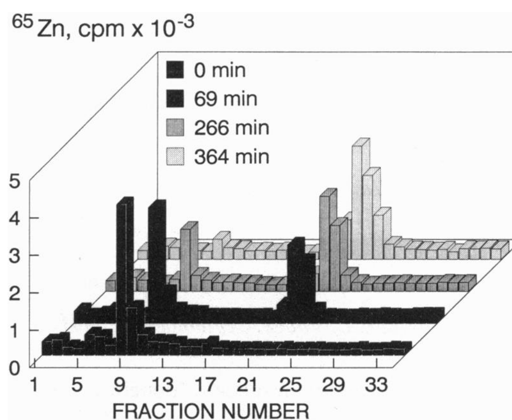

FIG. 1. Reaction of ^{65}Zn-MT-2 with GSSG as followed by radiochromatography. The reaction mixture contained 1 μM ^{65}Zn-MT-2 and 20 mM GSSG in 10 mM Tris·HCl, pH 8.6. At the times indicated, the mixture was applied to a DEAE MemSep-1000 chromatography cartridge and separated with a linear 10-min gradient from 0 to 75 mM NaCl in the same buffer. During the 10-min gradient, 34 fractions were collected. The chromatogram at time "0" represents the elution profile of ^{65}Zn-MT-2 in the absence of GSSG.

Zinc forms a 1:1 complex with GSSG with a dissociation constant of 5.9 × 10^{-8} M in the pH range 6–7.5 (16). The stability at pH 8.6 might even be higher, based on a pK_a of 8.6 which has been assigned to one of the α-amino groups of GSSG (16). For the higher pH range, a 1:1 complex was proposed as well (17). Further, ^{1}H and ^{13}C NMR spectroscopy have supported the earlier proposal (16) that the carboxyl and amino groups of both glutamate moieties coordinate to zinc. Complex formation between GSH and zinc has been studied more extensively, although a consensus about the species distribution and stability constants as a function of pH and about the molecular structures of the complexes is still lacking (18). The multiplicity of zinc complexes with GSH/GSSG precludes a definite assignment of the molecular species that are represented by the chromatographic peaks (Figs. 2 and 3). These data, however, indicate that the new chromatographic peak (Fig. 1) observed in the interaction between MT and GSSG represents a complex between ^{65}Zn and GSSG.

GSSG is the first physiological molecule of low molecular weight to effect release of zinc from MT. All of the metals are deeply buried in the clusters of MT, and hence—if accessible at all—may be reached by only a very limited number of chelating agents (9). It is, therefore, unlikely that metal release is mediated by ligand binding to the metals. Impor-

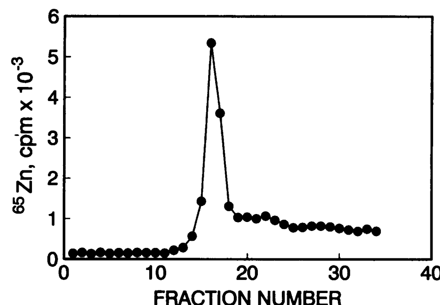

FIG. 2. Radiochromatography of a ^{65}Zn/GSH complex. The mixture contained 30 μM ^{65}Zn and 300 μM GSH and was analyzed under the same conditions of chromatography as described in the legend of Fig. 1.

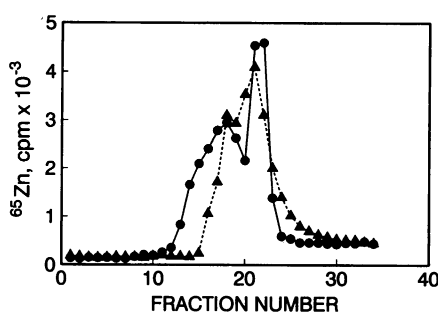

FIG. 3. Radiochromatography of a ^{65}Zn/GSSG complex. The mixture contained 30 μM ^{65}Zn and 300 μM GSSG (●) (or 10 mM GSSG, ▲) and was analyzed under the same conditions of chromatography as described in the legend of Fig. 1.

