Principles Governing Mg, Ca, and Zn Binding and Selectivity in Proteins

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I. Introduction

Metal ions are required for the growth of *all* life forms.[1−4] Currently, about half of all proteins contain metal ions,[5] and most ribozymes *cannot* function without metal ion(s).[1−3,6−11] Metal ions perform a wide variety of specific functions associated with life processes.[1−3] One function uniquely performed by metalloproteins is respiration, whereby an iron center in the hemoglobin-myoglobin family and hemerythrins or a copper center in hemocyanins binds an oxygen molecule reversibly. In many cases, metal ions, e.g., Zn(II), Mg(II), Ca(II), stabilize the structure of folded proteins, while in other cases they help to fix a particular physiologically active conformation of the protein. Metal ions are an integral part of many enzymes and are indispensable in several catalytic reactions, e.g., hydrolytic, redox and isomerization reactions. In particular, transition metals, such as Fe, Cu, and Mn, are involved in many redox processes requiring electron transfer. Alkali and alkaline earth ions, especially Na(I), K(I), and Ca(II), play a vital role in triggering cellular responses.

Among all naturally found metalloproteins, those containing Mg(II), Ca(II), and Zn(II) appear to be the most abundant and well studied both experimentally and theoretically.[1−3,12−63] Hence, these three cations are the focus of this review. Here, our goal is *not* to carry out an exhaustive review of the ever-growing literature on these divalent ions. Instead, we attempt to delineate some fundamental principles governing Mg(II), Ca(II), and Zn(II) binding and selectivity in proteins based on the body of recent computational results and experimental data (see end of Introduction). In what follows, we first summarize the known biological roles of Mg(II), Ca(II), and Zn(II), their coordination chemistry, and sequence motifs and/or structural characteristics of the metal-binding sites before presenting the specific questions that are addressed here.

Magnesium: This is one of the most versatile metal cofactors in cellular biochemistry, serving both intra-

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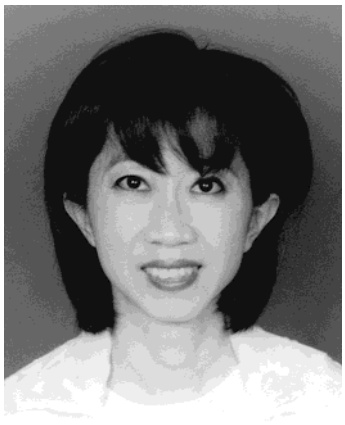

Carmay Lim obtained her Ph.D. degree in Chemical Physics from the University of Minnesota at Minneapolis in 1984 working with Prof. Donald Truhlar. After carrying out postdoctoral research with Prof. John Tully at AT&T Bell Laboratories, Murray Hill, and Prof. Martin Karplus at Harvard University, she joined the faculties of the Departments of Medical Genetics, Biochemistry and Chemistry at the University of Toronto. In 1995, she moved to the Institute of Biomedical Sciences at Academia Sinica in Taiwan where she is Professor/Research Fellow with a joint appointment at the chemistry department in National Tsing Hua University. Her research interests include (1) unraveling the principles underlying protein–metal, protein–protein and protein–nucleic acid recognition, (2) designing antibiotics, anticancer and antiviral agents with therapeutic potential, and (3) developing new computational techniques/algorithms.

and extracellular roles.[25] It is used to stabilize a variety of protein structures. For example, Mg binds at the interface of the ribonucleotide reductase subunits, stabilizing the interfacial domain,[64] and helping, in some cases, to form the active trimer structure.[65−67] It is also used to stabilize nucleic acids, by alleviating electrostatic repulsion between negatively charged phosphates. Furthermore, Mg(II) and Ca(II) stabilize biological membranes by charge neutralization after binding to the carboxylated and phosphorylated headgroups of lipids. Mg also plays a catalytic role. It activates enzymes that regulate the biochemistry of nucleic acids such as restriction nucleases, ligases, and topoisomerases,[25] and is essential for the fidelity of DNA replication.

Divalent Mg is a "hard" ion and prefers "hard" ligands of low polarizability, with oxygen being the most preferred coordinating atom, followed by nitrogen.[43,46,68] All the Mg-binding sites in the Protein Data Bank (PDB) structures contain at least one *carboxylate* ligand, which coordinates Mg predominantly in a *monodentate* fashion.[20,25,46,69] Among the noncharged protein ligands that bind Mg, the side chains of Asn/Gln and the backbone carbonyl groups are the most common, followed by the Ser/Thr, His, and Tyr side chains.[46] The rest of the *octahedral* coordination sphere is complemented by water ligand(s). The Mg−O internuclear separation ranges from 2 to 2.2 Å.[20,43,46,70] Although in proteins Mg tends to bind *directly* to the side-chain ligand (*inner-sphere mode*), in nucleic acids it prefers to bind *indirectly* via water molecule(s) (*outer sphere mode*).[25]

Unlike Ca- and Zn-binding sites (see below), only a few Mg-binding sequence motifs have been identified. These include -NA**D**F**D**G**D**- observed in different RNA polymerases, DNA Pol I, and HIV-reverse transcriptase,[71,72] and -YX**DD**- or -LX**DD**- found in reverse transcriptase[73] and telomerase[74] (the bold residues are the ones ligated to Mg). For some proteins that lack close sequence homology, three-dimensional binding motifs have been found.[69]

Calcium: Like Mg, Ca ions serve both intra- and extracellular roles. Outside cells, Ca ions are known to play roles in maintaining the rigidity of whole plants, in joining certain proteins in the blood-clotting system with membrane surfaces of circulating cells, in extracellular enzyme activity, and in increasing the thermal stability of proteins.[1,75] Inside eukaryotic cells, Ca concentration levels regulate a wide range of biological processes including muscle contraction, secretion, glycolysis and gluconeogenesis, ion transport, cell division and growth.[75] To maintain the correct Ca ion concentration in the intra- and extracellular space, the body uses Ca pumps. Consequently, the system is able to respond appropriately to signals that occur in the form of sudden changes in the Ca ion concentration. Most Ca-binding proteins possess a highly conserved Ca-binding motif, the EF-hand, which selectively binds Ca against the background of up to 10^5-fold higher concentrations of Na(I), K(I), and Mg(II).[16] This specificity is related to the *regulatory* role that Ca plays in the signal transduction process: the binding sites are engineered in such a way so that they remain unoccupied until a Ca signal appears, but they can eliminate Ca when it is no longer needed.[75]

Ca, like Mg, prefers to bind to "hard" oxygen-containing ligands but, generally, with lower free energy gain.[21,27,43,52,68] In order of decreasing prevalence, these ligands are carboxylates, carbonyls, water, and hydroxyl oxygen atoms.[68,76] Unlike Mg, which nearly always occupies the center of an octahedron, the observed coordination number (CN) of Ca in proteins varies from 6 to 8.[68,76] The ionic radius of a Ca ion with a given CN is always higher than that

of Mg with the same CN. The ionic radii of 6- and 8-coordinated Ca are 1.00 and 1.12 Å, respectively, whereas the corresponding values for Mg are 0.72 and 0.89 Å.[77] Carboxylate and carbonyl ligands exhibit Ca−O internuclear separations ranging between 2.1 (monodentate) and 2.8 Å (bidentate), whereas coordinating water molecules exhibit Ca−O distances of 2.3−2.9 Å.[70,76,78] Compared to Mg, Ca shows a greater tendency toward bidentate carboxylate binding, but a lower affinity for water.[28]

Three general types of Ca-binding sites have been identified:[79−81] class I sites provide Ca ligands from a short, continuous sequence (classical EF-hands); class II sites differ from class I in that one of the Ca ligands comes from a sequence distant from the classical EF-hand sequence (lipase, subtilisin); and class III sites supply Ca ligands from different parts of the sequence (cellulase, adamalysin). Most of the Ca-binding ligands are provided by loops/turns.[81] The EF-hand motif is one of the most common Ca-binding motif:[82] in the *Drosophila melanogaster* genome the EF-hand family is the 12th most abundant protein domain.[81] The classical EF-hand motif consists of a 12-residue Ca-binding loop flanked by two helices forming a conserved helix−loop−helix structure.[82−84] Asp/Glu side chains and Asn/Gln/backbone carbonyls from the loop bind Ca in a pentagonal bipyramidal geometry.[78,85,86] The monodentate-bound aspartate at the first loop position and the bidentate-bound glutamate at the last loop position are highly conserved in the EF-hand superfamily.[86]

Zinc: This is one of the most abundant divalent metals in living organisms (2.3 g of Zn for an average person[87]). It is an essential cofactor of many metabolic enzymes and transcription factors.[3,13,15,18,30,32] Zn-binding sites in proteins can be divided into two categories: (1) sites that play predominantly a catalytic role, and (2) sites that serve only a structural role. The most common Zn-chelating sphere found in the first category is His_3Water, although catalytic sites containing <3 His, Asp/Glu, or Cys side chains have also been observed.[13,18,44,88] The best studied "structural" Zn-proteins are those of the Zn-finger family, which is involved in nucleic acid binding and gene regulation.[18,34] Commonly accepted classes of Zn-fingers include: (a) the cellular or transcription factor type, characterized by a Cys_2His_2 metal-binding site;[89,90] (b) the retroviral type, possessing a Cys_3His chelation sphere;[91] and (c) the steroid receptor type, having a Cys_4 metal-binding site.[92] These classes can be further divided into different subclasses depending on the local fold and spacing between ligating residues.[93,94] In contrast to the "catalytic" Zn-binding sites, which are partially exposed to solvent, the "structural" Zn-sites are deeply buried and are surrounded by an elaborate network of hydrogen bonds provided by the second-coordination layer.[60]

