Effect of Carboxylate-Binding Mode on Metal Binding/ Selectivity and Function in Proteins

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ABSTRACT
We delineate the factors governing the carboxylate-binding mode (monodentate vs bidentate) in metalloproteins. We reveal how the carboxylate-binding mode affects the binding affinity and selectivity of a metal ion as well as the function of a metalloprotein using Ca^{2+}-binding proteins and enzymes (ribonuclease H1, phosphoserine phosphatase, and ribonucleotide reductase) as examples. The collected data indicate that a carboxylate monodentate ⇆ bidentate switch, in addition to other structural factors, could be used to fine tune the metal-binding site affinity and/or selectivity, thus modifying the function/properties of the metalloprotein.

1. Introduction

Almost one-half of all known proteins contain metal cofactor(s),[1] which perform a variety of tasks ranging from protein structure stabilization to enzyme catalysis, activating many essential life processes such as respiration and photosynthesis.[2−5] Among the metal ions, Na, K, Mg, Ca, Zn, Cu, Fe, Co, and Mn are most frequently found to bind to proteins under physiological conditions.[2,3,5] Because of the critical roles of these metal cofactors in protein function, many studies have been carried out to understand the factors governing metal binding and selectivity in metalloproteins. We recently summarized the fundamental principles governing Mg^{2+}, Ca^{2+}, and Zn^{2+} binding and selectivity in metalloproteins,[6,7] while others reviewed the role of Li^+, Cu^{2+}, and Fe^{2+} in binding, selectivity and metal-induced folding in proteins.[8−10] This Account differs from previous reviews in summarizing recent experimental and theoretical evidence on how the carboxylate-binding mode (monodentate vs bidentate) of aspartate (Asp^-) or glutamate (Glu^-) amino acid residues plays an important role in the binding affinity and/or selectivity of a metal cofactor and thus function of a metalloprotein.

Aspartate and glutamate side chains are unique among the 20 amino acids in possessing a carboxylate group that can bind the metal cation via one of the carboxylate oxygen atoms (i.e., monodentate meaning 'one-toothed', Figure 1a) or both oxygen atoms (i.e., bidentate, Figure 1b), forming a four-membered ring. The carboxylate side chain can also bind to two metal ions by bridging them via an oxygen to each metal ion, forming a 'chelate ring'.

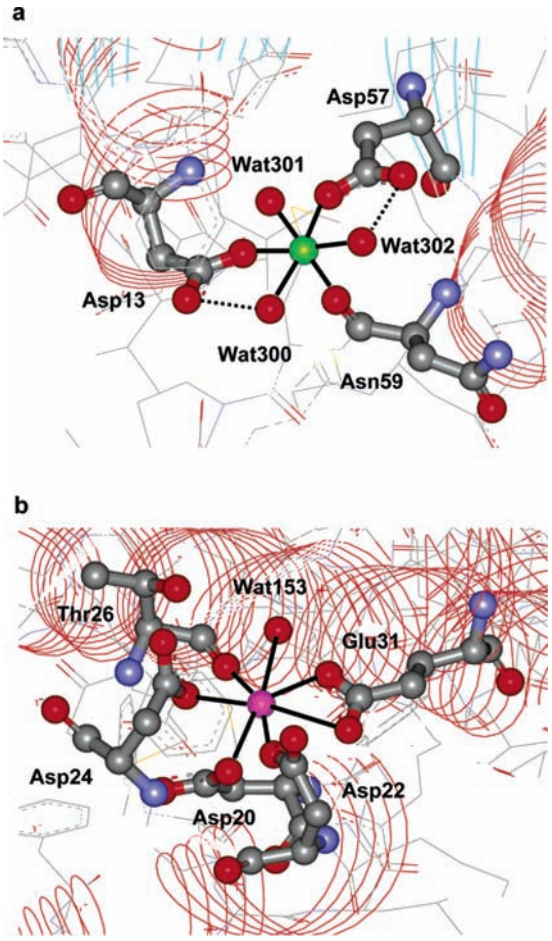

FIGURE 1. Monodentate vs bidentate carboxylate binding to a metal cation. (a) Monodentate binding of Asp13 and Asp57 to hexacoordinated Mg^{2+} in bacterial chemotaxis protein CheY (1CHN, 1.76 Å), where water molecules in the first shell stabilize the metal-free carboxylate oxygen atoms of Asp13 and Asp57. (b) Bidentate binding of Glu31 to heptacoordinated Ca^{2+} in site I of calmodulin (1OSA, 1.68 Å).

Todor Dudev was born in Troyan, Bulgaria, in 1959. He obtained his M.Sc. (1984) and Ph.D. (1989) degrees from the University of Sofia, Bulgaria. He subsequently specialized at the Tokyo Institute of Technology, Japan (1990), and the Instituto de Estructura de la Materia, CSIC, Spain (1993). He was a visiting scholar at the University of Salford, U.K., Technical University of Dresden, Germany, and University of Missouri—Kansas City. In 1989 he was appointed Assistant Professor at the Department of Chemistry, University of Sofia, and in 1997 was promoted to Associate Professor. In 1997 he joined the Institute of Biomedical Sciences, Academia Sinica, Taiwan, as a Senior Research Associate. His current research interests are in applying computational methods to study various aspects of the metal−protein interactions and function.