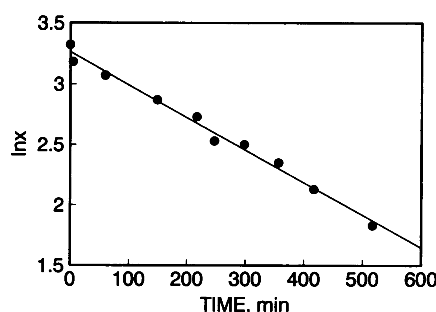

FIG. 4. Rate of the reaction between ^{65}Zn-MT-2 and GSSG. The reaction mixture contained 1 μM ^{65}Zn-MT-2 and 10 mM GSSG and was analyzed as described in the legend of Fig. 1. The rate reflects the decrease of radioactivity in the MT-2 fractions in relative units. The line drawn is based on a linear regression analysis (R^2 = 0.989).

tantly, the crystal structure of rat liver Cd_5Zn_2-MT offers an alternative possibility: Each domain has one cleft, in which the sulfur atoms of three cysteines are exposed to solvent (8). These cysteines are close to one another and provide one bridging and two terminal cysteine sulfur ligands to the metals in each cluster. We postulate that the zinc–sulfur bonds of these cysteines, in particular those of the two bridging cysteines, are residues pivotal for disruption of the clusters, and that zinc release is initiated by S-thiolation of the cysteine metal ligands in the clefts of both MT domains. For this reaction to occur, the disulfide would react directly either with the zinc–sulfur bond or with a potential fraction of free thiolate (19). In MT, the cluster dynamics could give rise to a fraction of thiolate in an amount sufficient to react with the disulfide. Inorganic analogs of the cluster structures lack stereochemical rigidity, and this allows the bridging and terminal sulfur atoms to "interchange" (20). Such a continuous making and breaking of thiolate bonds has been suggested to occur in the clusters of MT itself (21). Although experimental evidence for a reaction of a disulfide with an intact zinc–sulfur bond has not been obtained thus far, it may be pertinent that sulfur atoms of zinc-coordinating cysteines in proteins can be chemically modified. Thus, one of the two cysteine sulfur ligands of the catalytic zinc atoms in liver alcohol dehydrogenase can be carboxymethylated selectively without disrupting the zinc–sulfur bond (22, 23). Further, it was found that one of the four cysteine ligands of the zinc site in the *Escherichia coli* Ada protein is a "metallo-activated cysteine nucleophile" that attacks the methyl phosphotriester in DNA repair (24).

The reaction of MT with GSSG is not without precedent in metalloproteins. GSSG activates the latent form of collagenases—i.e., activation proceeds without proteolysis (25). When a zinc–thiolate bond was proposed in the latent collagenase, it was reasoned that the fraction of free thiol present in equilibrium with the intact zinc–sulfur bond in the zymogen would be the target for thiol/disulfide interchange with GSSG (26). If verified, this would be additional evidence that (*i*) zinc–sulfur bonds endow zinc in proteins with a special reactivity and (*ii*) thiol/disulfide interchange has an important role in controlling the biological function of zinc sites in proteins.

Reaction of MT with Other Disulfides. The reactivity of other disulfides with MT was tested by incubating reaction mixtures containing 1 μM ^{65}Zn-MT-2 and 10 mM disulfide for 60 min under the conditions employed to study the interaction between MT and GSSG and subsequently determining the relative metal content of the MT-2 fractions. Disulfides derived from monothiols were reactive, whereas those derived from two dithiols—i.e., *trans*-4,5-dihydroxy-1,2-dithiane and 1,2-dithiolane-3-pentanoic acid (lipoic acid)—were not. Based on chromatographic peak height, the percentage of metal released from MT increases in the order GSSG (20% of original ^{65}Zn dissociated from MT), mixed disulfide between glutathione and coenzyme A (44%), coenzyme A disulfide (77%), and cystamine (93%). The kinetics of the interaction of MT with the most abundant cellular disulfide, GSSG, have been studied in greater detail.

