Equilibrium Metal Binding of the Translational Activating Protein, COM

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The control of *mom* (COM) protein from bacteriophage Mu, a translational activator of the *mom* gene, is 62 amino acids in length. We have shown previously that COM binds a single Zn(II) ion using four cysteine residues as ligands. COM regulates the translation initiation of the *mom* mRNA in bacteriophage Mu. In this study the metal specificity of COM for Zn(II), Cd(II), and Co(II) was determined using nuclear magnetic resonance and UV/vis spectroscopic methods. The conditional stability constants were obtained and compared to those of other zinc fingers. The results show that the relative metal specificity is quite similar to that of other classical zinc fingers. (Zn(II) ≫ Cd(II) ≫ Co(II), Fe(II)). However, COM shows an unusually high relative affinity for Zn; it binds Zn(II) 100 000-fold more strongly than it binds Co(II). Thus, strong binding is retained at pH 4, where Zn(II) binding is abolished for the other zinc finger binding domains. We speculate that this affinity is important for the physiological function of COM, where the protein may have to compete for a limited pool of "free" zinc in a critical stage in the phage growth cycle.

Introduction

Zinc plays a key role in organizing and stabilizing the structure of many nucleic acid binding proteins.[1−5] These include not only the DNA-binding "zinc fingers" but also the RNA-binding proteins.

The present work examines equilibrium metal binding of the zinc-dependent translational activator protein, control of *mom* (COM).[6a,b] The COM protein controls translation initiation of the *mom* gene of phage Mu by binding to *mom* mRNA and altering the secondary structure in the region containing the ribosome binding site and the start codon. COM binds a single Zn(II) ion, using a Cys4 ligation site.[7]

In an elegant series of papers,[2−5] Berg and co-workers have examined metal binding characteristics of the $\beta_2\alpha$ "classic zinc finger" domain peptides. Their results show a high specificity for Zn(II): $K_{Zn}/K_{Co} \cong 10^4$. This relative specificity can be understood by a crystal field model.[5] The zinc binding constants in the zinc finger peptides examined range from 10^8 to 10^{11}. The stability of metal binding is coupled to the stability for overall folding of the domain structure since metal binding and folding are coupled into a single overall step.[3] Thus, amino acid substitutions that destabilize the domain also destabilize metal binding.[3] Following this analysis, Berg designed a "consensus peptide"[2] which attains maximum stability and an associated maximum binding constant, $K_{Zn} \approx 10^{11}$ M^{-1}.

Given the range of metal ion stabilities within this single $\beta_2\alpha$ domain, it was of interest to investigate the stabilities for metal binding to other, structurally unrelated regulatory zinc proteins that bind RNA or DNA. We report here the (conditional) equilibrium binding constants for COM binding to Zn(II), Cd(II), and Co(II). These values are compared with other zinc nucleic acid binding proteins. Analysis of these binding constants can provide insight into aspects of the folding of this protein.

Experimental Section

Wild-type (WT) COM and two mutants, I22V and C39S,E23Q, were used in this study. The growth, purification, and preparation of the protein samples with Zn(II) and Cd(II) were as described previously.[7] Metal chelate titrations were performed by titrating COM with small aliquots of a primary standard EDTA solution. The titrations were followed by monitoring changes in the one-dimensional (1D) ^{1}H spectrum of COM, which unfolds as the metal is removed. All NMR data were acquired on a Varian VXR spectrometer operating at 500 MHz. The data were processed on an SGI Indigo workstation using FELIX software (Biosym Technologies). From the known Zn(II) binding of EDTA and the relative fractions bound and unbound at a given COM/EDTA ratio, a conditional COM Zn(II) stability constant could be calculated. Conditional stability constants were determined for WT COM at pH 4.0 and 5.0, for the I22V mutant at pH 4.9, and for the E23Q,C39S double mutant at pH 5.0 by using conditional stability constants for EDTA.[8]

Metal ion concentrations were determined by titration with EDTA using Eriochrome Black T as an end-point indicator. Co(II)COM was prepared by adding stoichiometric aliquots of a cobalt(II) nitrate solution

