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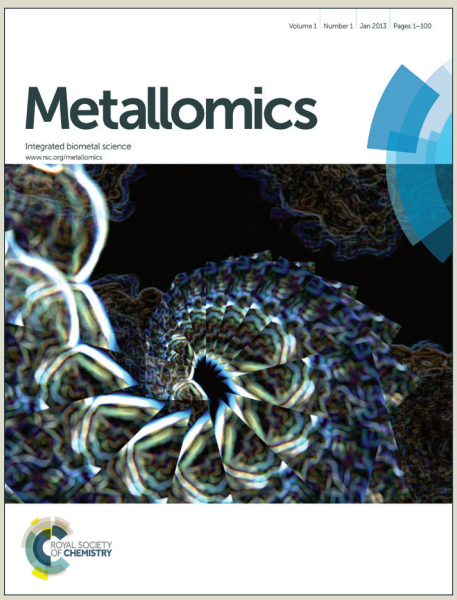

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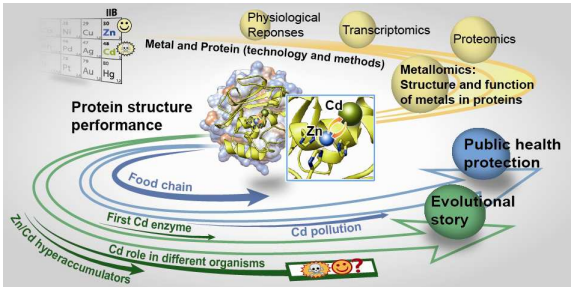

Here we give an overview of ongoing work on discovering the structural mechanisms of Cd-Zn exchange and the potentially diverse roles of Cd at Zn functional sites in proteins.

Cadmium-zinc exchange and their binary relationship in the structure of Zn-related proteins: a mini review

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Abstract

Research on cadmium-zinc exchange in proteins is important for understanding one of the main sources of Cd's biological toxicity. Because of the similar properties of these two elements, most living organisms must prevent Cd from replacing Zn in Zn-requiring proteins in order for those proteins to function normally. Recent structural studies of a variety of proteins associated with the versatile physiological functions of Zn have revealed widespread instances of Cd-Zn exchange in proteins of a large number of living organisms. Ongoing work is focused on discovering the structural mechanisms of Cd-Zn exchange and the potentially diverse roles of Cd at Zn functional sites in proteins. This research is a prerequisite to understanding the evolution of Cd-tolerant species (*e.g.*, Cd hyperaccumulating plants) and to the engineering of optimal strategies for protecting the public heath against Cd pollution.

1. Introduction

Cadmium (Cd) and zinc (Zn) are two metallic elements in group IIB with similar physical, chemical, and geological properties.[1] Zn is an ideal partner for proteins, enabling them to drive catalysis, as in the enzyme carbonic anhydrase (CA), or to form structures, as in zinc-fingers.[2,3] Living organisms have evolved to use the diversity of Zn-binding proteins in a variety of life processes, from long-term growth to instantaneous responses to environmental changes.[2-5] The Cd^{2+} ion is heavier and larger than the Zn^{2+} ion, but their chemical similarity allows Cd to substitute for Zn relatively easily in biological systems, which can destabilize the functional sites of Zn-containing proteins.[1,6] With the notable exception of the marine diatom *Thalassiosira weissflogii*, which has been shown to require Cd as a catalytic factor in the specific cadmium enzyme CDCA, almost all biological species use various strategies to exclude Cd from the active sites of proteins.[1,6,7-9] Cd is considered to be one of the most toxic heavy metals in contaminated soils, where it can cause phytotoxicity or be bioaccumulated in the food chain and then pose a threat to humans and other animals.[1,6]

The biological relationships between Cd and Zn, and especially the effects of Cd on Zn-related proteins, have been studied extensively.[10-13] Nevertheless, a more complete picture of how Cd-Zn exchange occurs at Zn-protein functional sites is needed in order to understand the evolution of Cd-tolerant species and allow the design of optimal strategies for protecting the public heath against Cd pollution. Through the determination of protein structures, researchers have recently observed widespread instances of Cd-Zn exchange in proteins of a large number of living organisms. Here we summarize these studies, and then give an overview of ongoing efforts to discover the structural mechanisms of the potentially diverse roles of Cd at Zn functional sites in proteins. We hope that this review, by advancing our current understanding of metallomics, will bring new insight and inspiration to researchers in evolutionary theory, public health, and pollution remediation, as well as to all readers interested in the behavior of metals in biology.

2. The versatility of Zn in proteins

The frequency of Zn use in protein structures can be revealed through a search of the

metalloproteins in the Protein Data Bank (PDB) with "zinc" as the keyword. Zn, together with the other three micronutrients for living organisms, iron (Fe), manganese (Mn) and copper (Cu), are well-known metallic elements utilized as cofactors by proteins. As of November 21st, 2013, there are 95, 582 structures in the PDB. Among the metalloproteins whose three-dimensional structures are known , Zn-proteins are the most abundant, with a total of 10,069 hits for "zinc" in the PDB, compared to 2,939 hits for "iron", 2,564 hits for "manganese", 1,551 hits for "copper" and 800 hits for "cadmium" (Fig. 1a). Approximately 75% of the protein structures containing Zn were determined and released after 2005, primarily using X-ray techniques. It is noteworthy that Zn-enzymes can occur in all six Enzyme Commission classes, accounting for 45.6% of hydrolases (3003 hits), 17.8% of transferases (1174 hits), 14% of oxidoreductases (924 hits), 13.1% of lyases (864 hits), 7.8% of ligases (514 hits), 1.6% of isomerases (104 hits), consistent with the versatile physiological functions of Zn (Fig. 1b). *Homo sapiens* was the source organism for 34.1% (3624 hits) of the Zn-proteins in the PDB, while a much smaller percentage was from the model plant *Arabidopsis thaliana*. The time is ripe to explore the roles that Zn plays in the structure of plant proteins, with the assistance of the techniques of bioinformatics and proteomics (Fig. 1b).

