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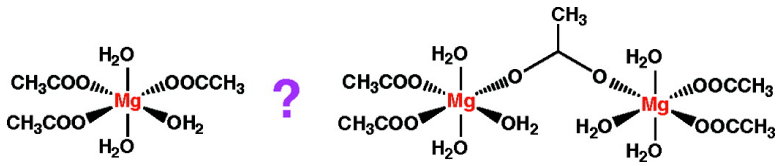

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Mononuclear versus Binuclear Metal-Binding Sites: Metal-Binding Affinity and Selectivity from PDB Survey and DFT/CDM Calculations

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Abstract: Binuclear metal centers in metalloenzymes are involved in a number of hydrolytic, hydration, isomerization, and redox processes. Despite the growing number of studies elucidating their structure, properties, and function, questions regarding certain aspects of the bimetallic proteins' biochemistry still remain, e.g., the following: (i) What are the general characteristics of binuclear sites found in 3D structures such as the range of metal−metal distances and the most common ligand bridging the two metal cations? (ii) How does the presence of a metal cation in one of the binuclear sites affect the metal-binding affinity/selectivity of the other site? (iii) How do the characteristics and metal-binding affinity/selectivity of binuclear sites compare with those of their *mono*nuclear counterparts? Here we address these questions by combining a Protein Data Bank survey of binuclear sites with density functional theory (DFT) combined with continuum dielectric method (CDM) calculations. The results reveal that, for homobinuclear sites, the metal separation depends on the metal's charge and electron-accepting ability, and Asp^-/Glu^-, bidentately bound to the two cations, is the most common bridging ligand. They also reveal that Mg^{2+} occupying one of the binuclear sites attenuates the metal-binding affinity but enhances the selectivity of its neighboring site, compared to the corresponding mononuclear counterparts. These findings are consistent with available experimental data. The weak metal binding of one of the binuclear sites would enhance the metal cofactor mobility in achieving the transition state, whereas the enhanced selectivity of Mg^{2+}−Mg^{2+} centers helps protect against unwanted substitutions by transition metal ions, which are generally stronger Lewis acids compared to Mg^{2+}.

Introduction

Metal cations are an integral and indispensable part of ≈40% of all known proteins.[1] This is due to their unique combination of physicochemical properties, namely, positive charge, rigid/adaptable coordination sphere, Lewis acidity, specific ligand affinity, and varying valence state and electron spin configuration combined with a small volume, simple structure, and high mobility. Thus, Nature has employed these building blocks, in addition to the 20 natural L-amino acid (aa) residues, to finely tune, enhance, and/or diversify the protein properties. Metal cofactors are engaged in performing a number of tasks spanning from protein structure stabilization to enzyme catalysis, signal transduction, nitrogen fixation, photosynthesis, and respiration.[2−5]

In most metalloproteins, one metal cation bound to a given (*mono*nuclear) binding site is capable of fulfilling the task—"catalytic" and/or "structural"—assigned to it by Nature. In some cases, however, two or even more metal cations, which are embedded in *bi*nuclear or *poly*nuclear binding sites, respectively, are needed to carry out the catalytic and/or structural function. They act synchronously during the catalytic process. Binuclear metal centers are further subdivided into *homo*binuclear, containing metal ions of the same chemical type, e.g., Zn−Zn, and *hetero*binuclear, where chemically different metal cations (e.g., Cu−Zn) are bound to the active-site ligands. In many bimetallic proteins the two metal centers (even in homobinuclear sites) are functionally and structurally not equivalent with different ligand surroundings, solvent exposure, and binding affinity.[4,6−20] The two metal cations are usually connected by

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one or more bridging ligands such as water, hydroxide, carboxylate (from Asp/Glu side chains), or imidazolate (from His side chain) which, apart from their structural role, may participate in the catalytic process.[7,21]

Binuclear metal centers in metalloenzymes are involved in a number of hydrolytic, hydration, isomerization, phosphorylation, and redox processes. Interestingly, there exists some mononuclear metalloenzymes where a single cation suffices to perform quite efficiently the same task as its bimetallic counterpart. Notable examples are the binuclear (Cu−Zn) and mononuclear (Ni, Fe, or Mn) superoxide dismutases,[22,23] mono- and dizinc metallo-β-lactamases,[24] and dimanganese and monoiron catalases,[7] as well as dimanganese (e.g., arginase) and monozinc hydrolases.[7] This fact raises the following intriguing question: why does Nature, which generally evolves efficient cellular/protein machinery to sustain life processes on Earth, require two metal cations instead of one to catalyze the same type of enzymatic reaction? In other words, do bimetallic centers offer any advantages over the mononuclear active sites, thus justifying their existence?

As far as the functionality of the metal-binding site is concerned, both experiment and theory have indicated that the extra metal ion in bimetallic centers has indeed beneficiary effects on the catalytic process. Thus, bimetallic centers have the advantages of (i) delocalizing charges better, which facilitates two-electron redox reactions, (ii) enhancing stabilization of the intermediate and transition-state structures by charge neutralization and/or polydentate metal binding, thus lowering the respective activation barriers, (iii) binding polyatomic substrates better,[25] and (iv) activating/ionizing the nucleophile (e.g., water) of the reaction more efficiently.[7] However, questions regarding other aspects of the bimetallic proteins' structure and biochemistry such as metal-binding affinity and selectivity still remain: (1) What are the general characteristics of binuclear sites found in 3D structures, e.g., the range of metal−metal distances, the most common ligand bridging the two metal cations, and the first-shell aa preference of a given metal cation? (2) How does the presence of a metal cation in one of the binuclear sites affect the metal-binding affinity/selectivity of the other site? (3) How do the characteristics and the metal-binding affinity/selectivity of binuclear sites compare with those of their mononuclear counterparts? For example, does a given metal cation exhibit the same aa preference in both mono and binuclear sites? Does it bind with comparable affinity to mono- and binuclear sites sharing the same set of nonbridging first-shell ligands?

In this paper we address the above questions by combining a Protein Data Bank (PDB) survey of binuclear metal-binding sites with density functional theory (DFT) to treat the metal cations and the ligands (to account for electronic effects such as polarization of the participating entities and charge transfer from the ligands to the metal cation) coupled with a continuum dielectric representation of the rest of the protein (see Methods). We first present the results of the PDB survey, which reveal the factors affecting the metal separation in binuclear sites as well as the most common bridging first-shell ligands in homobinuclear sites. We then evaluated how the free energies for Mg^{2+}/Zn^{2+} binding in bimetallic centers depend on (i) the ligand composition and overall charge of the binding site, (ii) the metal type and coordination number, and (iii) the solvent exposure and flexibility of the binding site. The advantages/disadvantages of the mono- and bimetallic centers in terms of their metal-binding affinity and selectivity were determined by comparing the data obtained for binuclear binding sites with those for the respective mononuclear counterparts possessing the same type of ligands and total charge. Our findings are consistent with available experimental data. Although Mg^{2+}/Zn^{2+}−carboxylate complexes have been used to model mono- and binuclear binding sites herein, the present findings appear to have broader implications and cover a larger spectrum of bimetallic binding sites.