Unlike Mg and Ca, Zn prefers "softer" ligands such as Cys and His, although it is also found coordinated to Asp and Glu side chains.[40,68,76] The Cys side chains are considered to be deprotonated when bound to the metal.[32,60,62] Although in aqueous solution Zn is octahedrally bound to six water molecules,[70] in both Zn-finger proteins and enzymes Zn is usually tetrahedrally coordinated, but it can also adopt a 5- or 6-coordinate geometry. The average Zn−ligand bond distances for a tetrahedral binding site are (in Å) as follows: Zn−N(His) 2.07−2.09; Zn−S(Cys) 2.21−2.35; Zn−O(Asp/Glu) 1.95−2.04; Zn−O(Water) 2.12−2.15.[88]

Although a wealth of information has been accumulated on the biochemical and physiological significance of Mg(II), Ca(II), and Zn(II), no generic rules on their binding and selectivity in proteins have been reported (to the best of our knowledge). Here, based on findings from our group and other research groups, we try to establish physical bases for the following aspects of metal binding and selectivity in proteins. (1) Why do metal ions tend to bind to proteins *directly* at centers with high hydrophobic contrast? (2) What is the most thermodynamically preferable set of inner-sphere ligands for a given metal cation (Mg(II), for example) in a protein? (3) What is the most thermodynamically preferable coordination geometry of a bidentate ligand (such as carboxylate) or a given metal (Zn(II), for example) in proteins? (4) How does a protein select a specific metal cation (Mg or Ca or Zn) from the mixture of ions in the surrounding fluids? In each of the following sections, we first present the background/rationale and outline the approach (depending on the original references to provide details of the methodology[95]). Then, the key results are summarized and the physical basis and/or implications of the findings are discussed.

II. Effect of Dielectric Medium

A. A Buried Cavity (Low Dielectric Medium) Favors the Inner-Sphere Binding of Protein Ligands to the Metal, Whereas a Solvent-Exposed Site (High Dielectric Medium) Favors the Outer-Sphere Binding of Negatively Charged Asp/Glu to the Metal

Metal ions have been found in proteins to bind generally to a shell of polar hydrophilic residues surrounded by a shell of nonpolar hydrophobic groups.[14] Furthermore, they tend to bind *directly* to the hydrophilic protein residues, instead of indirectly via a metal-bound water molecule. However, it is not clear why metal ions tend to bind to proteins in an inner-sphere fashion at centers of high hydrophobic contrast. To answer this question ab initio and continuum dielectric methods (CDM) have been used to compute the free energy (ΔG_{ex}) of exchanging a metal-bound water for ligands of biological interest in various dielectric media:[52]

$$[M\cdot W_6]^{2+} + L^z \rightarrow [M\cdot W_5\cdot L]^{2+z} + H_2O \qquad (1)$$

where M = Mg, Ca or Zn, W = H_2O, and L^z ($z = 0, -1$) are models of protein ligands that are most frequently found to be coordinated to the metal cations. These are (a) imidazole for the His side chain, (b) formate for deprotonated Asp or Glu side chains, and (c) formamide mimicking the backbone peptide group or Asn or Gln side chains.

Table 1. Calculated Free Energies (ΔG^{X}_{ex}) for the Reaction, $[M(H_2O)_6]^{2+} + L^z \rightarrow [M(H_2O)_5L]^{2+z} + H_2O$ in Media of Different Dielectric Constant X (in kcal/mol)[a]

M	L^z	ΔG^{1}_{ex} [b]	ΔG^{2}_{ex}	ΔG^{4}_{ex}	ΔG^{80}_{ex}
Mg	$HCOO^-$	−190.3	−90.0	−40.0	7.6
Ca	$HCOO^-$	−181.7	−85.5	−37.6	8.1
Zn	$HCOO^-$	−195.1	−95.1	−45.3	2.0
Mg	$HCONH_2$	−12.7	−4.1	0.4	4.2
Ca	$HCONH_2$	−12.7	−5.0	−0.9	2.8
Zn	$HCONH_2$	−13.1	−5.2	−1.3	1.8
Mg	imidazole	−18.5	−9.7	−5.4	−1.3
Ca	imidazole	−16.3	−8.6	−4.8	−1.1
Zn	imidazole	−24.7	−16.0	−11.8	−7.8

[a] From Dudev & Lim, 2000.[52] [b] Calculated at the MP2/6-311+G*//HF/6-311+G* level.[52]

Table 1 lists the computed free energies (ΔG^{ϵ}_{ex}) for eq 1 in the gas phase ($\epsilon = 1$), in buried or partially buried sites characterized by a dielectric constant ϵ equal to 2 or 4, and in fully solvent-exposed sites where the $\epsilon \sim 80$.[52] In the gas phase, the water−formate exchange reactions are characterized by a high free energy gain (−182 to −195 kcal/mol) due to the strong attractive Coulombic interactions between the oppositely charged metal and formate ions. As ϵ increases, the magnitude of the exchange free energy decreases rapidly, but the free energy is still negative when the metal site is buried or partially buried (ΔG^{4}_{ex}= −38 to −45 kcal/mol). However, in aqueous solution, the exchange free energy becomes positive (ΔG^{80}_{ex}= 2−8 kcal/mol), implying that the exchange reaction may not occur.

In analogy to exchanging a metal-bound water for a formate, a neutral ligand can replace water in the gas-phase, although the weaker charge−dipole interactions result in a smaller free energy gain (−13 to −25 kcal/mol) compared to that for the water−formate exchange reactions. Although the absolute value of ΔG_{ex} decreases with increasing ϵ as for the water−formate exchange reactions, replacing a water with formamide in Mg, Ca, and Zn complexes becomes thermodynamically unfavorable at a smaller value of ϵ; i.e., $\epsilon > 4$. In contrast to exchanging a metal-bound water for formate or formamide, imidazole can displace a water molecule from the hexahydrated metal over the entire ϵ range due mainly to the more favorable ΔG_{ex} in the gas-phase. This is consistent with the fact that His−x_3−His sites on *surface exposed* α-helices have been successfully used in protein purification due to their binding to metal dications immobilized on resins.[96,97]

The results in Table 1 suggest that metal ions tend to bind to proteins in an inner-sphere fashion at centers of high hydrophobic contrast because the hydrophobic outer sphere provides a relatively solvent-inaccessible, low-dielectric cavity that enhances electrostatic metal−protein ligand interactions, thus favoring the exchange of a metal-bound water for a protein ligand.[14,52] In structures in which the metal dications in Table 1 are found to be directly bound to *negatively charged* residues, the binding process to the protein probably occurs in a stepwise (as opposed to a one-step) process: the hydrated metal ion initially positions itself in a solvent-inaccessible pocket, and subsequently, some of its inner-shell waters are replaced by negatively charged side-chains.

III. Coordination Stereochemistry

A. Hexahydrated Mg Has High Affinity for Asp/Glu Side-Chains in Protein Cavities, but There Is an Upper Limit to the Number of Asp/Glu Residues That Can Be Coordinated to Mg

Although Mg is known to bind to oxygen-containing ligands (see Introduction), its most thermodynamically preferable set of inner-sphere ligands (i.e., number of each residue type) has not been experimentally determined. In particular, it is not known if Mg will exchange all its first-shell water molecules for protein ligands, and if it will still prefer negatively charged ligands after its positive charge has been neutralized by binding to two Asp/Glu side chains to form a neutral complex. To address these issues, density functional theory (DFT) and CDM have been used to compute the free energy (ΔG_{ex}) of successive water exchange reactions in Mg complexes:[46]

$$[Mg{\cdot}W_{6-n}{\cdot}L_n]^{2+nz} + L^z \rightarrow [Mg{\cdot}W_{5-n}{\cdot}L_{n+1}]^{2+(n+1)z} + H_2O \quad (2)$$

where L = HCOOH, CH_3OH, $HCONH_2$, $HCOO^-$ are models of ligands that are most frequently found to be coordinated to Mg and $n = 0-5$. These reactions mimic the water-amino acid exchange reactions in Mg-binding sites. Since Mg will exchange its first-shell water for a formate anion only if it is bound in a relatively solvent-inaccessible, low-dielectric cavity (see section IIA), the free energies have been computed for $\epsilon \leq 4$.