3. Cd-Zn exchange in proteins

The similarities between Cd and Zn allow the Zn-binding sites of some proteins to incorporate Cd as a replacement for Zn. Determination of protein structures has shown that this Cd and Zn exchangeability often occurs at sites in the protein where Zn plays a crucial catalytic or structural role. A search for "zinc and cadmium" in the PDB yields a total of 110 protein structures from animal (including human), plant, and bacterial species. Thus Cd-Zn exchange in proteins appears to be a widespread phenomenon, occurring when certain Zn-proteins are exposed to an environment with some quantity of Cd (Fig. 2; Table 1).

3.1 Microorganisms

Substitution of Cd for Zn at the active sites of various proteins has been observed in a number of

microorganisms in recent years. Three such proteins in the enzyme class of hydrolases include an α-toxin from *Clostridium perfringens*, a metallo-β-lactamase from *Bacteroides fragilis* and a phosphotriesterase from *Pseudomonas diminuta*.[14-19] The *C. perfringens* α-toxin, a Zn metallophospholipase, was the first bacterial toxin to be identified as an enzyme. It is the key virulence determinant in gas gangrene and has also been implicated in the pathogenesis of sudden death syndrome in young animals. A crystallographic study of the structure of this α-toxin carried out with a relatively high concentration (0.05 M) of cadmium ions in the crystallization buffer showed the presence of Cd in active sites normally occupied by Zn.[14] Surprisingly, in this case Cd can replace Zn without resulting in loss of enzyme activity. The phosphotriesterase from the soil-dwelling bacterium *P. diminuta* has attracted significant research attention as a potential bioremediation tool in light of its ability to catalyze the detoxification of organophosphate-based insecticides and chemical warfare agents. The two bridged Zn ions in the phosphotriesterase can be replaced with Cd ions without loss of enzymatic activity.[15] Little influence of Cd-Zn substitution on protein structure possibly contributed to the unchanged activity in these two enzymes. Concha *et al.* found that the *B. fragilis* metallo-β-lactamase requires Zn or Cd for hydrolyzing β-lactam antibiotics.[16] The crystal structure of the Zn^{2+}-bound enzyme showed that Zn atoms were positioned in a binuclear center at the active site. The Cd^{2+}-bound enzyme exhibited the same active-site architecture as that of the Zn^{2+}-bound enzyme but had a 10-fold reduction in activity, which was attributed to fine changes in the charge distribution due to the difference in the ionic radii of these two metals.[16] Non-hydrolases that have exhibited Cd-Zn exchange at the metal binding site include the staphylococcal enterotoxin SEA and the metalloregulatory protein CadC, both from *Staphylococcus aureus*.[20-22] SEA was the first Zn^{2+} binding enterotoxin to have its structure determined and is unique for having a Zn^{2+} coordination site involved in MHC class II binding and for its unusual octahedral coordination geometry.[20] In both the native Zn-bound SEA and its Cd derivative, the metals were coordinated by the same residues. The CadC protein is physiologically a cadmium-sensing transcriptional regulator, which de-represses synthesis of the cadmium-transporting P-type ATPase CadA. CadC has two types of metal binding site present at the interface between monomers, a regulatory physiological inducer binding site (termed Site 1) and a

non-regulatory site (termed Site 2). Kandegedara *et al.* demonstrated that both sites can bind Cd or Zn but differ in selectivity and affinity for them.[21] Additionally, in the cyanobacterium *Gloeobacter violaceous*, Cd^{2+} acts as an inhibitor blocking a membrane channel, whereas Zn^{2+} remains permeant.[23] Both ions can bind to a site at the intracellular end of the pore, but Cd^{2+} also binds at a nearby site within the pore.

3.2 Plants/Algae

The first report on the structural effects of Zn replacement by Cd in Zn fingers in plants was by Malgieri *et al.*,[24] who carried out high resolution structural characterization of the single Zn finger domain (SUP37) from the *Arabidopsis thaliana* protein SUPERMAN. Although the two peptides Cd-SUP37 and Zn-SUP37 adopted similar conformations in solution and Cd-SUP37 retains the *ββα* fold, replacement of Zn by Cd resulted in a global structural rearrangement affecting both the relative orientation of secondary structure elements and the position of side chains involved in proper functioning of the domain. Ser17 is known to play a key role in the DNA recognition mechanism of zinc finger domains. Malgieri *et al.* demonstrated that Ser17 side chain, solvent exposed in Zn-SUP37, moves toward the backbone chain in Cd-SUP37 forming a H-bond with Arg16 backbone carbonyl.[24] By quantifying Cd-Zn exchange at the binding site, this study added to our understanding of the mechanism of Cd toxicity to these crucial Zn proteins. Spontaneous exchange of Cd and Zn has been reported for the Cd-containing carbonic anhydrase enzyme (CDCA1) from the marine diatom *T. weissflogii*. Although initially isolated as a Cd enzyme, it is a cambialistic enzyme capable of using either Zn or Cd as its metal center for catalysis, with the Cd-Zn exchange occurring at much greater speed compared with previously described cases of metal exchange and metal binding.[8,9] This phenomenon appears to be an unusual adaptation to oceanic life, and is structurally explained by a stable opening of the metal coordinating site in the absence of metal.[9] "The remarkable ability to make use of an element previously known only for its toxicity is presumably a significant competitive advantage for diatoms in the metal-poor environment of the oceans," suggested Xu *et al*,[9] referring to the presence of Cd in this enzyme.