Methods

Database Survey. The PDB[26] was surveyed for ≤3.5 Å resolution X-ray and NMR structures of proteins containing native binuclear binding sites. Binding sites containing different types of native metal cofactors such as Zn^{2+}, $Cu^{+/2+}$, Ni^{2+}, Co^{2+}, $Fe^{2+/3+}$, $Mn^{2+/3+}$, Ca^{2+}, and Mg^{2+} were studied. These were checked against available experimental (kinetic) data to confirm that both metal ions are indeed needed for proper functioning of the respective binuclear metalloproteins. However, proteins containing metal clusters like Fe_2S_2, Cu_4S_4, and/or nonbiogenic metal ions like Cd^{2+}, Al^{3+}, Hg^{2+}, Pd^{2+}, and Li^{+} in the binding site were excluded from the survey. Mutant protein structures were not considered either. For a given type of bimetallic center, the protein sequences were aligned by the ClustalW[27] program and those with sequence identities >35% were considered to belong to the same protein family. Only one representative from each protein family was included in the survey, namely, the free protein (not bound to any substrate/transition state analogs, inhibitors, or products) structure solved at the highest resolution or, if no free structure is available, the ligand-bound structure solved at the highest resolution.

Defining First-Shell Ligands in the Binuclear Metal-Binding Sites. Analysis of high-resolution X-ray structures of small complexes in the Cambridge Structural Database[28] has shown that the first-shell M−O, M−N, and M−S distances (where M denotes the metal ion) do not exceed 2.6 Å.[29] To account for the lower resolution of some of the PDB structures, a slightly larger cutoff of 3.0 Å was used to locate the first-shell ligands, which were defined as residues with a donor atom (e.g., N, S, or O) within 3.0 Å from the metal.

Models Used. The side chains of Asp^{-}/Glu^{-} were modeled by acetate anion, CH_3COO^{-} (ACE^{-}). In aqueous solution, both Mg^{2+} and Zn^{2+} are experimentally determined to be hexacoordinated,[30] so their aqua complexes were modeled as $[Mg(H_2O)_6]^{2+}$ and $[Zn(H_2O)_6]^{2+}$, respectively. In proteins, Mg^{2+} is still octahedrally coordinated, while Zn^{2+} exhibits higher flexibility upon protein binding and is usually tetracoordinated.[31] Thus, representative structures of hexacoordinated mono- and binuclear hexacoordinated Mg^{2+}-binding sites from the PDB

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Scheme 1

$$\begin{array}{ccc} & \Delta G^1 & \\ \text{Reactants } (\varepsilon = 1) & \rightarrow & \text{Products } (\varepsilon = 1) \\ \Delta G_{solv}^{\ x}\text{ (Reactants)} \downarrow & & \downarrow \Delta G_{solv}^{\ x}\text{ (Products)} \\ \text{Reactants } (\varepsilon = x) & \rightarrow & \text{Products } (\varepsilon = x) \\ & \Delta G^x & \end{array}$$

surveys were modeled (see Results). Upon replacement of Mg^{2+} with Zn^{2+}, both octahedral and tetrahedral Zn^{2+} complexes were considered, mimicking rigid binding sites (prohibiting ligand rearrangement upon metal exchange) and flexible binding sites (allowing for ligand rearrangement upon metal exchange), respectively.

DFT Calculations. (a) Geometries. Full geometry optimization for each complex studied was carried out using the Gaussian 03 program[32] employing the S-VWN functional and the 6-31+G* basis set. This functional/basis set combination was chosen as it reproduces the experimentally observed metal−ligand distances in a number of metal−ligand complexes within experimental error.[33] For each fully optimized structure, S-VWN/6-31+G* vibrational frequencies were computed to verify that the molecule was at the minimum of its potential energy surface. No imaginary frequency was found in any of the metal complexes.

(b) Gas-Phase Free Energies. On the basis of the fully optimized S-VWN/6-31+G* geometries, the electronic energies, E_{elec}, were evaluated using the B3-LYP functional in conjunction with the large 6-311++G(2df,2p) basis set. The latter was chosen from among several other basis sets as the gas-phase formation energy of $[Mg(H_2O)_6]^{2+}$ has been found to converge at this level of theory.[34] Since the 6-311++G(2df,2p) basis set employed is relatively large, basis set superposition error (BSSE) in the reaction energies is not expected to be significant. Indeed, for the successive binding of hexahydrated Mg^{2+} to two Asp^-/Glu^- side chains (modeled by acetate) in mononuclear sites (see Results), the BSSE comprise only 0.6% and 0.3% of the total reaction energies, respectively. Therefore, the BSSE was not considered in the present calculations. The thermal energy including zero point energy (E_T), work (PV), and entropy (S) corrections were evaluated using standard statistical mechanical formulas,[35] where the S-VWN/6-31+G* frequencies were scaled by an empirical factor of 0.9833.[36] The differences ΔE_{elec}, ΔE_T, ΔPV, and ΔS between the products and reactants were used to compute the reaction free energy in the gas phase at room temperature, $T = 298.15$ K, according to the following expression:

$$\Delta G^1 = \Delta E_{elec} + \Delta E_T + \Delta PV - T\Delta S \quad (1)$$

Continuum Dielectric Calculations: Solution Free Energies. The reaction free energy in a given environment characterized by a dielectric constant $\epsilon = x$ can be computed according to Scheme 1.

ΔG^1, the gas-phase free energy, was computed using eq 1, as described above. $\Delta G_{solv}^{\ x}$, the free energy for transferring a molecule in the gas phase to a continuous solvent medium characterized by a dielectric constant, x, varying from 4 for totally buried metal-binding sites to 80 for fully solvent-exposed sites, was estimated by solving Poisson's equation using finite difference methods.[37,38] Thus, the reaction free energy in an environment modeled by dielectric constant x, ΔG^x, can be computed from

$$\Delta G^x = \Delta G^1 + \Delta G_{solv}^{\ x}(\text{products}) - \Delta G_{solv}^{\ x}(\text{reactants}) \quad (2)$$

The solvation free energies were evaluated using the MEAD (macroscopic electrostatics with atomic detail) program,[39] as described in previous works.[33,39] The effective solute radii, which were obtained by adjusting the CHARMM (version 22)[40] van der Waals radii to reproduce the experimental hydration free energies of Mg^{2+} and Zn^{2+} and model ligand molecules, are as follows (in Å): $R_{Mg} = 1.50$; $R_{Zn} = 1.40$; $R_C = 1.88$; $R_O(CH_3COO^-) = 1.575$; $R_O(H_2O) = 1.78$; $R_O(H_2O-Mg/Zn) = 1.70$; $R_H = 1.468$; $R_H(H_2O-Mg) = 1.16$; $R_H(H_2O-Zn) = 1.09$. These effective solute radii reproduce the experimental hydration free energies of Mg^{2+}, Zn^{2+}, and model ligands within 1 kcal/mol.[33]