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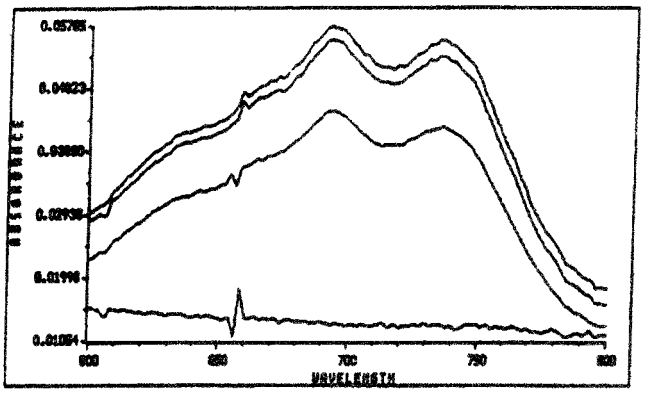

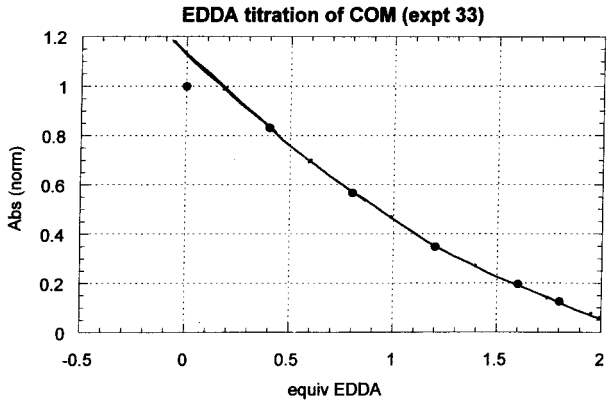

Figure 1. (a) Typical absorption spectra of E23Q,C39S COM (1×10^{-4} M) titrated with cobalt(II) nitrate at pH 5.4. Final Co(II) concentration is 5×10^{-4} M. (b) Back-titration of Co(II)COM with ethylenediaminediacetic acid (EDDA) pH 4.9, [COM] = 1.6×10^{-3} M.

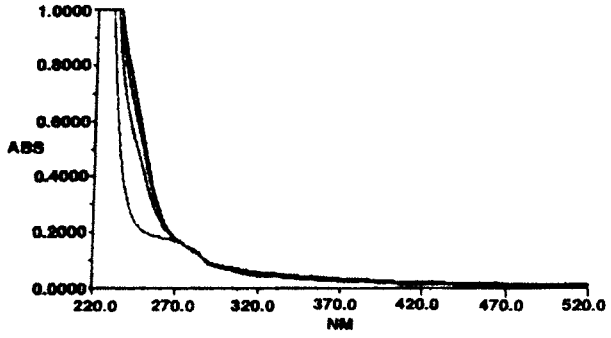

Figure 2. Titration of wild-type COM with Cd(II) chloride followed by the UV band at 250 nm. The spectra were taken at Cd(II)/COM ratios of 0, 0.5, 1, and 2.

to a buffered solution of apoCOM. The reaction of C39S,E23Q COM at pH 5.4 with Co(II) was monitored optically at 695 nm as shown in Figure 1. The stability constant for Cd(II) was determined by direct titration at 250 nm, in a 1-cm path length cell (Figure 2). The reaction of Cd(II)COM with Zn(II) at pH 4.0 was monitored by back-titrating Cd(II)COM with Zn(II). Cd(II)COM was prepared by adding stoichiometric amounts of cadmium chloride (Baker) to apoCOM. Zinc chloride was used as the titrant.

Results

Equilibrium Zinc Binding. It was previously shown that COM protein spontaneously folds in the presence of Zn(II).[7] A representative chelation titration of Zn(II)COM followed by ^{1}H NMR is shown in Figure 3. An expansion of the aromatic and downfield shifted α regions is shown. Upon addition of

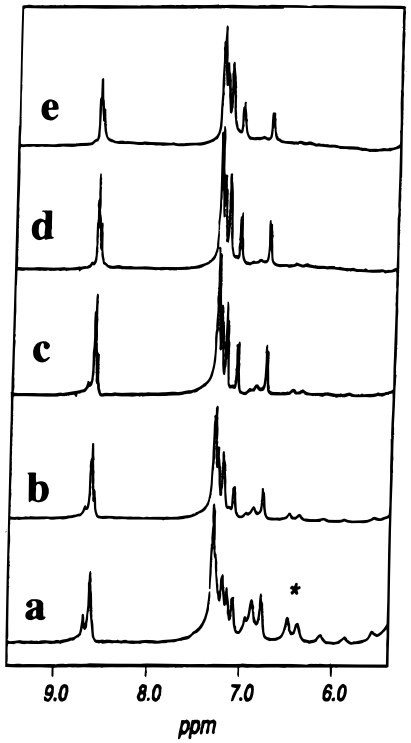

Figure 3. Aromatic and downfield α regions of ^{1}H NMR spectra of 0.5 mM ZnCOM (pH 4.0 in ^2H_2O) titrated with EDTA. The asterisk (*) denotes peaks from a phenylalanine residue indicative of the folded state. The spectra are labeled as follows: (a) 0 mM EDTA, (b) 1 mM EDTA, (c) 2 mM EDTA, (d) 3 mM EDTA, (e) 5 mM EDTA.