3.3 Animal/Human

Studies of Zn-Cd exchange in animals have focused on a set of Zn-dependent alcohol dehydrogenases in liver.[25-27] In a comparison of Co-, Ni-, and Cd-substituted horse liver alcohol dehydrogenase (LADH) derivatives of the native Zn enzyme using X-ray structural determination, Schneider *et al.* reported that the electronic configuration of Cd was most similar to that of Zn, and that there was no substantial difference in metal coordination between the native and the Cd-substituted enzymes.[25] Nevertheless, substitution of Cd for Zn reduced enzyme activity to only 2.5% of that of the native enzyme.[25, 27, 28] Meijers *et al.* suggested that lower activity of Cd-LADH compared to Zn-LADH resulted from the much weaker Lewis acid properties of Cd.[27] The enzyme cytochrome C oxidase (CcO), which is also an oxidoreductase, transfers electrons and protons for dioxygen reduction coupled with proton pumping that is tightly associated with energy transduction by unknown mechanisms.[29] CcO is distinctive in having Zn-binding sites on both its positive and negative sides that slow down or abolish proton release and uptake. X-ray structural determination revealed seven Zn^{2+}-binding sites when crystals of bovine heart CcO were treated with Zn^{2+} ion (0.5-5 mM $ZnSO_4$), of which two were replaced by Cd^{2+} when the crystals were treated with Cd^{2+} ion (0.5 mM $CdSO_4$). One of these Zn^{2+}/Cd^{2+}-binding sites, a well-conserved histidine, is near the active site of CcO and may be directly involved in the coupling of proton-pumping with the proton transfer process used in O_2 reduction.[29] Cd-Zn exchange has also been observed in human amyloid precursor protein (APP), which has been closely linked with the development of Alzheimer's disease and is essential for neuronal development and cell homeostasis in mammals.[30] Of eight intermolecular and intramolecular sites at which Cd can bind to APP, Cd-Zn exchange was observed at two sites, with bound Cd ion being replaced by Zn ion completely at one site and partially at the other.[30]

3.4 Other cases

Zn^{2+} is spectroscopically silent, while Cd^{2+} can usually be used as a probe in various spectroscopic techniques such as NMR.[31] Thus, some researchers have substituted Cd ions for Zn ions in structural

and kinetic studies of Zn enzymes in an attempt to understand the mechanistic role of the Zn center in catalysis or to elucidate additional details of enzymatic function. For example, CREB-binding protein (CBP) is a large, multi-domain protein that provides a multitude of binding sites for transcriptional coactivators. Using NMR spectroscopy of the ZZ domain where Zn was replaced by ^{113}Cd, Legge *et al.* resolved details of CBP's structure, identifying which side-chains constitute the Zn ligands as well as the composition of each Zn coordination sphere.[32] In bovine pancreatic carboxypeptidase A (CPA), the role of the Zn ion is to activate the coordinating water molecule to make it suitable for nucleophilic attack at the peptide bond. In a study that compared Cd^{2+}-substituted CPA with the native enzyme, Jensen *et al.* proposed a more elaborate model for the function of this enzyme, elucidating additional details of substrate recognition and water transport and providing a structural explanation of the observed Cl^- inhibition.[33]

4. The diverse roles of Cd at Zn functional sites in proteins

Although Cd and Zn have some similar properties, their differences are crucial. The substitution of Cd for Zn can negatively affect the structure and function of many proteins, and the distinct roles of Cd and Zn in living organisms have been well demonstrated. In spite of a few exceptional reports of functioning Cd-substituted derivatives of Zn proteins, as a rule, substitution of Cd at a Zn binding site induces changes in the character of a protein (Fig. 3a). In order to understand what is meant by biological "toxicity" of Cd, at least at the level of protein structure, one needs to examine all of the changes brought about by the presence of Cd instead of Zn at a Zn functional site.

4.1 Effects of Cd substitution on protein structure and function

In the *T. weissflogii* enzyme CDCA described previously, Cd is located at the bottom of a funnel-shaped active site pocket, playing a Zn-like function and coordinated by three invariant residues: Cys 263, His 315 and Cys 325.[9] A comparison of protein structures showed that replacement of Zn by Cd in CDCA induced moderate structural changes in the active pocket and in the substrate channel, and consequently altered the catalytic efficiency (value of k_{cat}/K_m) of the enzyme. Though

less efficient than the Zn form, the authors concluded that the value of k_{cat}/K_m for the Cd-bound CDCA was sufficient to satisfy a substantial fraction of the catalytic needs of a fast growing diatom.[9] Tetrahedral coordination is by far the most common geometry for Zn^{2+} binding proteins. At the Zn^{2+} binding site of SEA from *S. aureus*, a substituted Cd^{2+} ion binds with tetrahedral coordination to the same ligands that bind to Zn^{2+}, even though small molecules often display 6-coordination for both Zn^{2+} and Cd^{2+}, and one would expect 6-coordination to be more common for Cd^{2+} than for Zn^{2+} due to the larger radius of the Cd^{2+}.[20,34] Naylor *et al.* showed that in addition to helping maintain structural integrity, Cd^{2+} that replaced Zn^{2+} in the active region of *C. perfringens* α-toxin appeared to be involved in catalysis.[14] The structure of the Cd-derivative α-toxin revealed an open active site cleft where the Cd^{2+} ion was in the correct conformation for enzyme function. In other cases, however, the difference between the proteins of the Cd-form and Zn-form can distort protein function and cause loss of function. For example, the substitution of Cd for Zn in a *B. fragilis* metallo-*β*-lactamase caused a 10-fold reduction in enzyme activity, although the Cd^{2+}-bound enzyme still exhibited the same active-site architecture as that of the Zn^{2+}-bound enzyme.[16] Thus the larger effective ionic radius of Cd^{2+} compared with the ionic radius of Zn^{2+} (0.95 Å, and 0.74 Å, respectively) had only a moderate effect on protein structure, but the notable difference in the distance between the shared hydroxide and Cd^{2+} or Zn^{2+} in the Cd- and Zn-bound forms, respectively, may have influenced the catalytic rate. Similarly, although the Cd^{2+}-bound form of horse liver LADH remains enzymatically active, replacement of Zn by Cd resulted in a 97.5% decrease in enzyme activity.[27] Finally, Zn to Cd replacement in the *A. thaliana* SUPERMAN Cys(2) His(2) zinc finger induced structural rearrangements of typical DNA base determinant positions, which possibly contributed to loss of the DNA binding capability of the protein.[24]