Figure 1 shows ΔG^{4}_{ex} for successively replacing a Mg-bound water molecule with formic acid, methanol,

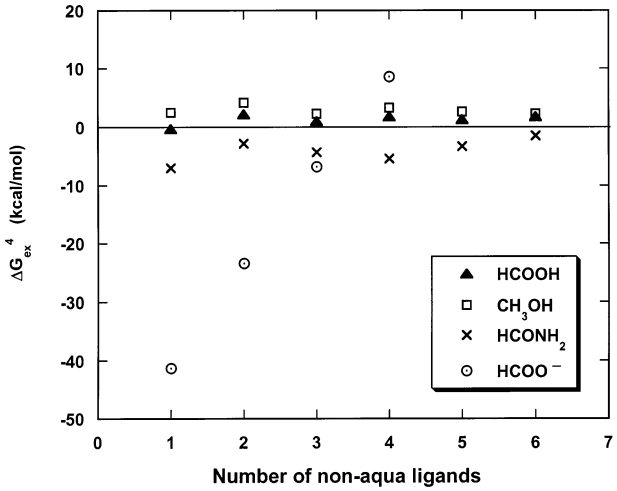

Figure 1. The free energy, ΔG^{4}_{ex}, for replacing a Mg-bound water molecule with formic acid (▲), methanol (□), formamide (×) and formate (open circle with dot) in a cavity characterized by $\epsilon = 4$ as a function of the number of nonaqua ligands bound. The gas-phase properties are evaluated at the B3LYP/6-31+G* level.[46]

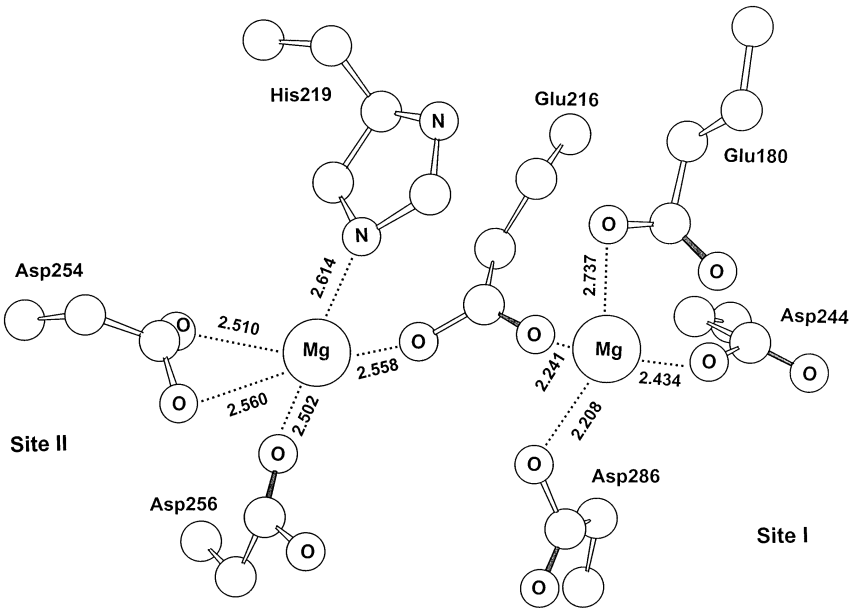

Figure 2. The Mg-binding site in the 1.8 Å X-ray structure of xylose isomerase (PDB entry 1XYA).

formamide, and formate in a cavity characterized by $\epsilon = 4$. For the first three reactions, the free energy gain upon exchanging water for a negatively charged formate greatly exceeds that for the other neutral ligands, but this gain is abolished upon protonating the formate as the neutral formic acid does not compete effectively with water for Mg. Thus, Mg has high affinity for negatively charged Asp/Glu in sites with low solvent accessibility. These results correlate with the finding that (i) all Mg-binding sites in the PDB contain at least one Asp or Glu[46] (see Introduction) and (ii) most enzymes containing Mg as a cofactor are active around pH 8, where Asp and Glu are probably deprotonated.[11,98]

Figure 1 also indicates an upper bound on the number of deprotonated acidic residues that can be coordinated to hydrated Mg: for complexes containing four (or more) formates, the exchange of a Mg-bound water for another formate becomes thermodynamically unfavorable. The finding that Mg can accommodate up to only three negatively charged ligands in its first coordination shell appears, at first glance, to be at odds with the four-carboxylate binding pocket (site I) found in the X-ray structure of Mg-bound xylose isomerase (PDB entry 1XYA). However, one of the ligands (Glu 216) is shared with a second Mg-binding site containing one His and three carboxylates (see Figure 2). Since the electron density on Glu 216 is shared, the net negative charge involved in Mg binding in site I will be less than −4. Alternatively, one of the acidic residues constituting binding site I may possess an abnormally high pK_a value such that it is protonated at the crystallization pH of 7.4, thereby reducing the total negative charge contribution to Mg binding. It is noteworthy that in the 1XYA structure the Mg−O distance of E180 (2.74 Å) is significantly longer than that of the other acidic side chains in binding site I (2.21−2.43 Å).

In sites with low solvent accessibility, Mg prefers to coordinate first to negatively charged Asp/Glu than to neutral oxygen-containing ligands because the former has more favorable electrostatic interaction energy with the metal than the latter. However, up to three carboxylates may bind Mg: the attack of a fourth anionic ligand ($HCOO^-$) to a negatively charged complex $[Mg(H_2O)_3(HCOO)_3]^-$ is predicted to be thermodynamically unfavorable, if there is no relaxation/reorganization of the protein matrix.

B. When Mg Is already Bound to ≤ 3 Negatively Charged Carboxylate Groups, It May Coordinate to Neutral Carbonyl Group(s) but It Will Not Exchange All Its First-Shell Water Molecules for Protein Ligands

Although the availability of a relatively solvent-inaccessible cavity containing negatively charged carboxylates appears to be important for Mg binding to proteins, the neutral amino acids, in particular carbonyl groups, may also contribute to the Mg-binding free energy. To verify this, the ΔG^4_{ex} values for successively replacing a Mg-bound water molecule with formamide once it is bound to one, two, or three formates have been computed.

Figure 3 shows that the complex with one formate favors exchange of all but its last inner-shell water with formamide, as the last exchange free energy is positive. The complex with two formates is almost indifferent toward exchange of the first water (ΔG^4_{ex} = −1.0 kcal/mol) but opposes the second water → formamide substitution. The complex with three formates opposes exchange of any of its three water molecules for a formamide. Thus, the most favorable thermodynamical configurations for the Mg-carboxylate complexes contain, as a rule, water, i.e., complete inner-mode binding of Mg bound to six protein ligands is an unlikely event. These results correlate with the observation that all Mg-binding sites in the PDB contain not only one Asp or Glu, but also at least one water molecule.[46]

As the number of formamides and formates bound to Mg increases, the metal prefers water to formamide due to increased steric crowding among the nonaqua ligands and decreased charge-dipole inter-

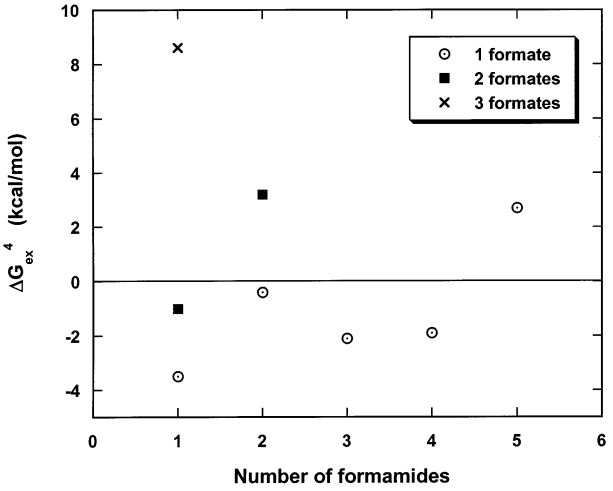

Figure 3. The free energy, ΔG^4_{ex}, for replacing a Mg-bound water molecule with formamide once Mg is bound to one (open circle with dot), two (■), or three (×) formates in a cavity characterized by $\epsilon = 4$ as a function of the number of formamides bound. The gas-phase properties are evaluated at the B3LYP/6-31+G* level.[46]

actions as the metal charge is neutralized by the formates and/or formamides.[46] The stability of water-containing Mg complexes may explain why nature has given priority to this form of bound Mg center whenever a metal cofactor is needed for an enzymatic hydrolytic reaction.[98,99]

IV. Coordination Mode and Number

A. The Carboxylate Coordination Mode (Mono- or Bidentate) in Zn Complexes Depends on Other Interactions within the Complex

Catalytic binding sites in Zn-enzymes often contain Asp or Glu residue(s).[15,30,68,76] The carboxylate side-chains generally bind to Zn in either monodentate or bidentate fashion with a Zn−O bond distance around 1.8−2.0 Å and 2.1−2.4 Å, respectively. In some proteins (e.g., bacillolysin and sonic hedgehog), they bind to the metal in an intermediate mode that is neither monodentate nor bidentate, as manifested by Zn−O bond distances around 2.0−2.1 Å and 2.5−2.8 Å.[30] To elucidate the factors determining the carboxylate coordination mode in Zn (and other metal) binding sites, DFT calculations have been performed on Zn bound to carboxylates (CH_3COO^-) and other ligands (see Table 2),[100] which model metal-binding sites in Zn-enzymes such as carboxypeptidase and thermolysin.

Table 2 summarizes the computed acetate coordination mode and relative energies for tetrahedral Zn complexes with H_2O (W), OH^-, imidazole (Im), and CH_3COO^- (Ace). For complexes with only inner-shell ligands, monodentate carboxylate binding is generally preferred except for $[Zn{\cdot}Im_3{\cdot}Ace]^+$ where mono- and bidentate binding appear to be isoenergetic, and an intermediate mode is the dominant species. Including second-shell water molecule(s) reverses the trend for the $[Zn{\cdot}Im_2{\cdot}Ace{\cdot}H_2O]$ complex due to favorable inter-ligand interactions, which favor the bidentate mode.

Table 2. Calculated Acetate Coordination Mode and Relative Energies for Tetrahedral Zn Complexes with H_2O (W), OH^-, Imidazole (Im), and CH_3COO^- (Ace)[a]

complex	coordination mode	relative energy (kcal/mol)
$[Zn{\cdot}Im_2{\cdot}Ace{\cdot}OH]^0$	monodentate	0.0
	bidentate	5.3[b]
$[Zn{\cdot}Im_2{\cdot}Ace_2]^0$	monodentate	0.0
	bidentate	3.1[b]
$[Zn{\cdot}Im_3{\cdot}Ace]^+$	monodentate	0.0[c]
	bidentate	0.0[b]
	intermediate	−1.0
$[Zn{\cdot}Im_2{\cdot}Ace{\cdot}W]^+$	monodentate	0.0
	bidentate	2.3
$\{[Zn{\cdot}Im_2{\cdot}Ace{\cdot}W]{\cdot}W\}^+$	monodentate	0.0
	bidentate	−3.8
$\{[Zn{\cdot}Im_2{\cdot}Ace{\cdot}W]{\cdot}W_2\}^+$	monodentate	0.0
	bidentate	−2.3

[a] From Ryde, 1999.[100] [b] The Zn−O−C angle was constrained to 90°. [c] The Zn−O−C angle was constrained to 120°.