Results

PDB Survey. The PDB search produced 213 structures containing 253 *homo*binuclear binding sites where the two metal ions have the same oxidation state, but only 36 structures containing *hetero*binuclear binding sites. The latter are insufficient for proper statistical analyses and were therefore excluded from analysis. Among the homobinuclear binding sites, the biMg^{2+}-binding sites appear to be the most abundant, followed by those containing Zn^{2+} and Mn^{2+} (Table 1). Since a characteristic feature of binuclear binding sites is the separation between the two cations, it is of interest to evaluate the average distance between the two metal ions, ⟨M−M⟩, and its dependence on the metal's properties. For homobinuclear binding sites, the metal ions are usually separated by 3−5 Å. Interestingly, for a given metal type, the higher its oxidation state (formal charge), the shorter the ⟨M−M⟩ distance (Table 1). For example, the $\langle Cu^{2+}-Cu^{2+}\rangle$ distance (3.29 ± 0.59 Å) is significantly shorter than the $\langle Cu^{+}-Cu^{+}\rangle$ distance (4.24 ± 0.35 Å). This implies that the charge−charge repulsion between the positively charged cations is effectively alleviated by the surrounding ligands, especially the bridging ligand(s). A cation in a higher oxidation state has a smaller ionic radius (see Table 1) and shorter metal−ligand distances, resulting in a more

Table 1. Average Distances (in Å) between the Two Metal Ions in Homobinuclear Binding Sites

metal, M^{q+}	R_{ion}[a] (Å)	N_{sites}[b]	⟨M–M⟩[c] (Å)	⟨M–O⟩[d] (Å)	q_M[e] (e)
Cu^+	0.910	4	4.24 ± 0.35	2.29	0.85
Ca^{2+}	1.140	30	4.56 ± 0.93	2.44	1.89
Mg^{2+}	0.860	57	4.36 ± 1.04	2.11	1.79
Mn^{2+}	0.970	52	4.07 ± 1.08	2.21	1.58
Co^{2+}	0.885	9	3.91 ± 0.89	2.12	1.54
Fe^{2+}	0.920	18	3.82 ± 0.47	2.16	1.56
Zn^{2+}	0.880	52	3.52 ± 0.38	2.12	1.69
Cu^{2+}	0.870	5	3.29 ± 0.59	2.11	1.56
Ni^{2+}	0.830	1	3.03	2.08	1.53
Fe^{3+}	0.785	24	3.42 ± 0.22	2.00	1.72
Mn^{3+}	0.785	1	3.03	2.04	1.74

[a] Shannon ionic radius for a hexacoordinated metal in a high spin state.[41] [b] The number of bimetallic sites containing the given metal ion in the set of nonredundant proteins. [c] The number after the distance is the standard deviation. [d] The average distance between the metal and the oxygen in $[M(H_2O)_6]^{q+}$ complexes optimized at the B3-LYP/6-31+G* level; high-spin configurations are considered for open-shell metal cations. [e] The NBO charge on the metal in the fully optimized $[M(H_2O)_6]^{q+}$ complexes evaluated at the B3-LYP/6-31+G* level.

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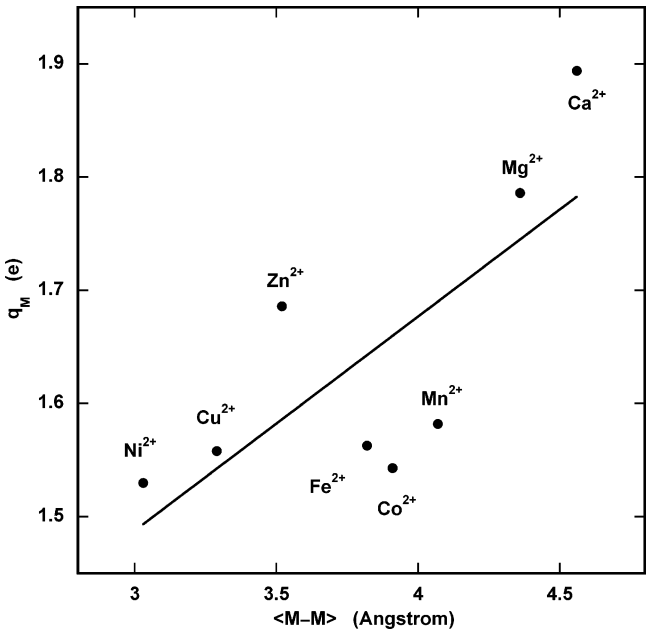

Figure 1. Plot of the average intermetallic distance in homobinuclear binding sites, ⟨M−M⟩, with respect to the metal charge, q_M. The Pearson correlation coefficient is 0.74.

compact structure of the binding sites, compared to the respective cations in a lower oxidation state.

Nontransition metal dications such as Mg^{2+} and Ca^{2+} seem to form less compact bimetallic sites than divalent transition metal ions such as Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}. In fact, the ⟨Ca^{2+}−Ca^{2+}⟩ distance is the longest among the dicationic bimetallic centers in Table 1, in line with its larger ionic radius, poorer charge accepting ability, and weaker binding, as compared to the other divalent cations. In general, the divalent transition metal ions seem to be better charge acceptors than Mg^{2+} and Ca^{2+}, as evidenced by the larger charge transfer from water ligands to the former, as compared to the latter (see q_M in Table 1). In fact, the trend in the distances between two homodications is generally in accord with the metal's charge-accepting ability (Table 1 and Figure 1).

In summary, the metal separation in homobinuclear binding sites, which ranges between 3 and 5 Å in the absence/presence of other ligands, depends on the charge and the electron-accepting ability of the metal ion.

Another characteristic feature of binuclear binding sites is the ligand bridging the two metal cations. To determine the most common bridging first-shell ligand in homobinuclear sites, the percentage frequency distributions of the bridging and non-bridging first-shell ligands in homobimetallic centers were computed (see Supporting Information, Figure S1). A negatively charged Asp/Glu carboxylate, bidentately bound to the two metal cations, is the most common bridging ligand, followed by H_2O/OH^- for the transition metal ions, regardless of whether other nonprotein ligands are bound to the metal ion.

Is the first-shell ligand preference found in mononuclear metal-binding sites[42] the same as that in the respective homobinuclear sites containing the same metal type? Since previous work[42] analyzed mononuclear sites containing Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} bound to only water and aa ligands, we analyzed the subset of homobinuclear sites containing the same four dications bound to only water and aa ligands. Comparison of the percentage frequency distributions of the first-shell ligands in mononuclear and homobinuclear sites in Figure 2 shows that Ca^{2+}, Mg^{2+}, and Mn^{2+} exhibit similar aa preference in both types of sites. These are Asp/Glu, followed by the backbone for Ca^{2+} (Figure 2a) and Mg^{2+} (Figure 2b), and Asp/Glu, followed by His for Mn^{2+} (Figure 2c). Water/hydroxide is also a common ligand for both mono- and bimetallic centers, especially in Mg^{2+}−Mg^{2+} sites, where it is more commonly found bound to Mg^{2+} than Asp/Glu (Figure 2b).