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Despite this unique feature in binding the metal cation monodentately or bidentately, Asp/Glu side chains have been assigned various roles in metal binding and selectivity based mainly on their charge rather than on their denticity. Due to the negative charge of the carboxylate group, interactions with the metal cation in a buried protein cavity are not only thermodynamically favorable but also generally more favorable than those of other neutral ligands; thus, Asp/Glu are thought to be mainly responsible for sequestering the metal cation from physiological fluids.[6,11−13] The negative charge of the carboxylate group also determines the net charge of a carboxylate-binding pocket, which contributes to the selectivity of the metal-binding site. For example, engineered EF-hand calcium-binding sites containing three Asp/Glu side chains (with a net ligand charge of −3) can selectively bind Ca^{2+} against a much higher background concentration of monovalent cations such as Na^+ and K^+.[14] Increasing the net negative charge of the protein cavity protects the metal-bound ligands from being dislodged by cellular anions; thus, the Asp^-/Glu^- lining the metal-binding pocket also plays a protective role against interactions with unwanted "alien" anionic species from cellular fluids.[15]

Recent studies have revealed that in addition to the negative charge of the carboxylate group, the *mode of carboxylate binding* also plays an important role in recognition of a native metal cofactor and thus the function of a metalloprotein. Previous studies on the carboxylate-binding mode,[16−18] which were confined to Zn proteins, focused on the thermodynamics and kinetics of the monodentate ⇆ bidentate equilibrium. In this Account we focus on the role of the carboxylate-binding mode in metal recognition and protein function based mainly on our previous theoretical results and literature pertinent to this topic. We first delineate the factors governing the carboxylate-binding mode in proteins and show how these principles can be used to rationalize the observed difference in the carboxylate-binding mode in Mg^{2+}- and Ca^{2+}-containing proteins. We then reveal how the carboxylate-binding mode affects not only the binding affinity but also the selectivity of a metal ion. Finally, we show how the carboxylate-binding mode affects the function of a metalloprotein using Ca^{2+}-binding proteins and several enzymes as examples.

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). The theoretical studies mentioned herein generally employed two approaches: in studying specific metalloproteins, all-atom molecular dynamics simulations were performed, whereas in unraveling general principles or guidelines, a combined quantum mechanical/continuum approach was employed where the effect of the protein matrix on the thermodynamics of metal binding/selectivity was implicitly taken into account (see section 2). We then summarize the key results and discuss the physical basis and/or implications of the findings.

2. Factors Governing the Carboxylate-Binding Mode to a Given Metal Cofactor in Proteins

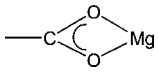
Of the two carboxylate-binding modes the monodentate mode is generally more common than the bidentate mode in both small molecules and metalloproteins, as shown from statistical analyses of structures in the Cambridge Structure Database (CSD) and the Protein Data Bank (PDB).[19−23] Although the former is predominant in Mg^{2+}- and Ca^{2+}-binding sites containing inner-shell Asp and Glu, significant differences between the preferred carboxylate-binding mode in Mg^{2+} and Ca^{2+} proteins were found:[23] Of the 27 Mg^{2+}- and 54 Ca^{2+}-binding sites with first-shell Asp/Glu ligands, only one Mg^{2+}-binding site (in human phosphatase, 1EW2) contains bidentately bound carboxylates while almost a third (29%) of the Ca^{2+}-binding sites have at least one bidentately bound acidic residue. Since previous theoretical studies on carboxylate mono/bidentate equilibrium have been carried out on only one type of metal-binding site, viz., catalytic zinc sites,[16−18] why Mg^{2+}- and Ca^{2+}-binding sites contain significantly different amounts of bidentately bound acidic residues was not clear. Furthermore, the physical principles determining the carboxylate-binding mode were also not clear.

To elucidate which factors favor monodentate carboxylate binding and which favor bidentate binding to the metal ion, we systematically investigated how the carboxylate-binding mode depends on (i) the immediate neighbors of the carboxylates, (ii) the type of metal and its coordination number, (iii) the total charge of the metal complex, and (iv) the relative solvent exposure of the metal-binding site.[23] For a given metal complex, we computed the free energy for converting a monodentately bound carboxylate to a bidentately bound one using density functional theory (DFT) combined with the continuum dielectric method (CDM). DFT was used to treat the metal and its ligands in order to incorporate electronic effects such as polarization of the participating entities and charge transfer from the ligands to the metal ion, while a dielectric constant ϵ was used to model the rest of the protein. The latter was used instead of an atomic representation of the protein matrix as our goal was to derive general principles *not* for a specific metal-binding site in a metalloprotein but rather for all sorts of metal-binding sites with varying degrees of solvent accessibility, represented by ϵ ranging from 2 to 80. The calculations predict metal−carboxylate oxygen distances and preferred carboxylate-binding modes in metal complexes that agree with those observed in the respective X-ray structures in the CSD or PDB.[23]

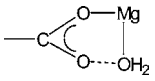
The theoretical results[23] suggest that the carboxylate-binding mode is determined mainly by competition between the metal cation, on one hand, and nonacidic neighboring ligands from the metal's inner or outer coordination sphere, on the other, for the second oxygen of the COO^- moiety. *Bidentate* carboxylate binding is preferred over the monodentate mode when the second carboxylate oxygen's interactions with the metal cation

are more favorable than those with first or second-shell ligand(s). This occurs when the following conditions occur.

(1) The metal cation has a high coordination number (high steric repulsion among the ligands) and is thus more tolerant to the less space-demanding bidentate motif



rather than the bulkier monodentate motif.



(2) The metal cation is a good Lewis acid that can accept charge from the second carboxylate oxygen.

(3) The metal's positive charge is not neutralized by charge transfer from negatively charged ligands in the metal complex and can thus attract the second carboxylate oxygen.