These results imply that the carboxylate coordination mode to Zn is governed *not* by the carboxylate group itself, but by other interactions within the complex. They also highlight the crucial role of hydrogen bonds (between both inner−inner and inner−outer shell ligands) in stabilizing the metal-binding site structure.[100] Since the energy barrier for switching between mono- and bidentate coordination is calculated to be only a few kcal/mol,[100] the protein may adopt a functional binding site configuration at a relatively low energy cost.

B. In Buried Zn Sites the Outer-Shell Asp/Glu Carboxylate May Act as a Proton Acceptor for the Inner-Shell His

PDB surveys of Zn-binding sites have shown that very often (in about 80% of the cases) a Zn-bound His is coordinated to a Asp or Glu carboxylate in the metal's second coordination sphere.[17,30,54,76,101−105] Experimental studies on carbonic anhydrase II and designed metal-binding sites indicate that the second-shell Asp/Glu ligands may be involved in (1) properly orienting the first-shell histidine for efficient metal binding, and (2) enhancing the affinity of the binding site for zinc.[101−108] To elucidate the role of this interaction, ab initio calculations have been performed on model systems containing imidazole (ImH), deprotonated imidazolate (Im^-), acetate (Ace^-), and protonated acetic acid (AceH).[47,54] Single-point electron energies of $ImH{\cdots}Ace^-$ and $Im^-{\cdots}AceH$ dyads with the distance between reaction centers fixed at either 5 or 8 Å have been calculated, and compared with those for the respective $Zn{\cdots}ImH{\cdots}Ace^-$ and $Zn{\cdots}Im^-{\cdots}AceH$ triads (Figure 4). Although these energies vary with the method used and the structure of the model system, the trends of changes are the same.

In the absence of Zn the dyad $ImH{\cdots}Ace^-$ is energetically more favorable than $Im^-{\cdots}AceH$, but upon Zn binding the trend reverses and $Zn{\cdots}Im^-{\cdots}AceH$ is more stable than $Zn{\cdots}ImH{\cdots}Ace^-$. These findings in conjunction with earlier results reported by Krauss and Garmer[17] imply that Asp/Glu may act as proton acceptors in solvent-inaccessible binding

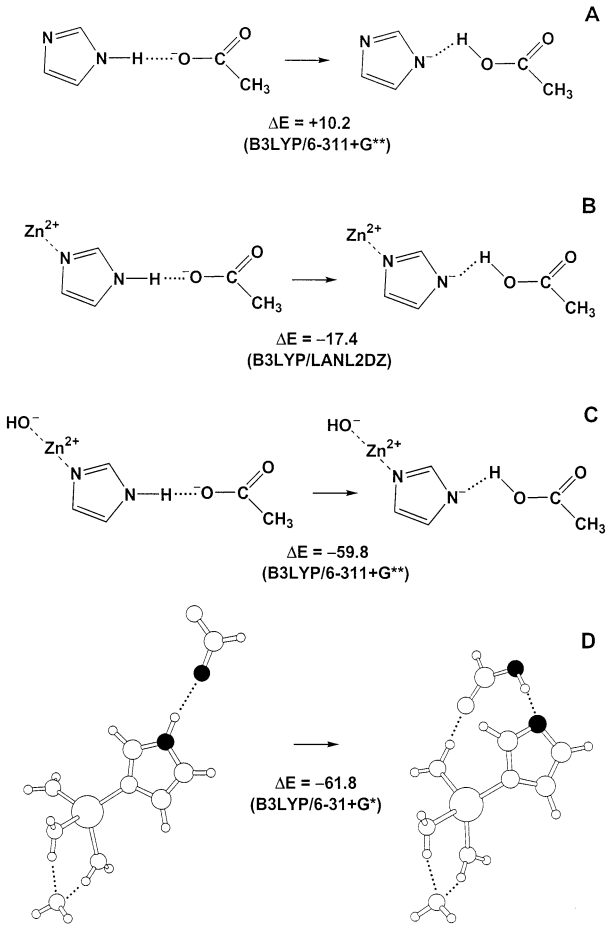

Figure 4. Schematic diagrams of (A) ImH···Ace$^-$ and Im$^-$···AceH, (B) Zn···ImH···Ace$^-$ and Zn···Im$^-$···AceH, (C) Zn(OH)$^+$···ImH···Ace$^-$ and Zn(OH)$^+$···Im$^-$···AceH. (D) Initial structure of $\{[Zn\cdot ImH\cdot(H_2O)_3]\cdot HCOO^-\cdot H_2O\}^+$ and the final fully optimized structure $\{[Zn\cdot Im^-\cdot(H_2O)_3]\cdot HCOOH\cdot H_2O\}^+$. Isomerization energies (in kcal/mol) computed at a given theory/basis level (in brackets) are listed below the arrows. The distance between reaction centers in (A), (B), and (C) has been fixed at 5 Å.

sites. Indeed, when Zn(OH)$^+$···ImH···Ace$^-$ was subjected to full geometry optimization, the proton on the imidazole nitrogen migrated to acetate and formed Zn(OH)$^+$···Im$^-$···AceH (Figure 4C).[54] The same effect of proton transfer has been observed in a tetrahedral Zn complex, $\{[Zn\cdot ImH\cdot(H_2O)_3]\cdot HCOO^-\cdot H_2O\}^+$, containing an inner-sphere imidazole hydrogen-bonded to an outer-sphere formate so that the final fully optimized structure was a Zn-bound imidazolate anion hydrogen bonded to formic acid (Figure 4D).[59]

It is not surprising that in the absence of a metal dication the dyad ImH···Ace$^-$ is energetically preferred over Im$^-$···AceH since the proton dissociation energy of ImH is slightly more positive than that of AceH (349 vs 345 kcal/mol; B3LYP/6-311+G(d,p) calculations).[47] However, upon Zn binding the gas-phase proton dissociation energy of ImH drops significantly (to 117 kcal/mol[47]) due to stabilization of the negatively charged imidazolate anion by the metal dication through charge−charge interactions and charge-transfer from the imidazolate to the metal. Furthermore, in a solvent-inaccessible cavity charge−charge-dipole electrostatic interactions in the triad, Zn···Im$^-$···AceH, are expected to be much more favorable than the charge-dipole-charge interactions in the alternative triad, Zn···ImH···Ace$^-$. The calculations thus reveal the important role of the second coordination layer in metal binding to proteins. In this case, the second coordination shell Asp/Glu may act as a *proton* acceptor for the Zn-bound-His rather than as a hydrogen bonding partner if the metal-binding site is solvent-inaccessible, and the deprotonated/protonated Asp/Glu is not strongly stabilized/destabilized by other protein interactions, respectively.

C. Tetrahedral Zn Complexes in Protein Cavities Are Generally Relatively More Stable Than Other Zinc Polyhedra

Zn is flexible with respect to the number of ligands it can adopt in its first shell. Although Zn is coordinated to six water molecules in aqueous solution,[70] it is usually *tetrahedrally* coordinated in Zn-finger proteins and most enzymes, but in some catalytic sites, it is found pentacoordinated and, rarely, hexacoordinated. A survey of Zn proteins in the PDB shows that for *structural* binding sites the ratio between tetra/penta/hexa-coordinated Zn is 79:6:12%, respectively, whereas for *catalytic* sites the ratio is 48:44:6%, respectively.[88] A survey of Zn complexes in the Cambridge Structure Database also shows that tetracoordinated Zn is the most preferred coordination mode (50% of the cases), followed by penta- (26%) and hexa-coordinated modes (24%).[88] Experimental studies on the complexation and coordination modes of single amino acids and oligopeptides to Zn[109−115] have also found that a tetrahedral ligand arrangement around Zn, especially with histidines and/or cysteines, is the most common among different Zn polyhedra. In sharp contrast to Zn, magnesium, which is also divalent with an ionic radius (0.72 Å) similar to that of Zn (0.75 Å), is usually octahedrally coordinated both in aqueous solution and in proteins.[43]

It is not clear if the observed decrease in the CN of Zn upon protein binding reflects (i) the constraints of the protein matrix on Zn,[116] or (ii) the specific physicochemical requirements of the metal and/or ligands. In other words, what is the lowest-energy, ground-state coordination geometry for Zn complexes in proteins? To address this question, two types of Zn complexes have been examined: octahedral $[Zn\cdot W_n\cdot L_{6-n}]^{2+}$ (n = 4, 5, 6) complexes with six ligands in the first coordination shell, and tetrahedral $\{[Zn\cdot W_n\cdot L_{4-n}]\cdot W_2\}^{2+}$ (n = 2, 3, 4) complexes with four ligands in the first shell, and two water molecules in the second shell ("4+2" structure). The free energies of isomerization between the two types of clusters have been computed for various dielectric media, and compared with the corresponding values between octahedral and tetrahedral Mg complexes in some cases (see Table 3).[53]