In contrast to Ca^{2+}, Mg^{2+}, and Mn^{2+}, Zn^{2+} exhibits aa preference in homobinuclear sites that is apparently different from that seen in mononuclear proteins[42,43] (Figure 2d). In mononuclear Zn-binding sites, Zn^{2+} prefers to bind to Cys (52%) followed by His (34%), whereas it prefers His and Asp/Glu (33% each) to Cys (14%) in binuclear Zn sites. Furthermore, the water content in binuclear Zn sites is roughly twice that in the mononuclear Zn sites. These findings can be rationalized by considering the different (catalytic or structural) roles of Zn^{2+}. In *catalytic* Zn sites, Zn^{2+} is found bound usually to a water molecule and preferentially to His, followed by Glu, Asp and then Cys, whereas, in *structural* Zn sites, it is found tetrahedrally bound preferentially to Cys rather than His.[44−46] The set of 74 nonredundant *mono*nuclear Zn-binding sites contains 50 structural and 24 catalytic Zn sites; hence, Zn^{2+} is found bound predominantly to Cys residues (see above). In contrast, the set of 52 nonredundant *bimetallic* zinc centers are mostly involved in catalytic reactions; hence, Zn^{2+} is found bound predominantly to His and Asp/Glu residues as well as water molecules.

In summary, the aa preference of a given metal ion in both homobinuclear and mononuclear proteins appear to be similar, provided the metal ion plays the same role in both types of sites.

Metal-Binding Affinities in Mononuclear and Binuclear Binding Sites. What is the difference between metal binding to a *mono*nuclear site and metal binding to a *bi*nuclear site where one of the sites is already occupied by a metal ion? To answer this question, we assume that Mg^{2+} has already entered the metal-binding cavity and subsequently binds to a carboxylate group lining the cavity. The nonbridging and bridging carboxylates are assumed to bind the metal ion in a monodentate and bidentate mode, respectively, in accord with the preferred binding mode found in the PDB structures (see Supporting Information, Figure S1). The free energies for Mg^{2+} binding successively to Asp^-/Glu^- side chains (modeled by acetate, ACE^-) in mononuclear sites and in the corresponding binuclear sites with the same overall charge of varying solvent exposure were computed (Figure 3). In the bimetallic sites, the anionic Mg^{2+}-occupied binding site containing three Asp^-/Glu^- side chains acts as a "super" ligand, $[(CH_3COO)Mg(H_2O)_3(CH_3COO)_2]^-$ (abbreviated as $[Mg{-}SL]^-$), in which one of the carboxylates bridges the two metal cations. Note that the biMg−carboxylate complexes in Figure 3 can be considered as derivatives of the respective Mg−carboxylate complexes where a CH_3COO^- ligand has been replaced by the anionic Mg^{2+}-

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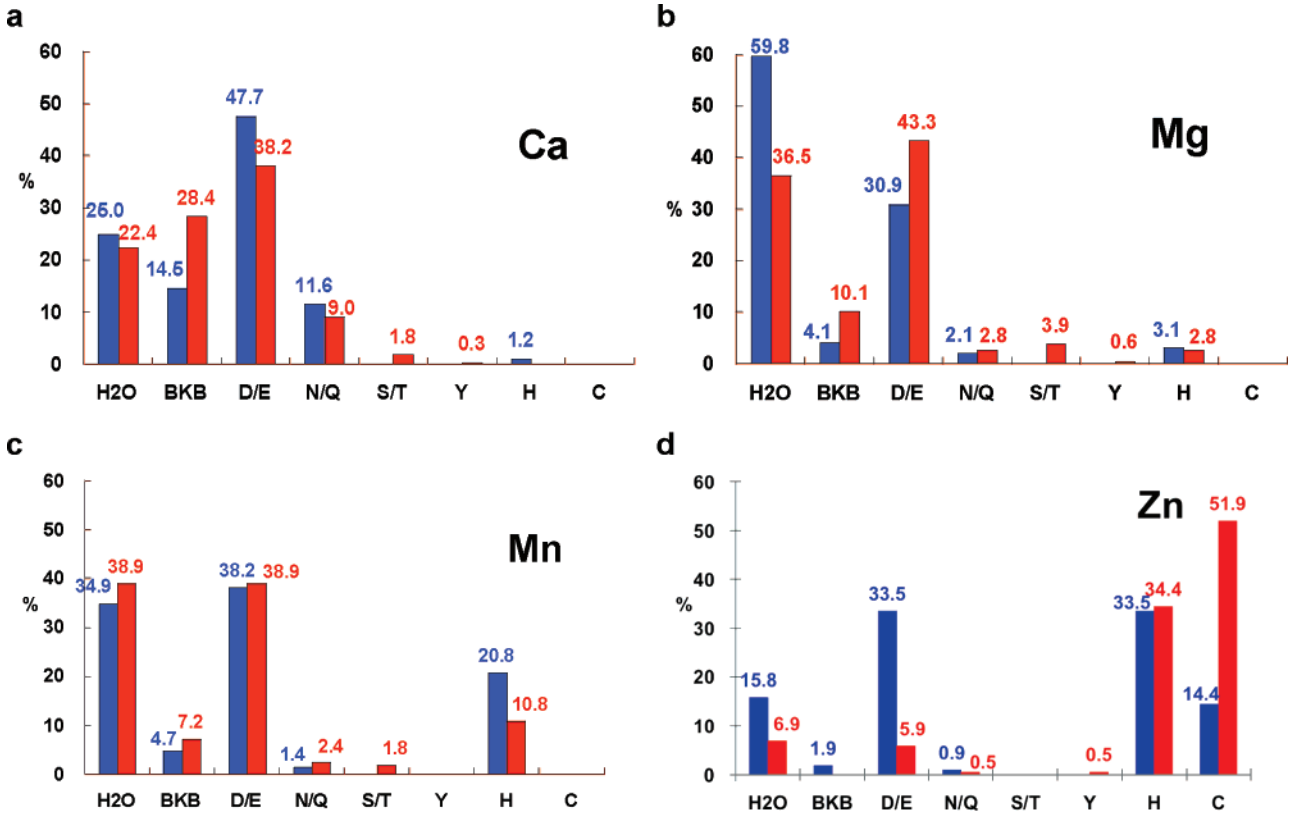

Figure 2. Comparison between the percentage frequency distributions of first-shell ligands in mononuclear and homobinuclear PDB sites. The blue and red bars represent the % frequencies observed in homobinuclear PDB sites (this work) and mononuclear PDB sites (from Dudev et al.[42]), respectively. H2O denotes water and BKB denotes the backbone group, while the other letters denote the aa side chain that is bound to the metal ion.

containing "super" ligand, $[Mg-SL]^-$. Increasing the solvent exposure of both mono- and binuclear binding sites attenuates the affinity for Mg^{2+}, as evidenced by less negative or even positive ΔG^x with increasing dielectric constant x (Figure 3).