4.2 Potential factors affecting the binary relationship between Cd and Zn in proteins

Exchange relationships between Cd and Zn have been demonstrated for a variety of Zn binding proteins in several species. Paracelsus, the father of toxicology, had the insight that whether or not a substance is poisonous depends on the dose. Hunter proposed a new understanding of the toxicity to

humans of some "poisonous metals," writing that "a growing body of evidence shows that poisonous metals might be essential nutrients in small doses," referring to Cd, lead (Pb), and even arsenic (As), the poison of choice for many murderers.[35] A biological role for the Cd ion was demonstrated, as discussed above, by the discovery of the first known Cd enzyme, CDCA, in the marine diatom *T. weissflogii.* Hunter suggests that this single-celled algae co-opted Cd as a replacement for Zn to catalyse the conversion of CO_2 to HCO_3^-, driven by its critical demand for Zn, the similarity between these two metals, and Zn deficiency in the marine environment.[35] However, it is possible that there are other cases of organisms exploiting the ease of Cd-Zn exchange in proteins. Hunter considers that the distinction between whether a metal is essential or toxic is, to some extent, only one of degree, and that the question of whether the substitution of Cd for Zn in proteins is beneficial or detrimental to an organism is probably not an absolute matter.[35] The relationship between Cd and Zn can be "friendly" or competitive depending on the site, the protein, and the species involved, based on structural variation between different metal-binding sites, protein species, and organisms. Approaches to answering this question can focus on the structural characteristics of proteins, evolutionary changes between species, the affinity of metal and protein, mechanisms of protein function, and other factors (Fig. 3b).

4.2.1 Structural characteristics of proteins

Meijers *et al.* showed that the difference in catalytic behavior between a Zn-enzyme and its Cd derivative could be attributed to the larger radius of Cd ion.[27] However, for a classical Zn finger structure, it appears that the effect of replacing Zn with the bigger Cd is dependent on specific sequence and structural characteristics (*e.g.*, the number of amino acids between the two coordinating histidines and between the two coordinating cysteines) of the domain under consideration.[24] For example, in the Zn finger domains of CP-1, TFIIIA-mF3, and SUP37, two amino acids are present between the cysteines (C-X_2-C) and three amino acids between the two coordinating histidines (H-X_3-H). When Zn is replaced by Cd in these domains, there is a loss of helical character associated with the DNA recognition helix resulting, in the case of TFIIIA, in a 10-fold decrease in binding

affinity for its DNA target. In contrast, when the number of intercysteine and interhistidine amino acids is four (C-X_4-C and H-X_4-H), as in the Zn finger domain Sp1, there is little difference in K_d between the Cd^{2+} and Zn^{2+} forms. Evidently, the structure of the Sp1 binding site allows Cd to be substituted for Zn without perturbing the structure of the domain sufficiently to interfere with DNA binding.

4.2.2 Evolutionary changes between species

The interactions between Cd and Zn in the carbonic anhydrase (CA) enzyme of the freshwater macrophyte *Ceratophyllum demersum* are very different from those described above for its fellow aquatic plant *T. weissflogii*.[8-9,11] Aravind and Prasad found that Cd exposure resulted in a significant decrease in the CA activity of *C. demersum*, which was reversed when Zn was added to the medium.[11] Furthermore, they purified the enzyme and determined the effect of the metal dose of Zn and Cd on the function and secondary structure of the pure protein, suggesting that Cd impairs CA activity by displacing Zn ions in the catalytic active sites of the enzyme. Notably, despite a lack of sequence homology and overall structure, the structure of CDCA in *T. weissflogii* is similar to that of the Zn-containing β class of CAs in higher plants in that there are two cysteines and a histidine at the metal-binding site.[9] In higher plants, a positive relationship between Cd and Zn-requiring CA is currently being investigated in Cd-hyperaccumulating ecotypes such as *Noccaea caerulescens* (formerly *Thlaspi caerulescens*) and *Picris divaricata*. Although there is not yet protein structural evidence from hyperaccumulator species, it appears that extraordinary environments (*e.g.*, Zn deficiency, Cd and Zn enrichment) have driven the evolution in protein structure that enables these unusual Cd-Zn relationships.