In a *protein* environment, Zn can adopt either octahedral or tetrahedral geometry depending on (i) the type of protein ligand it is bound to and (ii) the solvent accessibility of the metal-binding site.[53] For Zn complexes containing one *neutral* imidazole (representing the His side-chain), tetrahedral geometry

Table 3. Calculated Enthalpies (ΔH^1_{isom}) and Free Energies of Isomerization (ΔG^X_{isom}) between Octahedral and Tetrahedral Complexes of Zn and Mg for Media of Different Dielectric Constant X (in kcal/mol)[a]

reaction	ΔH^1_{isom} [b]	ΔG^1_{isom}	ΔG^2_{isom}	ΔG^4_{isom}	ΔG^{80}_{isom}
$[Zn{\cdot}W_6]^{2+} \leftrightarrow \{[Zn{\cdot}W_4]{\cdot}W_2\}^{2+}$	−2.3	−3.7	1.0	3.4	6.8
$[Zn{\cdot}W_5{\cdot}Im]^{2+} \leftrightarrow \{[Zn{\cdot}W_3{\cdot}Im]{\cdot}W_2\}^{2+}$	−8.2	−8.4	−3.1	−0.4	2.7
$[Zn{\cdot}W_4{\cdot}Im_2]^{2+} \leftrightarrow \{[Zn{\cdot}W_2{\cdot}Im_2]{\cdot}W_2\}^{2+}$	−12.9	−13.9	−8.1	−5.3	−3.7
$[Zn{\cdot}W_5{\cdot}HCOO]^{+} \leftrightarrow \{[Zn{\cdot}W_3{\cdot}HCOO]{\cdot}W_2\}^{+}$	−5.4	−9.6	−8.1	−7.3	−6.3
$[Mg{\cdot}W_6]^{2+} \leftrightarrow \{[Mg{\cdot}W_4]{\cdot}W_2\}^{2+}$	5.3	4.3	9.6	12.3	15.5
$[Mg{\cdot}W_5{\cdot}Im]^{2+} \leftrightarrow \{[Mg{\cdot}W_3{\cdot}Im]{\cdot}W_2\}^{2+}$	1.2	1.7	6.8	9.4	12.4
$[Mg{\cdot}W_5{\cdot}HCOO]^{+} \leftrightarrow \{[Mg{\cdot}W_3{\cdot}HCOO]{\cdot}W_2\}^{+}$	7.2	3.1	4.9	5.9	7.0

[a] From Dudev & Lim, 2000.[53] [b] Evaluated by B3LYP/6-31++G(2d,2p) calculations using fully optimized B3LYP/6-31++G(2d,2p) molecular geometries.[53]

is preferred if the binding site is buried ($\epsilon < 4$), but both tetrahedral and octahedral structures may exist if the site is characterized by $\epsilon > 4$ (see Table 3). For Zn complexes containing two imidazoles or one *negatively charged* formate, tetrahedral coordination is favored over octahedral coordination in both buried and solvent-exposed binding sites (negative ΔG_{isom} for reactions 3 and 4 in Table 3). *Tetracoordinated* Zn structures have also been found to be more stable than the respective pentacoordinated species by 24−48 kcal/mol in the alcohol dehydrogenase active site.[32,117] Furthermore, the 4-coordinate Zn structures have shorter metal−ligand distances than the respective 5- or 6-coordinate all-inner-sphere structures. In contrast to Zn, Mg prefers to be octahedrally coordinated to an imidazole or a formate in both buried and solvent-exposed binding sites (positive ΔG_{isom} for the last two reactions in Table 3).

Two factors favor Zn to be tetracoordinated. The first is a solvent-inaccessible, low dielectric medium, as evidenced by the greater stability of the "4+2"-hydrated Zn complex relative to the octahedral complex in the gas phase (by −3.7 kcal/mol, Table 3). In a high dielectric medium such as a solvent-exposed site or aqueous solution, solvation effects favor the octahedral structure. The second is the charge transfer from the ligand(s) to the metal cation. The amino acid side-chains, being more polarizable than water, transfer more charge to Zn than water, resulting in a greater neutralization of the positive charge on Zn, which reduces charge−charge and/or charge-dipole interactions.[53] The low positive charge on *penta*- and *hexa*-coordinated Zn and the steric bulk of the protein ligand probably disfavors binding of water molecule(s) in the inner sphere compared to that in the outer sphere. On the other hand, Mg prefers to be octahedrally coordinated in the gas-phase, in proteins, and in aqueous solution. This is because each of the water or nonaqua ligands transfers more charge to vacant (hybridized 3d4s) Zn orbitals, which are closer in energy to the highest occupied orbital, than to vacant (3s) Mg orbitals, which are much higher in energy than the highest occupied orbital. Thus, for a given set of protein ligands, the positive charge on Mg is not as neutralized as that on Zn, thus Mg can remain octahedrally coordinated in proteins as in aqueous solution.

The finding that in protein cavities *tetracoordinated* Zn is relatively more stable than penta- or hexa-coordinated Zn has two implications. First, tetrahedral Zn-binding sites do not significantly contribute "coordination strain" to the catalytic activity of Zn enzymes. Second, tetrahedral Zn-binding sites with shorter metal−ligand bond lengths are well suited for stabilizing a given protein fold or configuration. This is in accord with the fact that all structural mononuclear Zn-binding sites found to date are tetrahedrally coordinated.[18,76,88]

V. Metal Selectivity in Metalloproteins

A. Mg-Binding Sites Are Not Specific for Mg: Other Divalent Metals, Especially Zn, May Dislodge Mg from Its Binding Site. Mg Cannot Displace Zn from Rigid Tetrahedral Zn-Binding Sites

A particularly intriguing question in metalloprotein chemistry is how a protein selects a specific metal cation (Mg or Ca or Zn) from the mixture of ions in the surrounding fluids. Is this selectivity due to (i) the natural abundance of the metal in the biological locality, or (ii) properties of the metal (e.g., its stereochemical and charge to size requirements), or (iii) properties of the protein (e.g., its unique set of amino acid residues forming the metal-binding pocket and the stereochemistry of this pocket)? Another interesting question is why some proteins (such as those with the EF-hand motif) bind only a specific metal, while others (such as CheY) bind several ions with similar affinity.[16,118] It is also not clear if metal-binding sites in proteins are generally rigid or flexible, and the extent to which the protein can adjust to the stereochemical requirements of the incoming metal ion, or, conversely, the metal ion can comply with the constraints of the protein matrix. To elucidate the factors governing metal cation selectivity by proteins DFT and CDM have been employed to evaluate the free energy of metal exchange in model binding sites.[59] The DFT/CDM study focused on Mg $\leftrightarrow$ Zn exchange, and considered metal exchange in *rigid* sites that constrain the incoming metal to adopt the coordination geometry of the outgoing metal, as well as in *flexible* sites that can accommodate some reorganization of the protein ligands upon metal substitution.[59]

For rigid or flexible binding sites in which Mg is typically bound to one or two Asp/Glu side-chains (see section IIIA), the free energies of exchanging Mg for Zn are negative over the entire range of ϵ, from 1 to 80 (Table 4, first two and last two reactions). This implies that Zn may dislodge Mg octahedrally bound

Table 4. Enthalpies (ΔH^1_{ex}) and Free Energies (ΔG^X_{ex}) of Mg → Zn Exchange in Mg-Binding Sites for Media of Different Dielectric Constant X (in kcal/mol)[a]

reaction[b]	ΔH^1_{ex}	ΔG^1_{ex}	ΔG^2_{ex}	ΔG^4_{ex}	ΔG^{80}_{ex}
Rigid Binding Sites					
$[Mg{\cdot}HCOO{\cdot}W_5]^+ + [Zn{\cdot}W_6]^{2+} \rightarrow [Zn{\cdot}HCOO{\cdot}W_5]^+ + [Mg{\cdot}W_6]^{2+}$	−3.3[c]	−2.3[c]	−2.3	−2.5	−2.7
$[Mg{\cdot}(HCOO)_2{\cdot}W_4]^0 + [Zn{\cdot}W_6]^{2+} \rightarrow [Zn{\cdot}(HCOO)_2{\cdot}W_4]^0 + [Mg{\cdot}W_6]^{2+}$	−5.4[d]	−5.8[d]	−5.7	−5.5	−5.0
$[Mg{\cdot}(HCOO)_2{\cdot}Fm{\cdot}W_3]^0 + [Zn{\cdot}W_6]^{2+} \rightarrow [Zn{\cdot}(HCOO)_2{\cdot}Fm{\cdot}W_3]^0 + [Mg{\cdot}W_6]^{2+}$	−4.3[d]	−3.7[d]	−2.2	−0.8	1.5
Flexible Binding Sites					
$[Mg{\cdot}HCOO{\cdot}W_5]^+ + [Zn{\cdot}W_6]^{2+} \rightarrow \{[Zn{\cdot}HCOO{\cdot}W_3]\cdot W_2\}^+ + [Mg{\cdot}W_6]^{2+}$	−8.6[c]	−11.9[c]	−10.4	−9.8	−9.0
$[Mg{\cdot}(HCOO)_2{\cdot}W_4]^0 + [Zn{\cdot}W_6]^{2+} \rightarrow \{[Zn{\cdot}HCOO)_2{\cdot}W_2]\cdot W_2\}^0 + [Mg{\cdot}W_6]^{2+}$	−11.0[d]	−10.1[d]	−8.2	−6.3	−2.1

[a] From Dudev & Lim, 2001.[59] [b] W = H_2O and Fm = $HCONH_2$. [c] Using the 6-31++G(2d,2p) basis. [d] Using the 6-31+G* basis.