Magnesium binding to the "super" ligand, $[Mg-SL]^-$, in bimetallic centers appears less favorable than Mg^{2+} binding to the CH_3COO^- ligand in mononuclear sites, regardless of the solvent accessibility of the site: the respective free energies, ΔG^x, are much less negative than those for the mononuclear counterparts and become positive in relatively exposed binuclear sites ($x \geq 10$). Thus, the calculations imply that the metal-occupied binding site decreases the affinity of the other binding site for a second metal ion. Atomic charge distribution analysis (MK scheme[47]) reveals that this is mainly due to the poorer charge-donating properties of the "super" ligand where Mg^{2+}, acting as a Lewis acid (electron acceptor), absorbs part of the electron density from the bridging acetate, thus rendering the "super" ligand, $[(CH_3COO)Mg(H_2O)_3(CH_3COO)_2]^-$, less effective in ligating a cation than CH_3COO^-. Consequently, the charge transfer from the ligands to the metal cation in monometallic centers is greater than that in their bimetallic counterparts. For example, the charge transfer to Mg^{2+} from 4 water molecules and 2 acetates in the mononuclear complex in Figure 3b is 0.22e greater than that from 4 water molecules, an acetate, and the $[Mg-SL]^-$ "super" ligand in the corresponding binuclear complex.

Along the same vein, a "super" ligand with Zn^{2+} instead of Mg^{2+} is expected to exhibit poorer ligating ability than its $[Mg-SL]^-$ counterpart because Zn^{2+} is a far better electron acceptor than Mg^{2+}. Indeed, when reaction 3c was modeled with $[(CH_3COO)Zn(H_2O)(CH_3COO)_2]^-$ (denoted by $[Zn-SL]^-$) instead of $[(CH_3COO)Mg(H_2O)_3(CH_3COO)_2]^-$, ΔG^1 became unfavorable (+13.9 kcal/mol), as compared to the original reaction ($\Delta G^1 = -0.2$ kcal/mol; Figure 3c). Correspondingly, the charge transferred from $[Zn-SL]^-$ to Mg^{2+} is 0.07e less than that from $[Mg-SL]^-$.

In summary, when one of the binuclear sites is occupied by a cation, the other site has lower affinity toward a given metal ion than the corresponding mononuclear site with the same nonbridging ligands.

Metal Cation Selectivity in Mononuclear and Binuclear Binding Sites. Does metal binding to one site affect not only the metal-binding affinity but also the metal selectivity of the other site? Our previous work[48] had shown that Mg^{2+}-binding sites do not appear to be specific for Mg^{2+}: other divalent metals, especially Zn^{2+}, may dislodge Mg^{2+} from rigid binding sites that do not allow for any ligand rearrangement. Would an extra metal-binding site inhibit/facilitate other dications such as Zn^{2+} to replace the native metal cofactor in binuclear sites? To address this question, we computed the free energies for replacing Mg^{2+} with Zn^{2+} in (i) *rigid* binding sites where the incoming Zn^{2+} retains the octahedral geometry of the outgoing Mg^{2+} and (ii) *flexible* binding sites that allow the ligands to rearrange so that the incoming Zn^{2+} can adopt its preferred tetrahedral geometry.[49]

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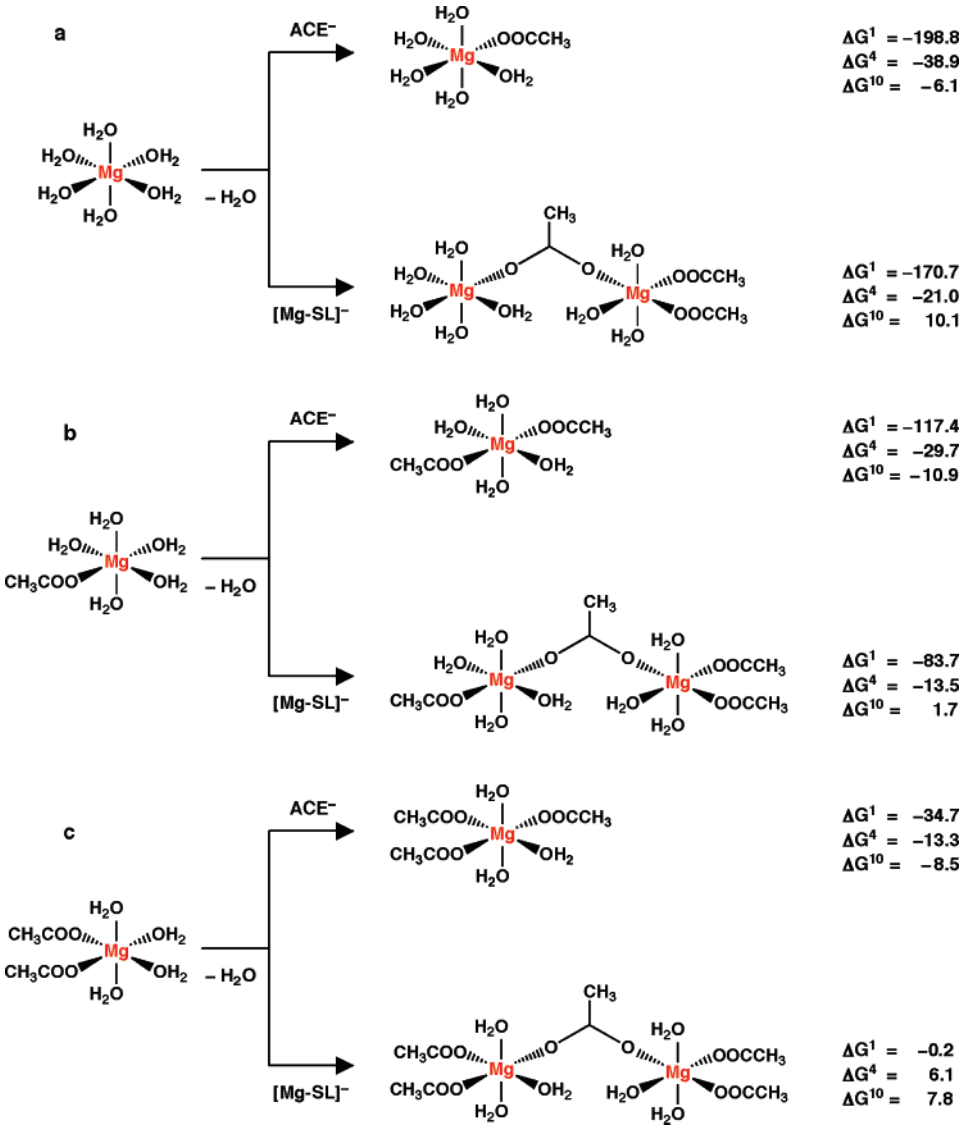

Figure 3. Free energies, ΔG^x (in kcal/mol), for replacing H_2O with CH_3COO^- (ACE$^-$) and $[(CH_3COO)Mg(H_2O)_3(CH_3COO)_2]^-$ (denoted by [Mg−SL]$^-$) in (a) $[Mg(H_2O)_6]^{2+}$, (b) $[Mg(H_2O)_5CH_3COO]^+$, and (c) $[Mg(H_2O)_4(CH_3COO)_2]^0$ complexes in a protein cavity characterized by dielectric constant, *x*.