(4) The metal cation is relatively *large* and can accommodate *bulky* protein main chain/side chain dipoles that do not stabilize the second carboxylate oxygen as well as the metal cation.

(5) The first- or second-shell ligands lack hydrogen-bond donors or provide only poor hydrogen-bond donors such as the peptide backbone that do not stabilize the second carboxylate oxygen as well as the metal cation.

In addition to competition between the metal cation and the first/second-shell residues for the second carboxylate oxygen, the relative rigidity of the metal-binding site structure can also affect the monodentate ⇆ bidentate equilibrium. This is exemplified in parvalbumin, which contains a canonical EF-hand motif, consisting of a contiguous 12-residue Ca^{2+}-binding loop flanked by two helices forming a conserved helix−loop−helix structure.[24,25] In the native protein (1PAL), Glu101 at the last position of the Ca^{2+}-binding loop binds bidentately to Ca^{2+} in a pentagonal bipyramidal geometry (Figure 2a), but in the E101D mutant (1B8L) Asp101 binds *monodentately* to Ca^{2+} in a distorted octahedral geometry (Figure 2b).[26] Molecular dynamics simulations of wild-type parvalbumin and the E101D mutant have shown that the inherent rigidity of the Ca^{2+}-binding loop prevents the Asp101 side chain from attaining a suitable orientation to coordinate the metal bidentately.[27]

The principles outlined above help to rationalize the observed difference in the preferred carboxylate-binding mode in different metalloproteins. For example, the size difference between Mg^{2+} and Ca^{2+} ions contributes in part to the observed difference in the preferred carboxylate-binding mode. Relative to Mg^{2+}, Ca^{2+} is much larger (the ionic radii of six-coordinated Ca^{2+} and Mg^{2+} are 1.00 and 0.72 Å,[28] respectively) and can better accommodate bulky protein main chain/side chain dipoles.[29] Consequently, Ca^{2+}−carboxylate complexes are "drier" than the respective Mg^{2+}−carboxylate complexes, in accord with X-ray structures showing that Ca^{2+}-binding sites have a much lower ratio of water:backbone ligands than Mg^{2+}-binding sites. When the metal's positive charge is reduced by coordination to negatively charged ligands, carboxylate interactions with the metal cation become weaker than

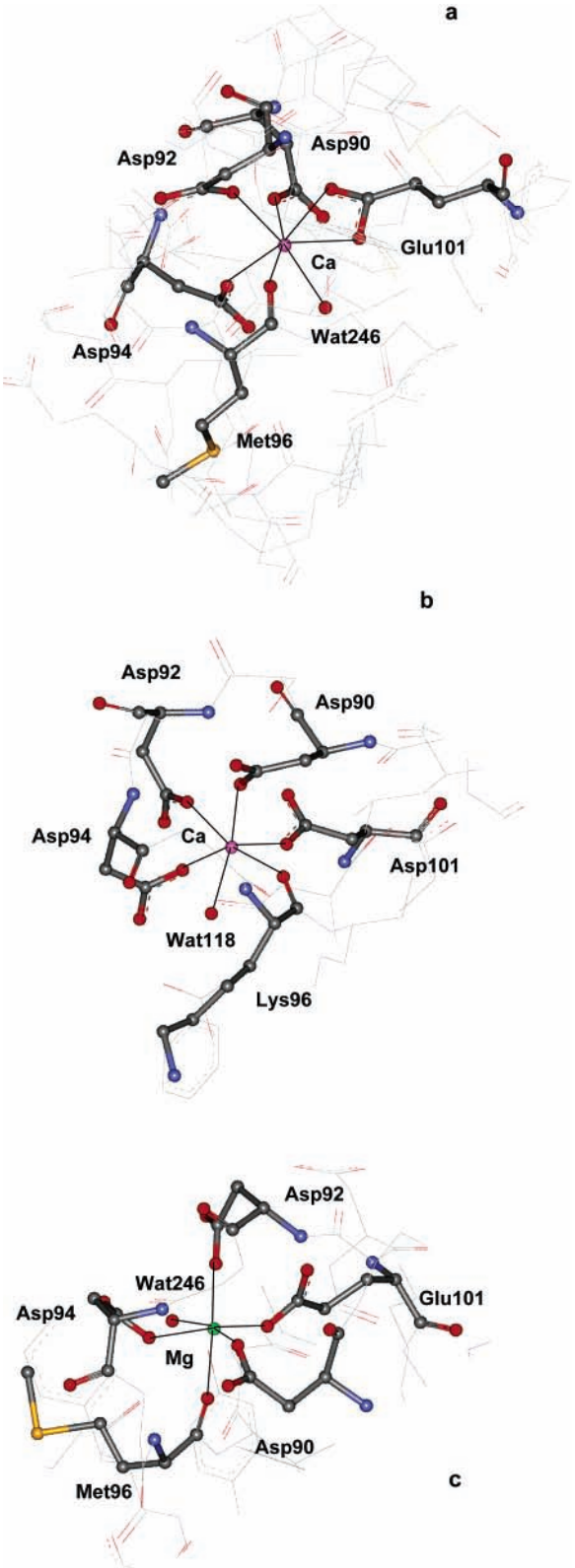


FIGURE 2. Bidentate vs monodentate carboxylate binding in parvalbumin. (a) Bidentate binding of Glu101 to heptacoordinated Ca^{2+} in pike 4.10 parvalbumin (1PAL, 1.68 Å). (b) Monodentate binding of Asp101 to hexacoordinated Ca^{2+} in mutant beta-carp parvalbumin (1B8L, 1.70 Å). (c) Monodentate binding of Glu101 to hexacoordinated Mg^{2+} in pike 4.10 parvalbumin (4PAL, 1.80 Å).