Table 5. Enthalpies (ΔH^1_{ex}) and Free Energies (ΔG^X_{ex}) of Zn → Mg Exchange in Zn-Binding Sites for Media of Different Dielectric Constant X (in kcal/mol)[a]

reaction[b]	ΔH^1_{ex}	ΔG^1_{ex}	ΔG^2_{ex}	ΔG^4_{ex}	ΔG^{80}_{ex}
Rigid Binding Sites					
$\{[Zn{\cdot}Im{\cdot}W_3]{\cdot}W_2\}^{2+} + [Mg{\cdot}W_6]^{2+} \rightarrow \{[Mg{\cdot}Im{\cdot}W_3]{\cdot}W_2\}^{2+} + [Zn{\cdot}W_6]^{2+}$	15.8[c]	15.6[c]	14.8	14.3	12.9
$[Zn{\cdot}(Im)_3{\cdot}W]^{2+} + [Mg{\cdot}W_6]^{2+} \rightarrow [Mg{\cdot}(Im)_3{\cdot}W]^{2+} + [Zn{\cdot}W_6]^{2+}$	29.0[c]	25.7[c]	25.1	24.5	23.1
$[Zn{\cdot}(Im)_2{\cdot}(CH_3S)_2]^0 + [Mg{\cdot}W_6]^{2+} \rightarrow [Mg{\cdot}(Im)_2{\cdot}(CH_3S)_2]^0 + [Zn{\cdot}W_6]^{2+}$	42.0[d]	39.9[d]	39.1	38.3	36.5
Flexible Binding Sites					
$\{[Zn{\cdot}Im{\cdot}W_3]{\cdot}W_2\}^{2+} + [Mg{\cdot}W_6]^{2+} \rightarrow [Mg{\cdot}Im{\cdot}W_5]^{2+} + [Zn{\cdot}W_6]^{2+}$	14.6[c]	13.9[c]	8.0	4.9	0.5
$\{[Zn{\cdot}Im{\cdot}W_3]W{\cdot}Fm\}^{2+} + [Mg{\cdot}W_6]^{2+} \rightarrow [Mg{\cdot}Im{\cdot}Fm{\cdot}W_4]^{2+} + [Zn{\cdot}W_6]^{2+}$	5.2[d]	6.4[d]	5.0	4.5	3.7
$\{[Zn{\cdot}Im{\cdot}W_3]{\cdot}W.HCOO\}^+ + [Mg{\cdot}W_6]^{2+} \rightarrow [Mg{\cdot}Im{\cdot}HCOO{\cdot}W_4]^+ + [Zn{\cdot}W_6]^{2+}$	−19.2[d]	−19.3[d]	−14.9	−12.4	−9.9

[a] From Dudev & Lim, 2001.[59] [b] Im = imidazole, W = H_2O and Fm = $HCONH_2$. [c] Using the 6-31++G(2d,2p) basis. [d] Using the 6-31+G* basis.

to one or two acidic residues from Mg-binding sites. On the other hand, for typical *rigid* Zn-binding sites modeled by the first three reactions in Table 5, Mg cannot displace Zn regardless of the solvent accessibility of the site (positive ΔG_{ex}). Even if the Zn-binding site was *flexible* Mg cannot, in general, displace Zn unless one or more Asp/Glu were present in the metal *second* coordination shell so that it could be added to the first shell of Mg in an octahedral geometry (negative ΔG_{ex} for last reaction in Table 5).

Zn complexes are more stable than corresponding Mg complexes because each of the ligands transfers more charge to Zn than to Mg[21,59] (see also section IVC). Thus, it is not surprising that Zn can successfully compete with Mg in rigid buried sites where the number of ligands coordinated to the metal does not change during the exchange reaction (Tables 4 and 5). It can also prevail over Mg in flexible Zn-binding sites with *neutral* ligands in the second coordination sphere. The trend is reversed, however, for flexible Zn-binding sites containing *negatively charged* ligand-(s) in the second coordination shell (Table 5, last reaction) because the free energy gain upon Mg binding to a *negatively charged* ligand is significantly higher (by more than 200 kcal/mol) than that for Mg binding to a neutral ligand (see also section IIIA).[59]

The finding that Zn(II) can dislodge Mg(II) octahedrally bound to one or two acidic residues from Mg-binding sites (Table 4) agrees with experimental observations. In the bacterial chemotaxis protein CheY, Mg is bound to two Asp side chains, one backbone carbonyl, and three water molecules (PDB entry 1CHN). This binding site is modeled by the third reaction in Table 4, which shows that Zn can replace Mg in a *buried* binding pocket (negative ΔG_{ex} for $\epsilon \leq 4$), in accord with the finding that Zn binds to CheY ($K_a = 1.0 \times 10^4$ M^{-1}) more tightly than its natural cofactor, Mg ($K_a = 2.0 \times 10^3$ M^{-1}).[119] Zn inhibits alkaline phosphatase catalytic activity by displacing its natural cofactor, Mg, which is octahedrally coordinated to the side chains of an Asp, a Glu and a Thr as well as three water molecules.[120−122] Zn also inhibits tyrosine Csk kinase catalytic activity by binding to the second Mg-binding site with a 13 200-fold higher affinity than Mg.[87] Furthermore, Zn inactivates some other enzymes, such as avian sarcoma virus integrase, beta-galactosidase, and casein kinase-II, which are active when bound to Mg.[123−125] Apart from Zn, other naturally occurring transition metal dications, such as Cu(II), Co(II), Ni(II), and Fe(II), also bind more tightly than Mg, often inactivating the respective enzyme.[87,122,123,125−133]

The finding that Mg(II) generally cannot displace Zn(II) bound tetrahedrally to one or more histidines from Zn-binding sites (Table 5) is supported by experimental observations. Zn, the physiological activator of L-ribulose-5-phosphate 4-epimerase, binds to three histidines and, presumably, a water molecule in the protein ($K_a = 5.9 \times 10^6$ M^{-1}) more tightly than Mg ($K_a = 7.4 \times 10^2$ M^{-1}).[134] The same type of Zn-binding site has been proposed for the hamster dihydroorotase domain, which binds Zn but not Mg under physiological conditions.[135]

The theoretical results together with available experimental data imply that Mg-binding sites are not very specific for Mg, whereas *rigid* Zn-binding sites generally prefer Zn to Mg. These findings raise some interesting questions: How do proteins, whose natural cofactor is Mg, select this cation from the surrounding fluids? How do these proteins prevent other cations, particularly Zn, from replacing Mg? Mg is the most abundant divalent cation in eukaryotic cells with concentrations of free Mg(II) ranging from 0.1 to 1 mM.[136] Zn is the second most abundant transition metal in living organisms after iron, but the intracellular concentration of free Zn(II) is kept

very low (estimated to be in the femtomolar range[137]) as it is postulated to be regulated by metallothionein[138] (a Cys-rich protein that traps up to seven Zn in its two binding domains).

In view of the aforementioned findings, it seems likely that during evolution some proteins have chosen Mg as a natural cofactor based mainly on its natural abundance in living cells. Mg-binding sites appear to be weakly protected against other divalent metals such as Zn, which can replace Mg and, in some cases, inhibit enzymatic activity. Therefore, it seems that it is not the *protein* that has evolved to select Mg from other cations. Instead, it is the cell machinery that regulates the process of metal binding by regulating appropriate concentrations of Mg and other cations (Zn in particular) in various biological compartments. Relative to Mg, Zn has higher affinity for a given protein ligand[59] and strongly prefers a tetrahedral geometry. Consequently, rigid Zn-binding sites, especially those lined by Cys residues, are more selective than Mg-binding sites. Thus, a protein can generally select Zn against the background of a much higher Mg concentration. In this case, the properties of the protein (the type of metal ligands, overall charge and shape of the cavity) as well as the properties of Zn (its greater charge-acceptor ability relative to Mg) govern the binding-site specificity.

B. Cys-Rich Zn-Binding Sites in Proteins Are Weakly Protected against Heavy Metals Such as Cd, Hg, and Pb

As discussed in the previous section, Zn-binding sites have higher specificity than corresponding Mg-binding sites, allowing the protein to sequester Zn from the cell fluids where other naturally occurring metal cofactors, such as Mg and Ca, are present. However, can these sites withstand attacks by "alien" heavy metal cations such as Cd(II), Hg(II), and Pb(II), which, like Zn, prefer "soft" Cys ligands? This question has been experimentally addressed by Krizek et al.,[139] and, more recently, by Hartwig et al.,[140−144] Petering et al.,[145,146] Razmiafshari et al.,[147] and Payne et al.[148] In Krizek's and Payne's studies, the experiments have been performed on three small peptides (26 amino acids each) based on the consensus sequence of 131 Zn-finger domains.[149] The "consensus" peptides (designated as CP-CCHH, CP-CCHC and CP-CCCC) model the three general classes of Zn-fingers and contain Cys_2His_2, Cys_3His, and Cys_4 Zn-binding motifs, respectively (see Introduction). Absolute association constants (K_a) for peptide complexes with different metal cations (Zn, Co, Cd, and Pb) have been measured in vitro by spectroscopic titration techniques, as well as K_a for other Zn-finger proteins; these are summarized in Table 6.

Despite differences in the absolute K_a numbers reported for different proteins/peptides, some general trends of changes in K_a emerge. The results in Table 6 show that all binding sites have stronger affinity for Zn than for the other natural metal cofactor, Co. Furthermore, the CP-CCHH, CP-CCHC, and TFIIIA-CCHH Zn-fingers bind Zn more tightly than heavier metals such as Cd(II) and Pb(II) and appear to be well protected against these metals. In contrast, the CP-CCCC, and ER-CCCC Zn-fingers bind Cd and Pb more tightly than Zn. Note that the K_a(Cd) in the CP-series increases (by one to two orders) with each additional cysteinate (Cys^-) in the binding site.