Kinetic Characterization of the Interaction Between MT and GSSG. The reaction of MT-2 with GSSG under conditions of pseudo-first-order kinetics is monophasic over three half-lives (Fig. 4). A replot of the slopes of reactions studied at GSSG concentrations between 5 and 30 mM (Fig. 5) is linear, indicating a simple rate law of the type $v = k$[GSSG]. The slope of this replot gives a second-order rate constant of $k = 4.9 \times 10^{-3}\ s^{-1} \cdot M^{-1}$. Since the second-order rate constant of the spontaneous reaction of GSH with disulfides is in the range 0.05–19 $s^{-1} \cdot M^{-1}$ (pH 7.6, 30°C) (27), the reaction rate of MT with GSSG is comparatively slow and probably reflects the low intrinsic reactivity of the zinc–sulfur bonds.

Previous studies have shown that 5,5′-dithiobis(2-nitrobenzoic acid) also reacts slowly with MT at physiological pH and under nondenaturing conditions (19). The biphasic nature of the kinetics of this reaction (19) was attributed to different reactivities for each cluster, since the isolated α-domain (4-metal cluster) of MT reacts with this reagent with a single rate (28) corresponding to the faster of the two rates. The terminal and bridging cysteine ligands in each cluster could also give rise to two reactivity classes of thiolates in MT. It is, therefore, somewhat surprising that we find monophasic kinetics for the interaction between GSSG and MT. Any

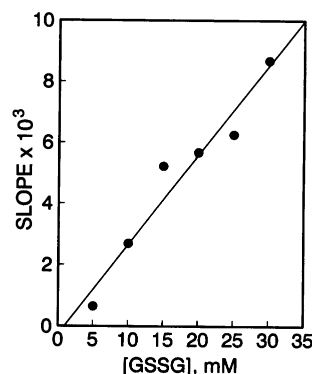

FIG. 5. Reaction of ^{65}Zn-MT-2 with GSSG under conditions of pseudo-first-order kinetics. The rates of reactions between GSSG and MT-2 in the range of GSSG concentrations given were measured as described in the legend of Fig. 1 and the slopes were determined from plots of the type shown in Fig. 4. Slopes are in units of −ln $x \cdot min^{-1}$. The line drawn is based on a linear regression analysis (R^2 = 0.945).

nonlinearity owing to different reactivities of the clusters or to the two classes of thiolate ligands should have been detected (Fig. 4).

Our data are compatible with a mechanism in which the target cysteines in each domain react at the same rate. Since there is no evidence for cooperativity of metal binding *between* the domains, the modification of one cysteine in each domain would appear to be responsible for the (non-rate-limiting) zinc depletion of each metal cluster. This interpretation is consistent with the absence of any detectable intermediates of the clusters with less than a full complement of metals (9). Therefore, neither the two-domain structure of the protein nor the difference in cluster structures has any obvious consequence regarding metal release.

How do our experimental conditions and findings relate to the possible situation *in vivo*? The range of concentrations of MT and glutathione employed here are comparable to those within the cell. For example, the constitutive concentration of MT (uninduced) in one particular cell line has been reported to be 13 μM (13), while that of glutathione is typically 0.1–10 mM (29); estimates for the cell nucleus are as high as 19 mM (30). To release zinc from MT under conditions analogous to those studied here, the GSH/GSSG redox couple must shift in favor of GSSG. Recent evidence indicates that the cytoplasmic GSH/GSSG redox status is subject to dynamic regulation under a variety of physiological conditions (31) and that in some cellular compartments the ratio differs significantly from that usually found in the cytoplasm—i.e., 1:300 (GSSG:GSH). In the endoplasmic reticulum, for instance, the ratio is 1:3 or even 1:1 (32). Thus, the driving force for the reaction between MT and GSSG could be generated intracellularly, as will be discussed.