those with first- or second-shell ligands. In such cases, first- or second-shell ligand−carboxylate interactions dictate the carboxylate-binding mode. In water-rich Mg^{2+} complexes containing two or more carboxylate groups, the metal-free carboxylate oxygen atoms can be stabilized by the metal-bound water molecules; therefore, Mg^{2+} prefers to bind the carboxylates monodentately rather than bidentately (see Figure 1a). On the other hand, in the respective "drier" Ca^{2+} complexes the second carboxylate oxygen may not be sufficiently stabilized by the peptide backbone; thus, Ca^{2+} could compete with the peptide backbone for the second carboxylate oxygen (see Figure 1b).

3. Bidentate Carboxylate Binding Favors a Trivalent Lanthanide Cation over a Divalent Cation

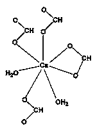
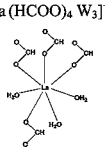
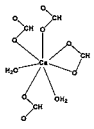
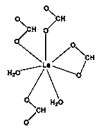
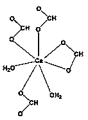
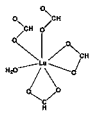
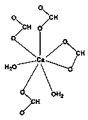
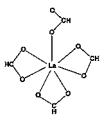
The principles outlined above could help not only to rationalize the preferred carboxylate-binding mode found in different metalloproteins but also in the design of metal-binding sites that are specific for certain metal cations. For example, they could be used to design binding sites that are specific for trivalent lanthanide cations, Ln^{3+}, instead of the natural divalent metal cofactors such as Mg^{2+} and Ca^{2+}. This is useful because alkaline-earth metal-binding sites in proteins (Ca^{2+}-binding sites in particular) have few chemical properties that can be used to explore their biochemistry in situ. In contrast, luminescent lanthanide ions can be used in bioanalytical assays[30] to determine the interdomain distance of proteins,[31] while paramagnetic lanthanides, whose large anisotropic magnetic susceptibility gives rise to large pseudocontact shifts that can be observed for residues as far as 40 Å from the metal center, can be used to obtain long-distance restraints for NMR protein structure determination.[32] Metal-bound carboxylates play a crucial role in determining the affinity of the Ca^{2+}-binding site for lanthanides.[33] According to the principles derived in the previous section, *trivalent* cations are better charge acceptors than divalent or monovalent ions; thus, relative to the latter, they should prefer to bind the carboxylate group bidentately.

To verify if indeed bidentate carboxylate binding could favor a trivalent lanthanide cation over a divalent cation, we computed the free energies for replacing a native divalent metal cofactor (Ca^{2+}) by a trivalent lanthanide cation (La^{3+}), retaining and changing the original carboxylate-binding mode in a pseudoclassical EF-hand Ca^{2+}-binding site, which is modeled by Ca^{2+} heptacoordinated to a bidentate formate (denoted by HCÕÕ), three monodentate formates, and two water molecules[34] (see Table 1). The four formates are heptacoordinated to La^{3+} in various combinations of monodentate and bidentate modes. Table 1 lists the free energies for the following

$$[Ca(HC\tilde{O}\tilde{O})(HCOO)_3(H_2O)_2]^{-2} + [La(H_2O)_9]^{3+} \rightarrow [La(HC\tilde{O}\tilde{O})_n(HCOO)_{4-n}(H_2O)_{3-n}]^{-} + (2+n)H_2O + [Ca(H_2O)_6]^{2+} \quad (1)$$

The results in Table 1 show that bidentate carboxylate

Table 1. Calculated ΔG^x (kcal/mol) for Replacing Ca^{2+} in $[Ca(HC\tilde{O}\tilde{O})(HCOO)_3W_2]^{-2}$ by $[LaW_9]^{3+}$ with and without Changing the Carboxylate-Binding Mode in Media of Dielectric Constant, x[a]

Reactant metal complex + $[La\,W_9]^{3+}$	Product metal complex + $[Ca\,W_6]^{2+}$	ΔG^4	ΔG^{10}	ΔG^{20}
	$[La(HCOO)_4W_3]^- + 2W$ 	−57	−8	+8
	$[La(HC\tilde{O}\tilde{O})(HCOO)_3W_2]^- + 3W$ 	−65	−17	−1
	$[La(HC\tilde{O}\tilde{O})_2(HCOO)_2W]^- + 4W$ 	−71	−24	−8
	$[La(HC\tilde{O}\tilde{O})_3(HCOO)]^- + 5W$ 	−71	−26	−12

[a] $x = 4$, 10, and 20 model protein-binding cavities with increasing degree of solvent exposure; $W = H_2O$.

binding facilitates the $Ca^{2+} \rightarrow La^{3+}$ exchange in fully or partially buried sites: the condensed-phase ΔG^x values ($x = 4-20$) in Table 1 become more favorable (more negative) with an increasing number of carboxylates bound *bidentately* to La^{3+}.[34] This is because, as compared to monodentately bound carboxylates, increasing the number of *bidentately* bound carboxylates to La^{3+} increases the number of water molecules that are freed from the metal's first coordination sphere, which, in turn, results in a gain in the gas-phase entropy and solvation free energy. The findings are consistent with available experimental data. That bidentate carboxylate binding facilitates $Ca^{2+} \rightarrow La^{3+}$ exchange is consistent with the observed switch from monodentate to bidentate carboxylate binding when Ca^{2+} is replaced by (a) Yb^{3+} in the EF-hand Ca^{2+}-binding site of carp parvalbumin[35] and (b) Eu^{3+} in thermolysin.[36] That La^{3+} prefers to bind at least one carboxylate bidentately in an EF-hand-like binding site is in line with the observation that all EF-hand-like lanthanide-binding sites in the PDB contain at least one bidentately bound Asp/Glu.