Table 1. Conditional Stability Constants of ZnCOM Obtained by Ligand Competition

COM	pH	log K^{app}
WT	4.0	7.6
WT	5.0	9.6
E23Q,C39S	5.0	7
I22V	4.9	9.5

chelator, the peaks that are hallmarks of the folded state decreased in intensity. This is indicative of protein unfolding due to the removal of Zn(II). The conditional stability constant, K_{Zn}^{app} for COM has been determined from chelate competition titrations and is given in Table 1. The binding constants for two site-specific mutants chosen for their favorable NMR properties were also examined. The zinc-binding affinity, K_{COMZn}^{app} of the COM proteins follows the order C39S,E23Q < WT ≈ I22V (Table 1). Since metal binding is coupled to proton release from the ligand atoms, the apparent binding constant is strongly influenced by pH. From the conditional constants at different pH values, the pH dependence of zinc binding can be assessed, along with the number of protons released on binding

$$\mathrm{Zn(II)} + \mathrm{COM\ H^+}_n \overset{K^{app}}{\rightleftharpoons} \mathrm{ZnCOM} + n\mathrm{H^+} \qquad (1)$$

EDTA, itself, is diprotonated at pH 5 and must release two protons on binding to Zn(II). Thus, pH variations within the range best studied by NMR (pH 4−6) will vary the conditional constant, K_{EDTA}^{app}, by 2 orders of magnitude for every unit pH change. The relevant equilibrium is

$$(n - 2)\mathrm{H^+} + \mathrm{ZnCOM} + \mathrm{H_2EDTA} \rightleftharpoons \mathrm{ZnEDTA} + \mathrm{COM\ H^+}_n \qquad (2)$$

Any additional pH dependence of K_{COM} beyond that of K_{EDTA} reflects additional changes in the proton stoichiometry of COM.

Table 2. Comparative Zinc Stability Constants (log *K*) for COM, COM Variants, and Some Other Zinc Finger Peptides at pH 7.0

zinc finger peptides	log *K*	zinc finger peptides	log *K*
COM (WT)[a]	13.6	CP-1[d]	12.3
COM (I22V)[a]	13.5	MoMuLV[e]	11.30
COM (E23Q,C39S)[a]	11.0	NC_1[f]	12
Sp1−3[b]	9.6	NC_2[f]	11
TFIIIA[c]	8.6		

[a] Extrapolated to pH 7 from the pH-dependent binding constants in Table 1. [b] D. Wilcox, personal communication. [c] Berg and Merkle, 1989. [d] Krizek et al., 1991 (ref 5). [e] Mely et al., 1996 (ref 11). [f] McLendon et al., 1996, unpublished results.

In the calculation of the apparent binding constants, the protons released by EDTA are already corrected, thereby accounting for two protons. The change in binding constant between pH 4 and 5 requires that two additional protons be released upon metal binding. Thus, four protons are released upon Zn(II) binding, consistent with four fully deprotonated cysteinate ligands. These values, corrected for pH, are compared with the available data for other zinc proteins in Table 2.

The zinc dissociation constants for the synthetic zinc finger peptides, Sp1, TFIIIA, and CP-1 at pH 7.0 are 2.8×10^{-10}, 2.8×10^{-9}, and 5.7×10^{-12}, respectively. Both Sp1−3 and TFIIIA peptides are zinc finger domains from naturally occurring transcription factors.[9,10] By contrast, CP-1 is a consensus zinc finger peptide in which the amino acid sequence has been optimized by using a 131-zinc finger domain database to select the residue with the highest frequency at each position. The 3 orders of magnitude difference between the dissociation constants for the zinc finger peptides was explained by assuming that the residues that occur with high frequency are those which the stabilize structure and in turn stabilize binding. After correcting for pH, we observed that the binding constant for ZnCOM is significantly larger than that observed for other zinc finger domains previously studied. This suggests that COM could successfully compete with endogenous zinc-binding proteins for the free zinc ions in the intracellular pool. Since COM is produced in the late stages of phage infection, for timely expression of its function in activating translation of the *mom* gene, successful competition for Zn(II) may be critical to phage replication. Thus the very high zinc binding constant is consistent with the functional role of COM.