4.2.3 Affinity between Cd and protein Zn sites

The Zn-Cd relationship in proteins is also influenced by the metal selectivity of each Zn site. Some proteins have more than one Zn site in their structures, allowing various combinations of metal binding (see the model in Fig. 4a). In the *S. aureus* protein CadC described above, there are two sites

which can bind Zn, a regulatory site (Site 1) and a site present at the interface between monomers (Site 2). Site 1 has three or four cysteine thiolates that prefer the softer metal Cd^{2+}, while Site 2, with two imidazole nitrogens and two carboxylate oxygens, would be expected to prefer the harder metal Zn^{2+}. In fact, both sites can bind Zn^{2+}, but addition of Cd^{2+} displaced only the Zn ion in Site 1.[21] Similarly, a phosphotriesterase isolated from the soil-dwelling bacterium *P. diminuta* is a dimeric enzyme, where each subunit contains a binuclear Zn center with α- and β- Zn-binding sites. Benning *et al.*,[15] who compared high-resolution X-ray structures of the Zn^{2+}/Zn^{2+}-, Cd^{2+}/Cd^{2+}-, and hybrid Zn^{2+}/Cd^{2+}-substituted forms of phosphotriesterase, reported that in the hybrid Zn^{2+}/Cd^{2+} form, Zn preferentially occupied the five-coordinate solvent-shielded α-site while Cd occupied the six-coordinate β-site. The influence of Cd-Zn substitution on enzyme function was different at the two sites, with the Zn^{2+}/Zn^{2+} and Zn^{2+}/Cd^{2+} forms being slower (lower k_{cat}) but more efficient (higher k_{cat}/K_m) than the Cd^{2+}/Cd^{2+} form.

4.2.4 Mechanisms of protein function

Whether the presence of Cd at a Zn site is detrimental or not can also be influenced by the role the site plays in the catalytic activity of the enzyme. For example, in the case of phosphotriesterase described above, a Zn ion at the α-site has more influence on catalytic activity than does one at the *β*-site. Thus, Cd substitution for Zn at the latter site in the Zn^{2+}/Cd^{2+}-substituted form did not induce a significant difference in the kinetic parameters, in comparison with the native Zn^{2+}/Zn^{2+} form. In contrast, in the case of Cd-substituted LADH described above, the larger radius of the cadmium ion stabilizes the conformation of the E68 side chain in the proximity of the metal, which is associated with as much as a 97.5% decrease in enzyme activity due to the critical role of E68 in the capture of substrates for catalysis.[27]

4.2.5 Other factors

As described above, for some kinds of proteins the role of Cd is dependent on its location in the structure, particularly when there are a number of metal-binding sites. Cd may be substituted at more

than one Zn site, and may influence enzymatic function through diverse effects on overall protein structure (see the model in Fig. 4b). The individual alterations of these sites and their distance from the active sites of the protein need to be taken into consideration. Moreover, Cd can induce changes in other components, e.g., subunits which have high affinity for Cd^{2+} or other metal ions which can also be replaced by Cd^{2+} such as Ca^{2+} or Cu^{2+}. These changes can potentially interfere with the function of Cd-substituted proteins.

5. Outlook

5.1 The Zn-Cd relationship in Zn/Cd hyperaccumulators

"Cd-CA may not be the only Cd enzyme, and marine phytoplankton may not be the only organism that synthesizes Cd-CA," suggested Lane *et al.*.[7] The plant species known as Zn/Cd hyperaccumulators have evolved to accumulate extraordinarily high levels of Zn and Cd in the aerial parts without causing visual toxicity symptoms.[36] Intriguingly, it has been hypothesized that Cd may stimulate the function of some Zn-binding proteins in the Zn/Cd hyperaccumulators *Noccaea caerulescens* (formerly *Thlaspi caerulescens*), *Picris divaricata* and *Sedum alfredii*. In these species, Cd may play a Zn-like role, enhancing the activities of enzymes such as carbonic anhydrase (CA), a typical Zn-requiring photosynthetic enzyme involved in the Calvin cycle of higher plants. Liu *et al.* and Ying *et al.* observed that Cd addition had a positive effect on the activity of CA in *N. caerulescens* and *P. divaricata*, respectively, and increased biomass in the former.[37-38] Other research has looked at the Zn/Cd relationship as it affects other growth processes of Zn/Cd hyperaccumulators. Optimization of hyperaccumulator growth may be dependent on their ability to match root system development with the distribution of soil elements in the field. In a study on the Zn/Cd hyperaccumulator *S. alfredii*, Liu *et al.* found that both Zn and Cd were actively foraged by roots. Moreover, in comparison with control plants, both metals increased the dry biomass of shoots (1.6-3.2 times) and diminished visible sublethal Zn deficiency symptoms such as chlorosis, internode shortening ('rosetting') and reduction in leaf size ('little leaf').[39] Zn is a vital factor for the growth of all plants, while Cd is normally

non-essential for higher plants.[6] It is possible that the growth promoting effects of Cd in Zn/Cd hyperaccumulators may be an indirect effect of interference with the plant's internal availability of 'real' nutritional elements (*e.g.*, Zn), noted by Verbruggen *et al.* in their review on plant mechanisms of coping with Cd.[40] In another review about "how plants cope with Cd," Qiu *et al.* collected the increasing evidence from biological and physiological studies that supports a positive role of Cd in these exceptional species.[41] Further study using enzymological and molecular methods is needed to elucidate the biological function of Cd in hyperaccumulators and the mechanisms by which it stimulates Zn-associated physiological functions of these plants.