The finding that freeing both carboxylate oxygen atoms so they could bind bidentately to the metal cation would enable lanthanide ions to replace a native divalent metal cofactor may be useful in NMR structure determination of Ca^{2+}/Mg^{2+}-binding proteins. The carboxylate oxygen atoms could be freed by replacing those wild-type residues that stabilize them with similar residues without hydrogen donors; i.e., bidentate coordination could be favored over monodentate coordination by "negative" design.

4. Carboxylate-Binding Mode Affects the Maximum Number of Metal-Bound Carboxylates

The number of metal-bound Asp^-/Glu^-, which determines the net charge of a carboxylate-rich metal-binding site, has been found to play an indirect role in enhancing the affinity and/or selectivity of a protein cavity for a given metal cofactor. For example, the three Asp^-/Glu^- side chains lining engineered EF-hand Ca^{2+}-binding sites contribute a net ligand charge of −3, which helps to selectively bind divalent Ca^{2+} against a much higher background concentration of monovalent cations such as Na^+ and K^+.[14] However, the maximum number of carboxylates that could bind to a metal ion of charge q, denoted by $Max^{COO}(M^{q+})$, was not known. Furthermore, if and how the carboxylate-binding mode affects this upper limit was also not known.

To evaluate if and how the carboxylate-binding mode affects the $Max^{COO}(M^{q+})$ in proteins, we computed the free energies for the successive exchange of metal-bound water molecules for carboxylates bound either *monodentately* or *bidentately*[37] using a combined DFT and CDM approach (see above). From the computed free energies (Figure 3) the maximum number of carboxylates that could bind to the natural metal cofactors, Mg^{2+}, Ca^{2+}, and Zn^{2+}, as well as non-natural metal cofactors, lithium (Li^+), lanthanum (La^{3+}), and zirconium (Zr^{4+}), were determined. The findings from the calculations were validated by comparison with the number of Asp/Glu coordinated to univalent (Li^+, Na^+, K^+, Rb^+, Cs^+), divalent (Mg^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}), and trivalent (lanthanides, Ln^{3+}) metal ions in <3.0 Å X-ray/NMR structures in the PDB. Complexes of tetravalent metals such as Zr^{4+}, Hf^{4+}, or Ce^{4+} were not found in any PDB structures. On the basis of the combined results from the DFT/CDM calculations and the PDB survey, guidelines were derived for estimating the $Max^{COO}(M^{q+})$ in metalloproteins.[37]

The results suggest that the carboxylate-binding mode could affect the $Max^{COO}(M^{q+})$.[37] If all the carboxylates are bound *monodentately* to the metal cofactor in a fully/partially buried protein cavity, a metal ion of charge q can bind no more than $q + 1$ Asp^-/Glu^- (see Figure 3), if the metal complex were not additionally stabilized by interactions with outer-shell ligand(s). This is because a metal ion of charge q bound to $q + 1$ Asp^-/Glu^- results in a *monoanionic* metal complex, which is unlikely to bind another Asp^-/Glu^- to form a *dianionic* metal complex due to repulsive charge−charge interactions between two negatively charged entities in a relatively buried cavity. However, if one or more acidic residues bind *bidentately* to the metal cofactor in a relatively buried protein cavity the $Max^{COO}(M^{q+})$ may be raised to $q + 2$, i.e., 4 for a divalent cation and 5 for a trivalent cation. Although a monodentately bound carboxylate cannot displace a metal-bound water molecule in the monoanionic $[Ca(CH_3COO)_3(H_2O)_4]^-$ complex (positive free energy, data not shown), a bidentately bound carboxylate could do so (negative free energy, data not shown) mainly because the release of an extra water molecule results in a gain in the gas-phase entropy and solvation free energy.[37] The finding that a divalent metal ion can bind as many as four Asp/Glu side chains if one of them is bidentately bound is consistent with the finding that 16 out of the 20 tetracarboxylate dication-binding sites in the PDB structures contain at least one Asp/Glu bound bidentately to the metal ion.[37]