Table 6. Experimental Binding Constants for Complexes between Zn-finger Domains and Divalent Metal Cations (in M^{-1})

peptide/ protein	K_a(Co)	K_a(Zn)	K_a(Cd)	K_a(Pb)
CP-CCHH	$1.6 \times 10^{7,a}$	$1.8 \times 10^{11,a}$	$5.0 \times 10^{8,a}$	$2.0 \times 10^{10,b}$
CP-CCHC	$1.6 \times 10^{7,a}$	$3.1 \times 10^{11,a}$	$1.6 \times 10^{11,a}$	$1.2 \times 10^{10,b}$
CP-CCCC	$2.8 \times 10^{6,a}$	$9.1 \times 10^{11,a}$	$2.5 \times 10^{13,a}$	$2.5 \times 10^{13,b}$
TFIIIA-CCHH[c,f]		1.0×10^8	3.6×10^5	
RMLV-CCHC[d]	5.0×10^7	1.0×10^{11}		
ERDBD-CCCC[e]	1.4×10^6	1.5×10^8	2.1×10^8	

[a] From Krizek et al., 1993.[139] [b] From Payne et al., 1999.[148] [c] Transcription factor TFIIIA from Hartwig, 2001.[142] [d] Rauscher murine leukemia virus from Hartwig, 2001.[142] [e] Estrogen receptor DNA-binding domain from Hartwig, 2001.[142]

The above findings can generally be rationalized in terms of the higher affinity of the heavier (and "softer") metals for cysteinate compared to Zn.[139,148] This is supported by ab initio calculations showing that Cd competes successfully with Zn for Cys^- in both octahedral and tetrahedral binding sites, although the energy gain is not very big.[57] On the other hand, Zn has higher affinity for His than Cd.[57] Apparently, the Zn/Cd selectivity of a given binding site is governed by the relative number of Cys:His residues: the more Cys there are in the metal-binding site, the more favorable the Cd binding. To the best of our knowledge, no quantitative data on Zn-finger binding to Hg(II) have been reported so far. However, experiments on the DNA repair protein, Fpg, containing a Cys_4-binding site, and a synthetic Cys_2His_2 peptide show that Hg can dislodge Zn from the respective binding sites.[144,147] These findings are in line with calculations predicting that the Hg dication binds Cys^- and His more favorably than Zn.[57]

There is growing experimental evidence that heavy metals, upon displacing Zn from the Zn-finger core, cannot maintain the proper conformation of the protein, thus disrupting the DNA-binding process.[140,142−144,147,148,150,151] Cd-substituted fingers, which are expected to keep the tetrahedral coordination geometry of the binding site,[40] cannot maintain the proper conformation of the protein probably because Cd has a larger ionic radius than Zn (r_{Cd}/r_{Zn} = 0.95:0.75 Å), resulting in longer Cd−N(His) and Cd−S(Cys) bond distances (2.3−2.5 and 2.6 Å, respectively[40]) compared to the "native" Zn−N(His) and Zn−S(Cys) bond distances (2.1 and 2.3 Å, respectively[40,88]). Hg- and Pb-bound proteins cannot maintain the proper conformation of the protein mainly because Hg and Pb prefer nontetrahedral coordination: Hg prefers linear complexes with Cys^-,[152−154] while Pb complexes are often characterized by a strongly distorted (hemidirected) coordination sphere, with the ligands occupying one-half of the sphere leaving the rest of it to the metal lone pair.[155]

Table 7. Experimental Divalent Metal Binding Constants for EF-Hand Proteins (in M^{-1})

molecule	metal site	K_a (Ca)	K_a (Mg)	ref
calmodulin	N-domain	3.5×10^6	2.7×10^3	Martin et al., 2000.[165]
	C-domain	2.0×10^7	5.8×10^2	
troponin C				
skeletal	N-domain	3.3×10^5	2.0×10^2	Potter & Johnson, 1982.[180]
	C-domain	2.0×10^7	5.0×10^3	
cardiac	N-domain	3.3×10^5	2.0×10^2	Potter & Johnson, 1982.[180]
	C-domain	1.4×10^7	1.7×10^3	
androcam	N-domain	1.2×10^4		Martin et al., 1999.[173]
	C-domain	2.9×10^7	$\sim 3 \times 10^3$	
S100P	N-domain	8.9×10^3 [a]	$\sim 10^2$	Gribenko & Makhatadze, 1998.[181]
	C-domain	2.5×10^7 [a]		
recombinant oncomodulin	CD-domain	1.2×10^6	6.6×10^2	Henzl et al., 1996.[182]
	EF-domain	2.2×10^7	3.8×10^3	
parvalbumin		2.7×10^9	9.5×10^4	Moeschler et al., 1980.[183]
VILIP		1.0×10^6	4.8×10^3	Cox et al., 1994.[167]

[a] Average values from five experimental data sets.

Formation of mixed Zn/heavy metal complexes in the Zn-finger cores could not be ruled out.[142]

These studies suggest a possible mechanism for the heavy metal poisoning in living cells.[142,143,150] Cys-rich Zn-finger cores are susceptible to attack by heavy metals. Hence, the Zn cation is ejected from the binding site by the incoming heavy metal, which cannot maintain the original fold, thus leading to Zn-finger inactivation. On the other hand, Cys-rich proteins are attractive targets for toxic metals, and have been utilized by living organisms to fight heavy metal intoxication. Cys-rich proteins such as metallothionein (see section VA) are used as traps to sequester nonbiogenic metals from the body fluids thus preventing the poisonous metals from damaging vital metal-binding sites.[145,146,156] Furthermore, there is increasing evidence that metallothionein plays a role in repairing damaged Zn-binding sites by abstracting the toxic metal such as Cd(II) from the respective binding site and delivering the essential natural cofactor (Zn) to the same binding site.[157]

C. The Metal Ligand Side Chain Interactions and Protonation States, and Carboxylate Binding Mode of the EF-Hand Binding Site Contribute to Its Specificity for Ca(II)

The EF-hand binding site is designed to bind specifically Ca cations. Ca binds to EF-hand proteins more favorably than its natural competitor, Mg (by a factor of 10^3-10^4, Table 7). Under physiological conditions, however, the competition between the two divalent cations for the EF-hand binding site depends also on their cytosolic concentration. In the *resting* cell the concentration of Ca ($10^{-7}-10^{-8}$ M)[158−160] is *10^4 times lower* than that of Mg ($10^{-3}-10^{-4}$ M, see section V.A), and does not promote Ca binding. Instead, Mg populates, at least partially, the EF-hand binding sites in the *resting* state of regulatory Ca-binding proteins.[161−165] However, Mg binding to EF-hand domains does not cause the large conformational changes characteristic of the Ca-activated proteins, and thus no signaling response is triggered.[161,162,165,166] Rather, Mg binding is thought to stabilize the structure of the resting EF-hand domains.[165] In the *activated* cell the concentration of Ca increases to $10^{-5}-10^{-6}$ M,[158−160] favoring Ca over Mg binding to the EF-hand domain. Ca binding to EF-hand domains causes significant conformational changes in the Ca-sensor protein, triggering a cascade of events along the signal transduction pathway.[160,167−174]

The roles of cavity size and binding-site total charge in controlling the Ca specificity in EF-hand motifs have been elucidated by mutational studies on a *Escherichia coli* galactose binding protein (GBP) that contains an EF-hand-like motif.[78,175−178] Although the GBP Ca-binding site with a helix−loop−β-strand structure differs from the classical EF-hand motif (helix−loop−helix structure), it appears to be a useful EF-hand model as the structure of its metal-binding pocket closely resembles that of the standard EF-hand sites.[177] In the EF-hand-like binding site, seven oxygen atoms from the side chains of Asp 134, Asp 138, Asn 136, Gln 142, bidentate Glu 205, and the mainchain oxygen of Gln 140 coordinate the Ca cation in a pentagonal bipyramidal geometry with Asp 134 and Gln 142 axial, while the other ligands occupy equatorial positions. Gln 142 was mutated to Asn, Asp, and Glu and the binding affinities of mono-, di-, and trivalent metal cations listed in Table 8 for the mutant proteins were measured.