The discovery of the biological function of a Cd-specific CA in the marine diatom *T. weissflogii* has generated considerable speculation about possible substitution of Cd for Zn at Zn functional sites in the proteins of vascular plants.[7-9] In approaching this question, the complexity of the system studied and the differences between single-celled algae and higher plants should be taken into consideration. For example, assuming that some Zn/Cd hyperaccumulator species do substitute Cd for Zn in some Zn functional sites, then these candidate sites must be closely linked with the roles Zn plays in these plants. Strasdeit has reviewed the biological chemistry of Cd, with all the facts from 1858 to 2000 in chronological order.[42] In his opinion, the utilization of Cd by *T. weissflogii* is a mechanism evolved for dealing with the very low Zn concentration in surface seawater, in view of the key role that CA-Zn plays in carbon acquisition. As Lane *et al.* explains,[8] "the enzyme carbonic anhydrase (CA) constitutes a major use of cellular Zn, and the expression of TWCA1, the major intracellular CA, depends directly on the Zn nutrition of the organism." In comparison, in higher plants, numerous studies have demonstrated a wide variety of functions of Zn. During photosynthesis, Zn functions not only in the enzyme CA mentioned above, but also in a considerable number of other photosynthetic proteins or steps.[2,43-44] Zn is required in plants for modulating proliferation and expansion of differentiating cells through affecting auxin metabolism or related transcription factors.[2,45-46] Moreover, Zn is essential to plants not only as a catalytic factor in enzymes, but also as a necessary structural component in hundreds of proteins.[4-5] In particular, many transcription factors in the nucleus are known to contain Zn fingers and similar domains, whose functions range from regulation of DNA-transcription and

RNA-processing to protein-protein interactions.[47-49] Thus, there are many possible sites other than CA where Cd may play a Zn-like functional role, as Liu *et al*. proposed in a study of the CAM Zn/Cd hyperaccumulator *S. alfredii*,[39] in which CA may play a limited role in photosynthesis. Given the variety of Zn functions in plants, it is possible that Cd may have an equally wide variety of nutrient-like behavior in Cd-tolerant plants (Fig. 5). The molecular understanding of this hypothesis is being tested by us and has not yet been published.

In the past two decades of research on Zn/Cd hyperaccumulators, many plant physiological and molecular investigators have used various techniques to identify the strategies these plants use for transporting and distributing Zn, due to their close relationship with the mechanisms of metal hyperaccumulation and hypertolerance. These techniques have included proteomics, transcriptomics, synchrotron X-ray fluorescence analysis (SXRF), fluorescence microscopy using metal-fluorophores, and so on (Table 2).[50] With respect to Zn-Cd exchange at the level of protein structure, non-denaturing conditions must be used during the experimental process if metal-related factors are to be retained in vitro as they are in vivo, and this can be technically challenging due to a potential for exchange between Zn and Cd leading to metal acquisition or loss during fractionation.[58] Recombinant expression of the involved Zn-proteins in vitro is a novel approach to determining the structures and the Zn-Cd exchange characteristics of these proteins, as exemplified by two recent studies, "Isolation and characterization of *Arabidopsis halleri* and *N. caerulescens* phytochelatin synthases" and "Metal binding properties and structure of a type III metallothionein from *N. caerulescens*".[56,57] In the search for potential Cd-enzymes in land plants, the larger goal is to characterize differences of protein structure and metal-protein relationships between non-hyperaccumulating and hyperaccumulating species. This work should yield greater understanding of the evolution in hyperaccumulators of increased metal tolerance and their possible use of Cd for Zn-like functions.

5.2 Cd toxicity and public health

The relevance of Zn to human health has been well documented since it was shown to be an essential nutrient for humans in 1963,[59] but, unlike the situation in plants, there have been no reports

of humans adapting to Cd toxicity. In contrast, abundant research has shown that Cd pollution is a major threat to public health. In China, levels of heavy metals (including Cd) in soil, food and humans have risen markedly during the rapid development of industrialization and urbanization that has occurred over the last two decades.[60] Consumption of food crops contaminated with heavy metals is a major food chain route for human exposure. In Ding Li, a suburban area of Tianjin City in northern China and the third largest industrial center in China, farmlands and fish ponds have become heavily polluted by heavy metals from atmospheric deposition, solid waste emissions, sludge applications, wastewater irrigation, and other sources.[61] Human intake of Cd and the associated health risk to adults in this area have been ascribed mainly to consumption of vegetables and fish, which contribute a substantial fraction (about 51%) of the total target hazard quotient, according to an estimate by the Tao group from the Laboratory for Earth Surface Processes at Peking University (Beijing, China).[62] Typically, the process of soil-to-plant transfer is one of the key sources of human exposure to metals through the food chain. In Beijing, "long-term wastewater irrigation has led to buildup of heavy metals in soils and food crops," according to results from the Zhu group at the Research Center for Eco-environmental Sciences, who found that pollution load index values indicated that wastewater-irrigated soils were strongly enriched with Cd, while Cd concentration of food crops exceeded SEPA limits.[63] Similarly, in the vicinity of Dabaoshan mine in southern China, heavy metal contamination of food crops grown around the mine has posed a significant health risk to the local population through its consumption of rice and vegetables.[64] In the industrial area of Huludao City in northeastern China, the non-carcinogenic health risk of Cd and other heavy metals to adults and children via dietary intake was estimated by Zheng *et al.*,[65-66] who found that the relative contribution of Cd to the Hazard Index (HI) was 24.0% for adults and 21.8% for children. Cereal, seafood, and vegetables were the main sources of heavy metal intake from foodstuffs for adults and children, while fruit, milk, beans, and egg were secondary contributors.[65] Together with heavy metal contamination in street dust due to metal smelting, the HI for Cd for children in Huludao City is close to 1, representing a substantial potential health risk.[66] Other research has shown that Cd may present a significant environmental and human health risk as a result of the recycling of printed circuit boards in e-waste

processing in southeastern China,[67] the eating of green-lipped mussels polluted by Cd,[68] and the application of Cd-containing phosphate fertilizers to urban park soils in Hongkong.[69] In light of the interchangeability between Zn and Cd and the importance of Zn in human biology, more research is needed on the molecular mechanisms of Cd toxicity, including quantification of Cd binding to proteins and the effect of Cd on protein structure. Continued study of Cd-Zn exchange in proteins will give us greater understanding of the threat Cd poses to human health and will contribute to the remediation and protection of China's environment in the future (Fig. 6).