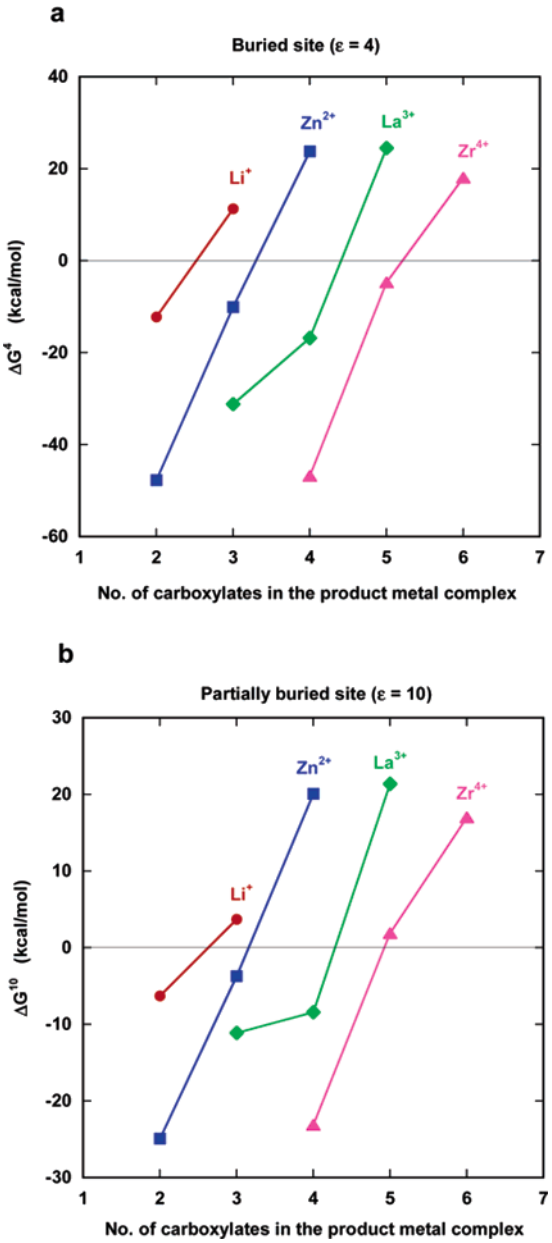


FIGURE 3. Calculated free energies (ΔG^ϵ) for the successive exchange of a metal-bound water molecule for monodentately bound acetate in $[M(H_2O)_m(CH_3COO)_n]^{q-n}$ complexes ($M = Li^+$, Zn^{2+}, La^{3+}, and Zr^{4+}) as a function of the number of carboxylates in the product metal complex. (a) ΔG^ϵ corresponding to a buried site ($\epsilon = 4$). (b) ΔG^ϵ corresponding to a partially buried site ($\epsilon = 10$).

Increasing the number of metal-bound Asp^-/Glu^- in *buried* sites facilitates exchange of a native divalent metal cofactor such as Ca^{2+}/Mg^{2+} for a trivalent lanthanide cation as the latter can accept more negative charge from the carboxylate side chains than the former.[23] It also

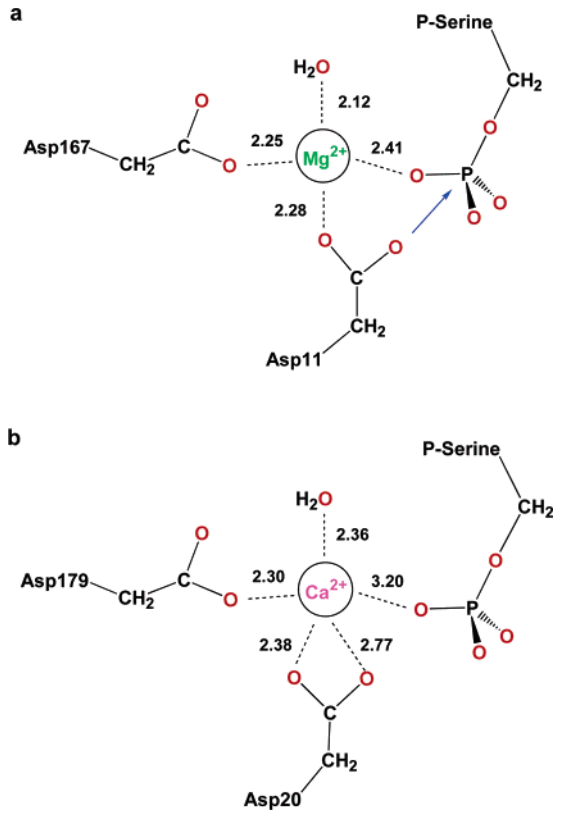


FIGURE 4. Monodentate vs bidentate carboxylate binding in substrate-bound phosphoserine phosphatase. (a) Monodentate binding of Asp11 to Mg^{2+} in PSP from *Methanococcus jannaschii* (1L7P, 1.90 Å). (b) Bidentate binding of Asp20 to Ca^{2+} in human PSP (1NNL, 1.53 Å). Adapted from Figure 5 in ref 43, where the substrate is modeled in the active site and a ligating water molecule and Asp13/22 were omitted for clarity only.

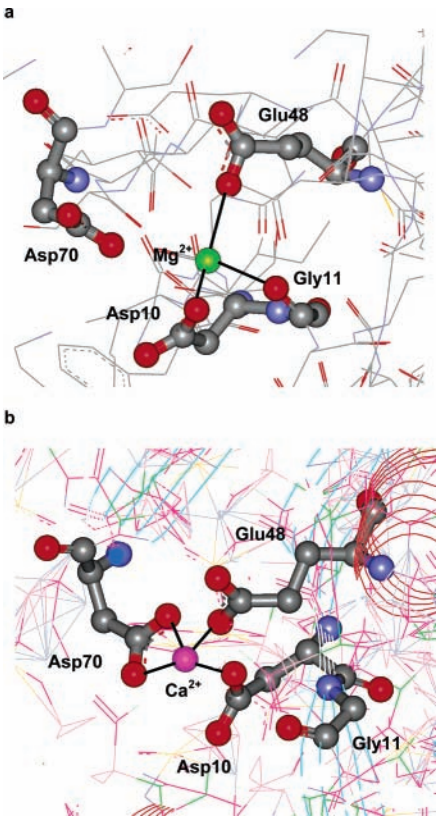


FIGURE 5. Different Mg^{2+}- and Ca^{2+}-binding sites in RNase H1. (a) Mg^{2+} bound to Asp10 and Glu48 monodentately (1RDD, 2.8 Å). (b) Ca^{2+} bound to Asp10 and Glu48 monodentately and additionally Asp70 bidentately (from average MD structure in ref 42).

increases the net negative charge of the metal-binding cavity, which in turn increases the protection level of the cavity against attacks from intracellular anions. Whereas Cl^- could displace both Mg^{2+}-bound amide and water ligands in a *neutral* cavity, it can displace only Mg^{2+}-bound water in an *anionic* cavity but none of the Mg^{2+}-bound ligands in a *dianionic* cavity.[15] Thus, by playing a role in determining the $Max^{COO}(M^{q+})$, the carboxylate-binding mode could indirectly (i) affect the affinity and/or selectivity of a protein cavity for a given metal cofactor and (ii) help protect the cavity from attack by "alien" cytoplasmic anions.