The rate for zinc release from MT calculated from the second-order rate constant determined here and assuming a steady-state concentration of 10 μM GSSG (31) is 2×10^{-4} h^{-1}. This rate is several orders of magnitude smaller than that estimated to obtain *in vivo*—i.e., 0.6 h^{-1} (13). Two possibilities, both invoking the participation of enzymes, are envisaged to reconcile the *in vivo* and *in vitro* observations. First, as shown here, in this type of reaction with MT other disulfides are more reactive than GSSG. Thus, the physiological reactant could well be another disulfide or the disulfides of a particular protein or proteins. Second, if GSSG is indeed the cellular disulfide involved, then its reaction with MT could be enzyme catalyzed. A growing number of glutathionylating enzymes—e.g., thioltransferases—with thus far unknown substrate specificities could be examined in this regard.

IMPLICATIONS

Redox Regulation of MT. Our experiments show that MT is redox regulated and that a natural oxidative chemical process mobilizes zinc from MT. This mechanism differs fundamentally from that discussed for release of iron from transferrin. In the latter case, metal release is modulated by the interaction of transferrin with its receptor and by a 60-fold difference in hydrogen ion concentration between the cell surface and the endosome (33). If an oxidative process releases metals from MT *in vivo*, this limits the cellular signals that could be involved and, by and large, the possible function of MT.

Redox regulation involving the sulfhydryl group of cysteine controls the activity of many enzymes (34) as well as the binding of proteins to their cognate RNA or DNA. Disulfide bonds have been observed in intracellular enzymes despite the reducing environment. For some proteins, it has been shown that thiol oxidation results in activation of biological function (34) and therefore, although MT is a zinc protein, it is not the only example of an intracellular protein that might be regulated by the oxidative action of GSSG *in vivo*. It is known, moreover, that under at least two metabolic conditions—i.e., phagocytosis and fertilization of echinoderm eggs—there is acute formation of H_2O_2 by a respiratory burst oxidase (35). Hence, enzymes that form GSSG, such as glutathione peroxidase, or other enzymes that generate cellular disulfides (34) might be coupled to events that signal release of metals from MT.

The only other small molecules that are known to release zinc from MT are all toxic—e.g., hypochlorous acid, peroxide, and superoxide (36)—the principal neutrophil oxidants at inflammatory sites. Interestingly, the target of these reagents is also thought to be the zinc–sulfur bond (37). These reactions would assist in mobilizing zinc under physical/oxidative stress.

A Role of Glutathione in Zinc Metabolism? While glutathione has been discussed widely in copper metabolism (38), there are few references to its participation in zinc metabolism (18). It was shown that a copper(I)/glutathione complex can reconstitute the apoforms of copper proteins (39), including the biosynthesis of copper-MT itself (40). Copper-MT, in contrast, is ineffective in this regard and must be activated by an oxidative process before it can reconstitute apoenzymes (41). Therefore, by analogy, it is tempting to speculate that the oxidative action of GSSG also mobilizes copper from copper-MT and, further, that zinc/glutathione complexes are similarly effective transfer vehicles in the reconstitution of zinc-requiring apoenzymes.

The importance of MT/glutathione interactions is underscored by the observation that GSH forms a complex with rabbit liver Cd_5Zn_2-MT (42). In the context of our finding that GSSG mobilizes zinc from MT, this could mean that complex formation between GSH and MT protects critical, oxidant-sensitive sulfhydryl group(s) in MT and blocks metal release from MT under normal physiological conditions where the glutathione redox status favors GSH. Too much GSH would counteract the electrophilic attack of GSSG on the zinc–sulfur bonds of MT by reducing intramolecular disulfides in both MT and its putative mixed disulfide with glutathione, thus regenerating free thiols in MT. Taken together, the results seem to establish a firm physiological relationship between MT and glutathione and suggest that cellular zinc is controlled by the glutathione redox cycle.

The extent to which zinc/glutathione complexes shuttle zinc between biomolecules remains to be established. The molecular complexity of the zinc/glutathione system and its pH dependence (18) would confer interesting properties as a biological carrier for zinc. The difference in stability constants between complexes of zinc with GSH and GSSG would further contribute to zinc mobilization when the redox ratio shifts toward GSSG, much as the flux of iron may be controlled by the effect of aconitase on the cellular citrate-to-isocitrate ratio (for a discussion, see ref. 43).

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