(a) Rigid Sites. The substitution of Mg^{2+} with Zn^{2+} in rigid binuclear binding sites is less favorable than that in mononuclear centers (Figure 4): the free energy for replacing Mg^{2+} with Zn^{2+} in a binuclear site is less negative than that in the corresponding mononuclear site, regardless of the net charge (0 for the complexes in Figure 4a and −1 for the complexes in Figure 4b) and solvent exposure of the binding pocket. As the metal substitution becomes less favorable with increasing solvent exposure of the bimetallic centers (ΔG^x becomes generally less negative/positive with increasing *x*), Zn^{2+} may *not* be able to replace Mg^{2+} in a solvent-exposed, rigid binuclear site.

(b) Flexible Sites. The substitution of Mg^{2+} with Zn^{2+} in *flexible* mono-and binuclear binding sites (Figure 5) is more favorable than that in the respective *rigid* binding sites (Figure 4). This is not surprising in view of the strong preference of Zn^{2+} toward tetrahedral coordination in carboxylate-containing complexes[49] and the favorable contribution of the two liberated water molecules to the reaction entropy and solvation free energy. However, as for the substitution of Mg^{2+} with Zn^{2+} in rigid binding sites, replacing Mg^{2+} with Zn^{2+} in flexible binuclear metal centers is less favorable than that in flexible mononuclear counterparts, regardless of the solvent exposure and total charge of the metal complex. This trend is mainly due to the poorer charge-donating ability of the "super" ligand compared to CH_3COO^-, which affects Zn^{2+} binding more than Mg^{2+} binding. The tendency becomes even more obvious when the substitution of Mg^{2+} with Zn^{2+} occurs in a trinuclear binding site containing two "super" ligands (Figure 6). As expected, the presence of another [Mg−SL]$^-$ in the binding site results in a further reduction of the free energy gain upon replacing Mg^{2+} with Zn^{2+}: ΔG^4 (−12.2 kcal/mol) and ΔG^{10} (−12.3 kcal/mol) for forming the binuclear $[CH_3COO{\cdot}Zn(H_2O)_2{\cdot}Mg{-}SL]$ complex (Figure 5a) decreases to −2.3 and 0.3 kcal/mol, respectively, in the trinuclear $[Mg{-}SL{\cdot}Zn(H_2O)_2{\cdot}Mg{-}SL]$ structure (Figure 6).

In summary, binuclear/trinuclear Mg^{2+}-binding sites are less prone to Zn^{2+} substitution and are thus more selective than the respective mononuclear centers. A second or third Mg^{2+}-binding site seems to play a protective role against unwanted metal substitutions. Conversely, the reverse of the reaction in Figure 6 indicates that tetrahedral Zn sites in mixed Zn−Mg trinuclear centers are not as selective as the respective mononuclear Zn

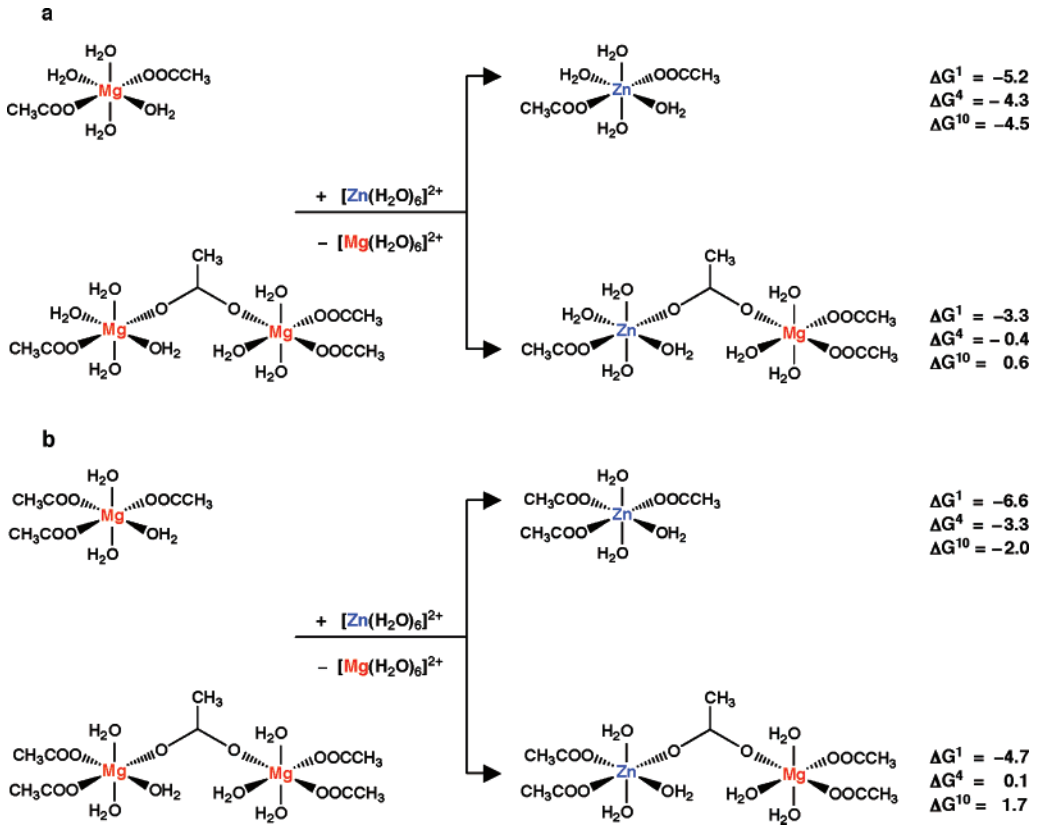

Figure 4. Free energies, ΔG^x (in kcal/mol), for replacing Mg^{2+} with Zn^{2+} in *rigid* (a) neutral and (b) anionic mono- and binuclear Mg^{2+}-binding sites characterized by dielectric constant, *x*.

sites (reverse of the reaction in Figure 5a) and Zn^{2+} could be vulnerable to substitution by Mg^{2+}, especially if the site substituted by Mg^{2+} was stabilized by hydrogen-bonding interactions from the second-shell ligands.[48]

Validation of the Models Used. Although the model binuclear Mg^{2+} complexes in Figures 3−5 had been fully optimized in the gas phase, they reproduce structural parameters of binuclear Mg^{2+}-binding sites found in the PDB: the average distance between Mg^{2+} and the bridging carboxylate oxygen in the fully optimized binuclear Mg^{2+} complexes depicted in Figures 3−5 (2.05 Å) is close to the average experimental value (2.13 ± 0.10 Å). Likewise, the calculated $\langle Mg^{2+}{-}Mg^{2+}\rangle$ distance (4.81 Å) falls within the error limit of the experimental value (4.38 ± 0.70 Å) determined for binuclear Mg^{2+}-binding sites bridged by Asp/Glu side chains.