5. Carboxylate-Binding Mode Can Control Protein Function

As the carboxylate-binding mode can affect the affinity and/or selectivity of a protein cavity for a given metal cofactor, it can also affect the protein's function. Perhaps the best known example of the effect of the carboxylate-binding mode on protein function is provided by EF-hand regulatory Ca^{2+}-binding proteins such as calmodulin, calcineurin, calpain, recoverin, S100, and troponin C, which are involved in a wide variety of physiological processes, including cell cycle regulation, signal transduction, second messenger production, muscle contraction, and vision.[26] The binding mode of a highly conserved Glu at the last position of the EF-hand Ca^{2+}-binding loop plays a crucial role in discriminating between Ca^{2+} and Mg^{2+} and their binding affinities in these proteins (Figures 1b and 2a).[14] *Bidentate* binding of this Glu to a hepta-coordinated Ca^{2+} (as observed in the X-ray structure of Ca^{2+}-bound parvalbumin; Figure 2a) is correlated with large conformational changes in the protein, which subsequently trigger a cascade of events along the signal transduction pathway[14,38,39] (so-called "calcium-induced molecular switch"[40]). In contrast, *monodentate* binding of this Glu to a hexacoordinated Mg^{2+} (4PAL; Figure 2c) resulted in a physiologically silent protein.[41]

The carboxylate-binding mode can not only regulate signal transduction but also abolish catalytic activity in some metalloenzymes such as *E. coli* ribonuclease H1 (RNase H1)[42] and human phosphoserine phosphatase (PSP).[43] Human PSP, which utilizes Mg^{2+} as a cofactor, catalyzes the hydrolysis of phosphoserine to yield L-serine and inorganic phosphate.[43] However, the enzymatic activity is abolished upon binding to Ca^{2+}, a potential competitor of Mg^{2+} in living cells. A comparison between the active-site X-ray structures of Mg^{2+}-bound (Figure 4a) and Ca^{2+}-bound (Figure 4b) PSP suggests that a switch in the carboxylate-binding mode of an essential Asp residue (from mono- to bidentate) contributes to the loss of catalytic activity. In the Mg^{2+}-bound PSP from *Methanococcus jannaschii* (Figure 4a), Asp11 is *monodentately*

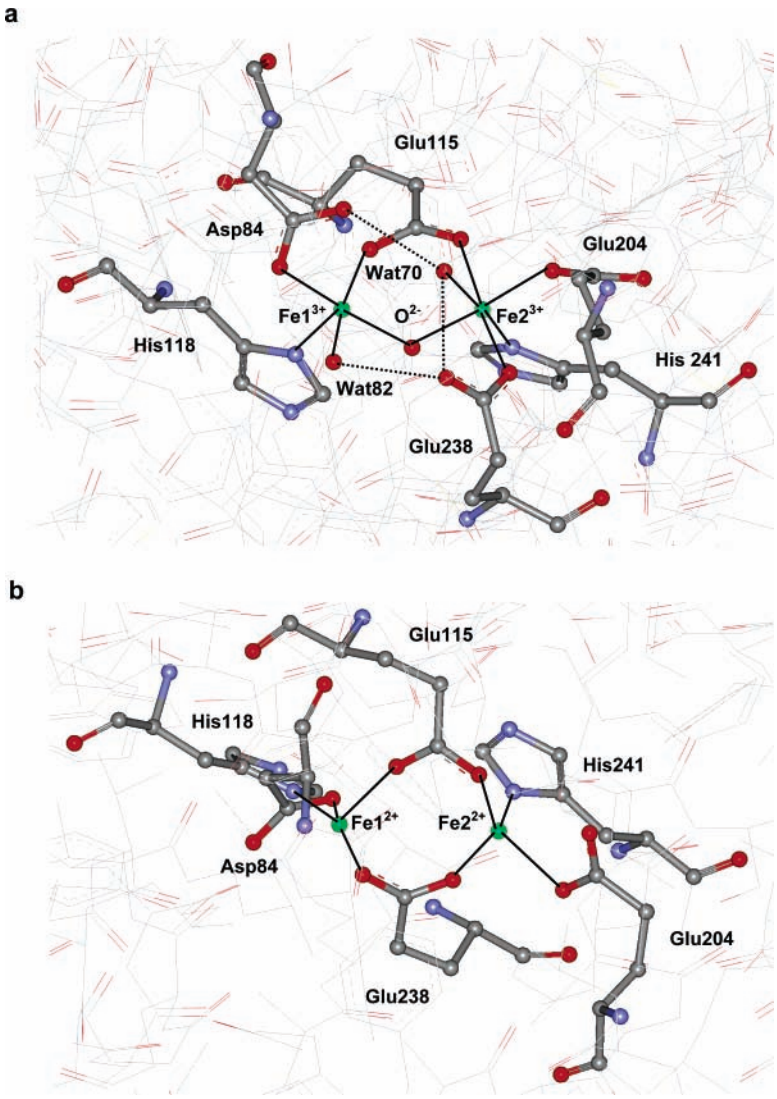


FIGURE 6. Bidentate vs monodentate carboxylate binding of Glu 238 in *E. coli* ribonucleotide reductase R2 domain. (a) Monodentate binding of Glu238 to $Fe2^{3+}$ in the oxidized diferric (met) form (1MXR, 1.42 Å). (b) Bidentate binding of Glu238 to $Fe1^{2+}$ and $Fe2^{2+}$ in the reduced diferrous form (1XIK, 1.70 Å).