Ionic charge selectivity: The metal binding constants (K_a) in Table 8 show that the wild-type protein effectively discriminates between Ca and the prevalent physiological competitors in the cellular fluids such as Na(I), K(I), and Mg(II), whose K_a values are more than 10^4-fold less than that of Ca. Trivalent metals, due mainly to stronger charge−charge interactions, have affinities comparable to that of Ca. Substituting Gln 142 by Glu (Q142E) increases the total negative charge of the binding site from −3 (see above) to −4 without affecting the cavity size significantly, as the side-chain volume of Glu is only 7% smaller than that of Gln.[179] This mutant effectively excludes all monovalent and divalent (including Ca) ions but still binds trivalent metals. Similarly, mutating Asn 136 to Asp yields a shift toward trivalent selectivity.[178] The finding that a binding site with a total charge of −4 (as in the Q142E and N136D mutants) has negligible affinity for all group IIa cations is consistent with the results in section IIIA, showing that "hard" divalent cations can bind up to

Table 8. Metal Ion Binding Constants of EF-Hand Binding Sites[a]

metal ion	effective ionic radius[b] (Å)	K_a (M^{-1}) WT	Q142E	Q142N
		Group Ia		
Li^+	0.84	$\leq 1.7 \times 10^{-1}$	$\leq 1.1 \times 10^{-2}$	$\leq 6.2 \times 10^{-1}$
Na^+	1.12	$\leq 3.7 \times 10^{-1}$	$\leq 3.8 \times 10^{-2}$	$\leq 7.7 \times 10^{-2}$
K^+	1.46	$\leq 2.6 \times 10^{-1}$	$\leq 2.3 \times 10^{-1}$	$\leq 5.5 \times 10^{-1}$
Rb^+	1.56	$\leq 3.0 \times 10^{-1}$	$\leq 1.1 \times 10^{-2}$	$\leq 6.2 \times 10^{-2}$
		Group IIa		
Mg^{2+}	0.81	1.8×10^{0}	$\leq 9.1 \times 10^{-1}$	$\leq 1.4 \times 10^{-1}$
Ca^{2+}	1.06	7.8×10^{4}	5.5×10^{0}	6.7×10^{4}
Sr^{2+}	1.21	1.6×10^{2}	$\leq 3.0 \times 10^{0}$	4.0×10^{2}
Ba^{2+}	1.38	$\leq 1.4 \times 10^{0}$	$\leq 1.3 \times 10^{0}$	$\leq 3.0 \times 10^{0}$
		Group IIIa		
Sc^{3+}	0.81	5.9×10^{3}	4.0×10^{3}	5.9×10^{3}
Y^{3+}	0.96	1.4×10^{4}	2.6×10^{3}	1.3×10^{4}
La^{3+}	1.10	5.3×10^{2}	9.1×10^{2}	2.5×10^{3}
		Lanthanides		
Ce^{3+}	1.07	1.0×10^{3}	2.0×10^{3}	7.1×10^{3}
Sm^{3+}	1.02	2.5×10^{3}	9.1×10^{3}	1.3×10^{4}
Lu^{3+}	0.92	8.3×10^{4}	2.0×10^{3}	1.1×10^{5}

[a] From Falke et al., 1991.[16] [b] Effective ionic radii corresponding to a 7-coordinated metal (see text) from Shannon, 1976.[184]

three negatively charged residues without compensating effects from the protein matrix, i.e., there is an optimal level of negative charge density for divalent cation binding.[178]

Ionic size selectivity: The metal binding constants in Table 8 also show that the wild-type protein effectively discriminates between multivalent cations of different size. For example, although the ionic radii of Ca and Ba differ by only 0.32 Å, the binding affinity of Ba is more than 10^4-fold less than that of Ca. Substituting Gln 142 by Asn (Q142N) decreases the mutant side-chain volume (by 31%) and length (by 1.9 Å) relative to the wild-type Gln.[179] Thus, the binding site cavity is thought to expand, and consequently it no longer binds Mg, although its affinity for Ca remains unchanged (Table 8). Replacing Asn 136 by neutral side chains differing in size, shape, and chemical properties each yielded similar changes in ionic size discrimination.[178] Falke and co-workers[178] attributed this finding to interactions between coordinating side chains constraining the metal-binding cavity to an optimal size that disfavors the binding of large cations. Replacing a coordinating side chain would disrupt the network of interactions constraining the cavity, which can thus bind ions of different size, as observed, thus weakening the size selectivity of the EF-hand binding site.

Ca vs Mg selectivity. In addition to interactions among the metal ligands optimizing the cavity size for Ca, the mode of Glu binding (mono/bidentate) appears to be another way of discriminating between Ca and Mg in regulatory Ca binding proteins. The bidentate Glu ligand (Glu 205 in GBP above), which is highly conserved and occupies the last position in the Ca-binding loop (see Introduction),[78,85,86] plays a crucial role in inducing the structural changes in Ca-sensors upon Ca binding: the bidentate Glu → Ala troponin C mutant remains "closed" after Ca binding and cannot trigger signal response.[168] It binds *bidentately* to Ca and other bulkier divalent cations such as Sr, Ba, and Cd, but *monodentately* to Mg.[166] Ca, Sr, Ba, and Cd, which are seven-coordinated in the EF-hand binding site, inflict large conformational changes in recoverin, whereas Mg, which is six-coordinate, cannot trigger signal response.[166]

The above results reveal that the carboxylate binding mode contributing to the pentagonal bipyramidal geometry of the EF-hand binding site, the metal ligand side chain interactions constraining the cavity size, and the metal ligand protonation states determining the cavity net charge play important roles in fine-tuning the Ca selectivity of EF-hand sites. Insufficient favorable electrostatic interactions between the EF-hand binding site (charge −3) and monovalent ions, such as Na(I) and K(I), abolish metal binding. On the other hand, the pentagonal bipyramidal geometry and relatively large size of the EF-hand binding site (due partly to electrostatic repulsion among the seven coordinating oxygens[178]) prevent the natural competitor Mg, which prefers octahedral geometry and has a smaller ionic radius than Ca (Table 8), from binding. Thus, as for Zn (see section V.A), it appears that the properties of the protein (the type of metal ligands, their coordination mode and side chain interactions, as well as the overall charge and size of cavity) can select Ca(II) against a background of much higher concentrations of Na(I), K(I), and Mg(II).

VI. Summary and Outlook

Metal binding sites are usually located inside cavities and crevices of the protein structure. These are usually solvent inaccessible, characterized by a low dielectric constant that enhances electrostatic metal-protein ligand interactions, thus favoring inner-sphere metal binding (section IIA). Metal binding on solvent-exposed surfaces, especially to negatively charged ligands, results in outer-sphere complexes.

Divalent metal cations have high affinity toward anionic protein ligands such as deprotonated Asp or Glu side chains due to the strong charge−charge interactions, and thus high free energy gain upon metal binding in a solvent-inaccessible, low dielectric

medium (section IIA and IIIA). There is, however, an upper bound on the number of carboxylates that can coordinate to the metal dication (section IIIA and VC). This number is predicted to be three for "hard" dications if the protein matrix does not undergo significant relaxation or reorganization. Other (neutral) protein ligands, such as the Asn, Gln, His, Ser, or Thr side chain and the backbone carbonyl oxygen, can also add to the stability of the metal complex, although the free energy gain is less than that upon binding a Asp/Glu side chain (section IIIA and IIIB). These ligands play an important role in shaping and fine-tuning the geometry of the binding site so that the protein can selectively sequester a particular metal cofactor from the cell fluids (section VC).

The metal coordination number in a given complex is dictated by three factors: (1) the dielectric medium or solvent accessibility, (2) properties of the metal (mainly by its ability to accept charge from its ligands), and (3) the chemical characteristics of the ligands (section IVC). In particular, the reason hydrated Zn may change its coordination number from six to four upon binding to amino acid residues in a protein cavity, whereas Mg does not, is related to the availability of vacant metal orbitals that can accept charge from the ligands as well as to the greater electron-donating ability of protein ligands compared to water. "Border-line" metal cations, e.g., Zn, in contrast to "hard" cations such as Mg, can adopt different coordination geometries at a relatively low free energy cost. In the case of Zn, *tetrahedral* complexes appear to be relatively more stable than other Zn polyhedra in proteins.

Interactions among ligands from the metal first- and second-coordination layers also play a role in the metal-binding process. Thus, the mode of carboxylate binding (mono- or bidentate) in Zn-binding sites appears to depend *not* on the properties of the carboxylate side chain itself, but on interactions with other ligands within the complex (section IVA). Furthermore, a second-shell carboxylate may act as a proton acceptor for the first-shell His in buried Zn sites if it is not significantly stabilized by interactions with other residues (section IVB). A second-shell carboxylate has also been considered to be responsible for ionizing a first-shell water in various catalytic metal-binding sites.[15,30,44]

Binding site selectivity appears to be anticorrelated with the natural abundance of the metal in living cells. Thus, Mg-binding sites are not very specific for Mg, which is the most abundant metal dication in the body fluids. These binding sites are weakly protected against other natural metal cofactors, in particular, Zn (section VA). On the other hand, binding sites that had evolved to utilize a given metal present in minute concentrations in the body are more specific, thus enabling the protein to selectively bind its natural cofactor against a background of usually higher concentrations of other metals (section VA and VC). In this case, the type of metal ligands, their coordination mode and side chain interactions, as well as the overall charge and shape of the cavity (section VC) govern the binding-site specificity. Some metal binding sites, however, are vulnerable to attacks by nonbiogenic "alien" cations such as Cd, Hg, and Pb. Replacing the natural cofactor, e.g., Zn, by a heavier metal may constitute one of the possible pathways for heavy-metal intoxication in living organisms (section VB).

However, many questions regarding the process of metal−protein recognition still need to be addressed. The mechanism of metal-Cys binding, which is concomitant with metal-assisted deprotonation of Cys, is not well understood. Furthermore, the detailed mechanism of metal-induced protein folding, typical of Zn-finger proteins, is not known. Limited information is available to date about metal binding in bi- and polynuclear metalloproteins, and the extent the bound cations influence each other's properties. Studies on the protein-binding characteristics of important transition metal cations, such as Cu(I), Cu(II), Ni(II), Mn(II), Co(II), Cd(II), and Hg(II), as well as nonbiogenic metals complexes with biological ligands, are scarce. The role of the outer coordination shell in metal binding and selectivity has to be further elucidated.

In conclusion, some basic principles and the physical bases governing metal binding and selectivity in proteins have been derived. It is the hope that the guidelines outlined above will be useful in further elucidating the mechanism(s) of protein−metal recognition as well as in designing metalloproteins with new properties.

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