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Figure 1-6; Table 1-2

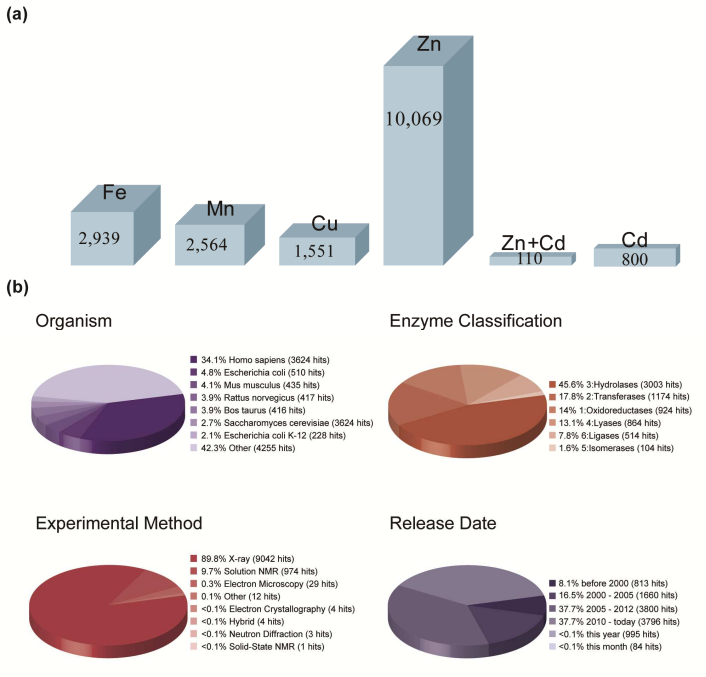

Fig. 1 Results of searching the Protein Data Bank (PDB) for metalloproteins whose 3-dimensional structures have been determined. (a) Number of hits using "zinc (Zn)", "iron (Fe)", "manganese (Mn)", "copper (Cu)", "cadmium (Cd)" or "zinc and cadmium (Zn and Cd)" as keywords. (b) Classification of Zn proteins in the PDB according to the categories "organism," "enzyme classification," "experimental method," and "release date." The database was searched on 21 November 2013.

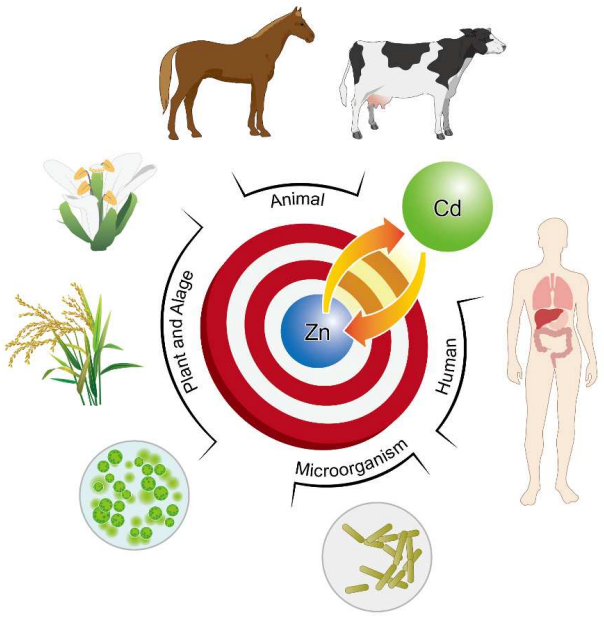

Fig. 2 Exchange between Cd and Zn can occur in Zn-proteins when humans or other animals, plants, and bacteria are exposed to an environment with some quantity of Cd.

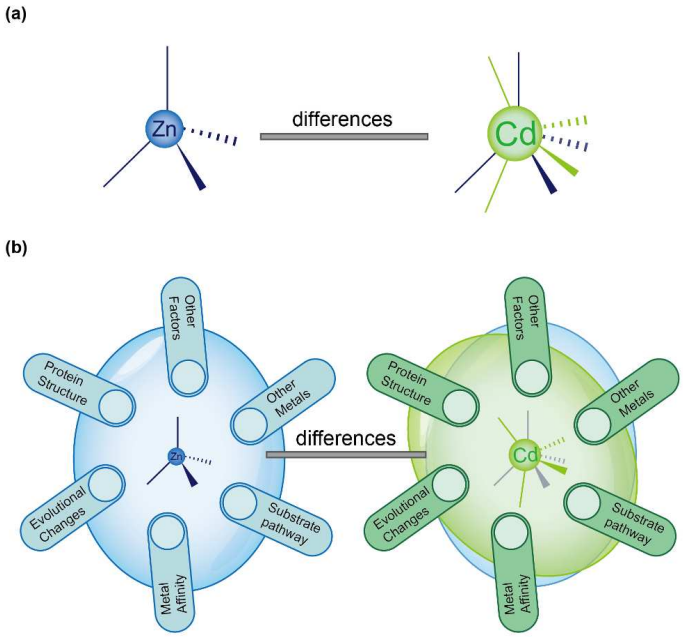

Fig. 3 Effects of Cd substitution on protein structure and function. (a) There are differences between Cd-substituted and native Zn functional sites in proteins. (b) Potential factors involved in the binary relationship between Cd and Zn in proteins.