bound to Mg^{2+} and its metal-free carboxylate oxygen can thus attack the phosphorus atom of the phosphoserine substrate. In the Ca^{2+}-bound human enzyme (Figure 4b), the corresponding Asp20 is *bidentately* bound to Ca^{2+}, thus preventing it from attacking the substrate, resulting in an inactive enzyme. In analogy, *bidentate* binding of Asp70, a hypothesized general base in RNase H1 catalysis, to Ca^{2+} in addition to the protein ligands of the smaller native cofactor, Mg^{2+}, enables Ca^{2+} to bind the enzyme tighter than Mg^{2+} in vitro but prevents Asp70 from deprotonating a water nucleophile for subsequent phosphate attack, thus abolishing enzymatic activity (Figure 5).[42]

In addition to mononuclear metal-binding sites, the interconversion between mono- and bidentate Asp/Glu in *polynuclear* metal-binding sites (so-called "carboxylate shift"[44]) has been postulated to play an important role in a number of catalytic processes.[45] Notable in this respect is the effect of the "carboxylate shift" on the function of ribonucleotide reductase (RNR), which catalyzes reduction of ribonucleotides to deoxyribonucleotides, which are essential for DNA synthesis.[46] Class I RNRs is a complex of two dimeric proteins—R1, containing binding sites for substrates and allosteric effectors, and R2, harboring a diiron redox center and the catalytically essential tyrosyl radical.[47] During the catalytic process the reduced diferrous ($Fe^{2+}Fe^{2+}$; redR2) form is oxidized by oxygen to an oxidized diferric ($Fe^{3+}Fe^{3+}$; oxiR2) form. In oxiR2 (1MXR; Figure 6a) pentacoordinated $Fe1^{3+}$ and hexacoordinated $Fe2^{3+}$ are bridged by a glutamate side chain (Glu115) and an oxo (O^{2-}) ion, while the rest of the metal coordination spheres are complemented by His, Asp/Glu, and water ligands. Upon reduction, the more weakly bound water ligands in oxiR2, which form hydrogen bonds with the metal-free carboxylate oxygen of Glu238, are lost, freeing Glu238 to bind bidentately to both Fe^{2+} ions in redR2 (1XIK; Figure 6b). The Glu238 carboxylate shift from binding one Fe^{3+} monodentately in oxiR2 to binding two Fe^{2+} bidentately in redR2 is accompanied by a Fe coordination number decrease from 5 or 6 in oxiR2 to 4 in

redR2; the resulting unsaturated, less crowded coordination sphere of Fe^{2+} facilitates O_2 binding.[48] Thus, a shift in the carboxylate-binding mode from mono- to bidentate creates an accessible reaction site for O_2.

6. Concluding Remarks

The results herein reveal that the carboxylate-binding mode plays an important role in the binding affinity and selectivity of a metal cofactor and thus function of a metalloprotein. It is determined mainly by the competition between the metal cation, on one hand, and neighboring nonacidic ligands (water, backbone carbonyls) from the metal-binding pocket, on the other, for the second oxygen of the COO^- entity. Several factors such as the immediate surrounding of the metal-bound carboxylate, the type of metal and its coordination number, the total charge of the metal complex, and the relative solvent accessibility and rigidity of the metal-binding pocket could affect the monodentate ⇆ bidentate equilibrium (section 2).

The carboxylate-binding mode to a metal ion could also play a dual role in metal-ion selectivity and protein function. By shifting the equilibrium toward bidentate carboxylate binding, a metal-binding site could become selective for trivalent (lanthanide) over divalent (Mg^{2+} or Ca^{2+}) cations (section 3) or be fine tuned to accommodate an extra Asp^-/Glu^- in the metal's first coordination sphere (section 4). The carboxylate-binding mode is of crucial importance in signal transduction: a switch in the carboxylate-binding mode of a highly conserved Glu in EF-hand regulatory proteins from bidentate to monodentate upon $Ca^{2+} \rightarrow Mg^{2+}$ substitution abolishes signal transduction (section 5). Conversely, a switch in the carboxylate-binding mode of a highly conserved Asp in human phosphoserine phosphatase from monodentate to bidentate upon $Mg^{2+} \rightarrow Ca^{2+}$ substitution abolishes enzymatic activity (section 5). Furthermore, by manipulating the mode of carboxylate binding at a relatively low-energy cost,[48] diiron protein R2 of ribonucleotide reductase can create an accessible coordination site for the incoming O_2 (section 5).

Both theoretical and experimental results summarized in this Account indicate that a carboxylate monodentate ⇆ bidentate switch, in addition to other structural factors, could be used to modulate the metal-binding site affinity/selectivity for a given metal cofactor, thus altering the function of the metalloprotein. Thus, switching the carboxylate-binding mode is potentially another design tool that could be employed to engineer new metal-binding sites with preprogrammed properties.

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