To ensure that the fully optimized model complexes do not bias the findings, we estimated the Mg^{2+}-binding affinity using more realistic mono- and binuclear Mg^{2+}-binding sites extracted from X-ray structures. We chose the mononuclear Mg^{2+}-binding site of avian sarcoma virus integrase (PDB entry 1VSD), as it resembles the mononuclear $[Mg(CH_3COO)_2(H_2O)_4]$ complex in Figure 3b with Mg^{2+} bound monodentately to two carboxylates (Asp64 and Asp121) and four water molecules. Unfortunately, no PDB structure contained a binuclear Mg^{2+} site with one of the sites containing the same Mg^{2+}-bound atoms as the 1VSD site. However, site I in the binuclear Mg^{2+} site of fructose-1,6-bisphosphatase (PDB entry 1EYI) resembles the 1VSD site, as the Mg^{2+} is bound monodentately to two carboxylates (Glu97 and Asp118), while site II resembles the "super" ligand, $[Mg{-}SL]^-$, with Mg^{2+} bound to 3 carboxylates (Asp118, Asp121, and Glu280). However, the Mg^{2+} in site I is also bound not only to a water oxygen but also the Leu120 backbone carbonyl oxygen and a phosphate oxygen.

To determine if site I in the 1EYI structure exhibits lower metal affinity than the respective mononuclear site with the same net charge and nonbridging ligands, as predicted by the model complexes (see above and Figure 3), we modified the Leu120 backbone oxygen to a water oxygen in the 1EYI structure and removed two water molecules from the 1VSD structure so that both site I in the 1EYI structure and the mononuclear site in the 1VSD structure have the same net charge and nonbridging ligands. The metal(s) and the Mg^{2+}-bound aa side chains and water oxygen atoms were then cut out from the respective PDB structures by truncating the aa side chains at the C_α position. The X-ray positions of all atoms were fixed and missing hydrogen atoms were added using GaussView in the Gaussian03 program. The resulting metal complexes, with and without Mg^{2+}, were then subjected to single-point electronic energy calculations at the B3LYP/6-311++G(2df,2p) level. The trend in the binding energies, $\Delta E = E$(binding pocket with Mg^{2+}) − E(binding pocket without Mg^{2+}) − $E(Mg^{2+})$, for the PDB-derived mono- and binuclear Mg^{2+}-binding sites is in accord with the predictions based on the model complexes: site I from the bimetallic center in the 1EYI structure exhibits lower metal affinity ($\Delta E = -563.2$ kcal) than the respective mononuclear site in the 1VSD structure ($\Delta E = -654.9$ kcal/mol).

To verify that the presence of second-shell ligands do not alter the above finding, second-shell aa side chains or backbone groups that hydrogen bond with the first-shell aa ligands were added to the inner-shell PDB structures. In 1VSD, the Asn160 side chain and the Gly123 backbone amide hydrogen bond with Asp64 and Asp121, respectively. In 1EYI, the Asn64 side chain

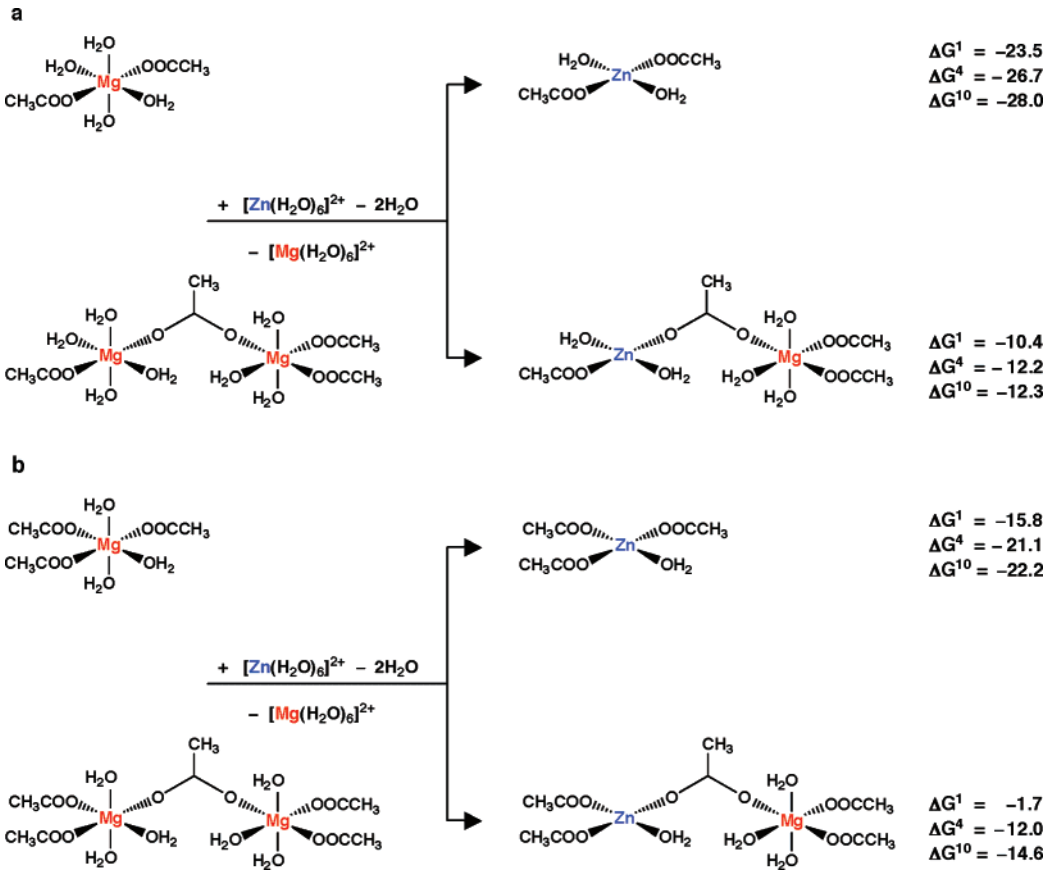

Figure 5. Free energies, ΔG^x (in kcal/mol), for replacing Mg^{2+} with Zn^{2+} in *flexible* (a) neutral and (b) anionic mono- and binuclear Mg^{2+}-binding sites characterized by dielectric constant, *x*.

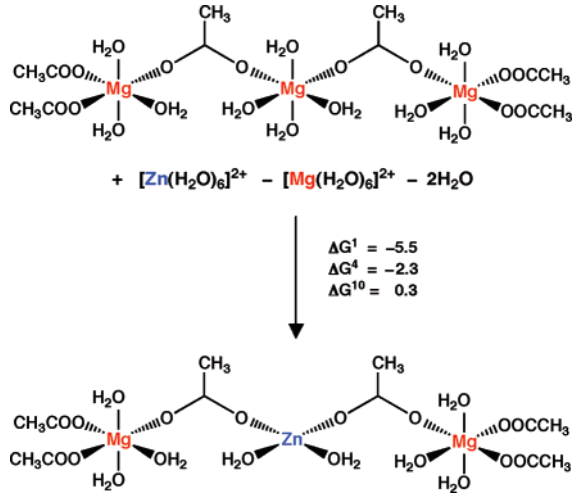

Figure 6. Free energies, ΔG^x (in kcal/mol), for replacing Mg^{2+} with Zn^{2+} in a flexible trinuclear Mg^{2+}-binding site characterized by dielectric constant, *x*.

hydrogen bonds with Glu97, while the Val249 and Arg276 backbone amides interact with Asp121 and Glu280, respectively. These second-shell ligands were cut out from the respective PDB structures by truncating the aa side chains and backbone amide at the C_α position. Missing hydrogen atoms were added using GaussView to the PDB-derived first- + second-shell complexes, and their B3LYP/6-311++G(2df,2p) energies in the presence and absence of Mg^{2+} were computed. The resulting binding energies show the same trend in that Mg^{2+} binds with lower affinity to the 1EYI site I ($\Delta E = -535.6$ kcal/mol) than the 1VSD mononuclear counterpart ($\Delta E = -639.0$ kcal/mol).