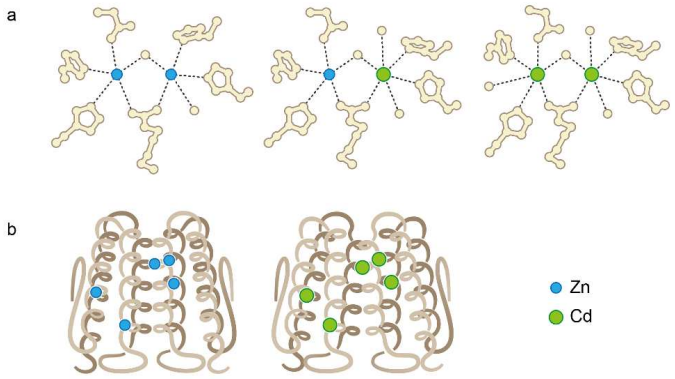

Fig. 4 Substitution of Cd for Zn can occur at more than one Zn-binding site for some proteins. (a) Model of the Zn^{2+}/Zn^{2+}-, mixed Zn^{2+}/Cd^{2+}-, and Cd^{2+}/Cd^{2+}-substituted forms of phosphotriesterase according to Benning et al. (2001). (b) Example of substitution of Cd for Zn at a number of metal-binding sites.

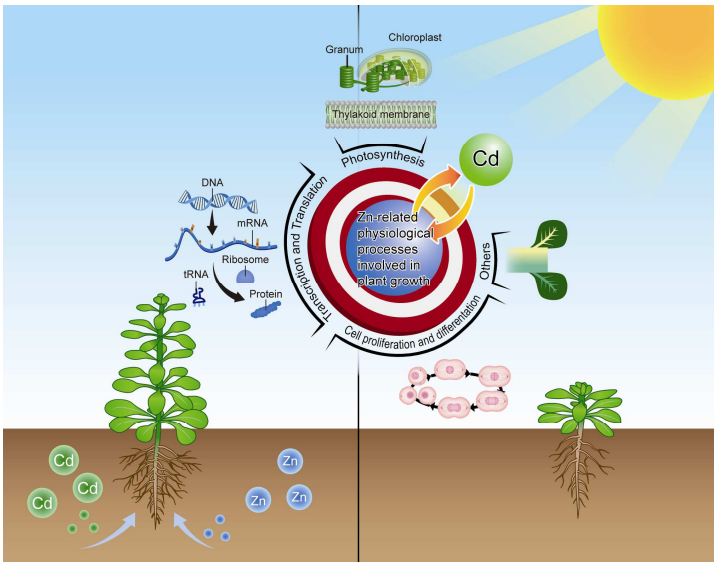

Fig. 5 Hypothesized positive effects of Cd-Zn substitution on a variety of Zn-related physiological processes involved in growth of Zn/Cd hyperaccumulators.

Fig. 6 Implications of Cd-Zn exchange in proteins for human health and the remediation and protection China's environment.

Table 1 Information from the PDB on Zn-Cd exchange cases mentioned in the text.

No	PDB code	Protein name	Species	Classfication
1	3BOC, 3BOE,3BOH,3BOJ	Carbonic anhydrase	*T. weissflogii*	Lyase
2	2WY6,2WXT,2WXU,1CA1	α-toxin	*Clostridium perfringens*	Hydrolase
3	1HZY,1IOB,1IOD	phosphotriesterase	*Pseudomonas diminuta*	Hydrolase
4	2ZNB,3ZNB, 4ZNB,3ZR9	metallo-β-lactamase	*Bacteroides fragilis*	Hydrolase
5	1ESF	staphylococcal enterotoxin type A	*Staphylococcus aureus*	Enterotoxin
				DNA Binding
6	3F72,1U2W	CadC	*Staphylococcus aureus*	Protein/Gene Regulation
7	2XQA,2XQ3-2XQ9	GLIC	*Gloeobacter violaceous*	Membrane Protein
8	2L1O	SUPERMAN Cys(2) His(2) zinc finger	*A. thaliana*	Metal Binding Protein
	2JHF,2JHG;			
9	1HET,1HEU,1HF3	Alcohol Dehydrogenase	*Horse Liver*	Oxidoreductase
10	2EIK, 2EIL	cytochrome C oxidase	*bovine heart*	Oxidoreductase
11	3UMI	amyloid precursor protein (APP)	*human*	Metal Binding Protein
12	1TOT	ZZ Domain of CBP	*human*	Transferase
13	1EE3, 1ELL, 1ELM	carboxypeptidase A	*bovine*	Hydrolase

Note: the data are from PDB before October, 2013.

Table 2 Recent studies on the relationship between Zn and Cd in Zn/Cd hyperaccumulators.

No	Species of Zn/Cd hyperaccumulators	Aspect/Technique used	Authors/References
6	*N. aerulescens*	Transcripitome	Van de Mortel *et al.*[12]
11	*N. caerulescens*	transport and distribution	Küpper and Kochian[13]
1	*N. caerulescens*	CA activity	Liu *et al.*[37]
2	*P. divaricata*	CA activity	Ying *et al.*[38]
4	*S. alfredii*	root growth (foraging)	Liu *et al.*[39]
3	*N. caerulescens*	root growth (foraging)	Whiting *et al.*[51]
10	*N. aerulescens and A. halleri*	transport and distribution	Cosio *et al.*[52]
9	*N. caerulescens*	transport and distribution	Ma *et al.*[53]
8	*Arabidopsis halleri*	Proteomics	Farinati *et al.*[54]
7	*Arabis paniculata*	Proteomics	Zeng *et al.* [55]
13	*N. caerulescens and A. halleri*	metal binding with PC	Meyer *et al.*[56]
12	*N. caerulescens*	metal binding with MT	Fernandez *et al.*[57]