In summary, calculations employing both first-shell and first- + second-shell complexes derived from PDB structures yield the same conclusion as the fully optimized model complexes.

Discussion

It is intriguing that Nature employs a single metal cation in a certain enzyme but two metal cations for the same enzymatic reaction in other proteins. For example, metallo-β-lactamase from *Aeromonas hydrophila* employs a single Zn^{2+} to catalyze the hydrolysis of β-lactam antibiotics, but that from *Bacteroides fragilis* uses two Zn^{2+} for the same enzymatic reaction.[50] Although the extra metal ion in bimetallic centers has been postulated to have beneficiary effects on the catalytic process, its advantages/disadvantages in terms of the metal-binding affinity and selectivity of the free binuclear site were not known (to the best of our knowledge). The calculations herein reveal the effects of the extra cation on metal-binding affinity and selectivity and the physical bases/origin of these effects. To further verify our findings, the experimental literature was searched for relevant data—our findings were found to be in line with available experimental data, as discussed below.

Effect on Metal-Binding Affinity. A given metal center in a binuclear binding site exhibits lower metal affinity than the respective mononuclear site possessing the same overall charge and number of negatively charged aa residues. This is because

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a metal dication occupying an adjacent site acts as a Lewis acid, absorbing some of the electron density of the bridging carboxylate/hydroxide, thus compromising the charge-donating ability of the bridging ligand, which thus coordinates another metal cation more weakly than a "metal-free" carboxylate/hydroxide. Note that the stronger the Lewis acidity of the metal cation occupying a binding site, the more pronounced this effect becomes. Thus Zn^{2+}, which is a stronger Lewis acid than Mg^{2+}, reduces the ligating ability of the bridging carboxylate/hydroxide more than Mg^{2+} (see Results). The charge-donating ability difference between a free and metal-bound carboxylate implies that the two metal-binding sites are unlikely to bind the same metal cation with similar affinity, if metal binding proceeds in a stepwise manner. The first metal cation would bind to one or more free carboxylates in a binuclear site with higher affinity than the next cation that binds to a bridging metal-bound carboxylate.

Experimental Support. Although Mg^{2+}/Zn^{2+}−carboxylate complexes were used to model mono- and binuclear binding sites herein, the present results seem to have broader implications and cover other bimetallic sites. That a metal-occupied site attenuates the affinity of its neighboring site for another metal cation of similar or reduced Lewis acidity is supported by several experimental observations. For example, a Fe^{2+}-occupied binding site (consisting of 1 His and 3 Glu residues) of the diiron ribonucleotide reductase has been shown to significantly decrease the affinity for both Fe^{2+} and Mn^{2+} of the adjacent binding site (comprising 1 His, 1 Asp, and 2 Glu residues).[20] Other bimetallic centers such as $Mg^{2+}-Mg^{2+}$ in glucose isomerase[9] and $Zn^{2+}-Zn^{2+}$ in D-aminoacylase,[16] metalloaminopeptidases,[13] and some metallo-β-lactamases[24] also possess different metal-binding affinities. It is interesting to note that even the higher affinity metal-binding site in binuclear metallo-β-lactamase from *B. cereus*, consisting of 3 His residues and a (bridging) water/hydroxide ligand, has lower affinity for Zn^{2+} ($K_d = 0.6-1.8$ nM[24]) than the respective mononuclear Zn^{2+}-binding site in carbonic anhydrase II with the same first-shell ligands ($K_d = 1-4$ pM[51]). Thus, some influence of the lower affinity binding site on its higher affinity neighbor in metallo-β-lactamase cannot be ruled out.

Effect on Metal-Binding Selectivity. The decreased metal-binding ability of the "super" ligand and the rigidity/flexibility of the binding pocket have important implications on the metal selectivity of the other binding site in binuclear metalloproteins. As ligand interactions with a strong Lewis acid such as Zn^{2+} are affected by changes in the ligand properties more than those with a weaker Lewis acid such as Mg^{2+}, replacing Mg^{2+} with Zn^{2+} in biMg^{2+} sites is not as favorable as in the respective monoMg^{2+} sites. Rigid Mg^{2+}-binding sites that do not allow the ligands to rearrange upon metal exchange are less vulnerable to substitution by Zn^{2+} (small exchange free energies; Figure 4) than the respective flexible binding sites (more favorable exchange free energies; Figure 5). This implies that rigid $Mg^{2+}-Mg^{2+}$ centers are better protected against substitutions from transition metal dications such as Zn^{2+} than their mononuclear counterparts, whereas *tetracoordinated* Zn^{2+} in solvent exposed trinuclear sites (reverse reaction in Figure 6) appear less specific for Zn^{2+} than their mononuclear counterparts (reverse reaction in Figure 5a).

Biological Implications. What types of advantages could the weak metal binding of one of the sites in binuclear enzymes confer? One plausible advantage for certain enzymes such as xylose isomerase is that the weak metal binding of one of the binuclear sites would enhance the metal cofactor mobility in achieving the transition state.[9] Furthermore, the additional metal center in binuclear Mg^{2+} enzymes may not only contribute to increasing the efficiency of the catalytic process (see Introduction) but it may also play a protective role against unwanted transition metal substitutions. This is especially helpful for a relatively weak Lewis acid like Mg^{2+}, which forms a weaker bond with a given ligand than Zn^{2+} and is therefore easily replaced if the concentrations of the two metal ions were similar. In contrast, additional metal centers in enzymes may facilitate a solvent exposed Zn^{2+} to be replaced by Mg^{2+}, as illustrated in Figure 6. One possible advantage of such promiscuity is that, in case of transition metal deficiency, the protein may easily bind another divalent cation available in the surrounding milieu (for example, Mg^{2+}, which is abundant in the cellular fluids) and utilize it in the enzymatic reaction. Indeed, some transition metal binuclear enzymes (metalloaminopeptidases, enolase, D-hydantionase, glucose (xylose) isomerase, and protein phosphatase 2C) are still active with Mg^{2+} bound to the active site.[7,9,13,52,53]

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Supporting Information Available: Complete ref 32 and percentage frequency distribution of first-shell ligands observed in the PDB structures of proteins containing homobinuclear binding sites (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org

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