Coupling of Metal Binding and Protein Folding. The NMR studies clearly show that, as for other zinc-binding proteins, the addition of a single metal ion converts COM from a random coil to a fully folded protein. The metal-binding and protein-folding steps are all formally thermodynamically coupled.[3] Thus, the equilibrium binding

$$\text{apoCOM (unfolded)} + \text{Zn(II)} \rightleftharpoons \text{Zn(II)COM (folded)} \quad (3)$$

provides a measure of relative protein stability for various amino acid replacements which are not directly involved as ligands in Zn(II) binding.

Consider two site-directed mutants, I22V and C39S,E23Q, which were prepared to minimize aggregation in NMR structural experiments. The overall folding appears constant in these variants, as both variants have virtually identical 1D ^{1}H NMR spectra as shown in Figure 4**.** This structural similarity is further confirmed by the similar pattern of cross-peaks in the two-dimensional 2D NOESY spectra of the WT and mutant proteins. Furthermore the local Cys4 metal-binding site in these variants is unchanged, as judged by the invariance of the sensitive ^{113}Cd frequency in the Cd(II)-substituted proteins shown in Figure 5. Thus, at the level of sensitivity of these NMR structural probes no differences in the structure are observed. The metal-binding affinity, K_{zn}, of I22V is indistinguishable from WT, while for E23Q,C39S, K_{zn} decreases by over 100-fold. Since neither overall structure nor local metal structure changes in the E23Q variant, we conclude that the difference in metal binding reflects a difference in the overall conformational stability of the E23Q,-C39S variant. This stability effect might reflect a difference in the relative stability of either the folded or unfolded states. Note that even if the overall folding pattern as judged by the NOE intensities is largely invariant, the forces which determine that pattern depend on the sequence, so that a significant stability change can accompany structural perturbation too small to detect by NMR. Consistent with this explanation, the apparent melting temperature of E23Q,C39S is decreased ($T_m \approx 60$ °C) relative to the WT COM ($T_m \approx 75$ °C). In this way, metal-binding measurements can provide sensitive measurements of protein-folding energetics. A similar argument has been used by Berg[3] to measure energetic parameters for β sheet formation in a classical $\beta_2\alpha$ motif.

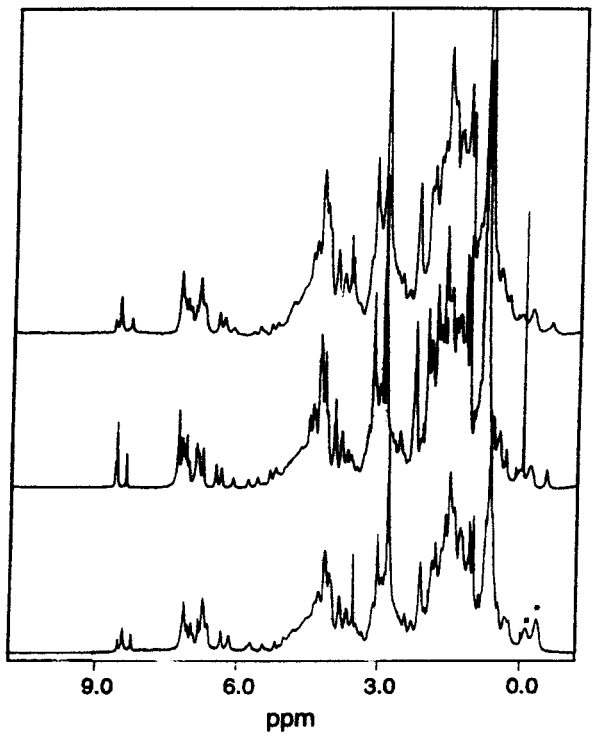

Figure 4. ^{1}H NMR spectra of (top) wild-type COM at pH 5.0, (middle) E23Q,C39S COM at pH 4.3, and (bottom) I22V COM at pH 4.2 in the presence of Zn(II) at 293 K in ^2H_2O.

Metal Ion Dependence. NMR experiments demonstrate that COM can specifically bind to and fold around Co(II) and Cd(II) (Figure 6). The work of Berg and colleagues[5] showed that classical zinc finger peptides preferentially bound zinc over divalent first-row transition metals. For comparison, conditional binding constants for Co(II) and Cd(II) derivatives of COM have been determined, using direct optical methods.

The COM protein binds Cd(II) with a similar but lower affinity than it binds Zn(II). At pH 4, the conditional stability constant for Cd(II) is an order of magnitude lower than for

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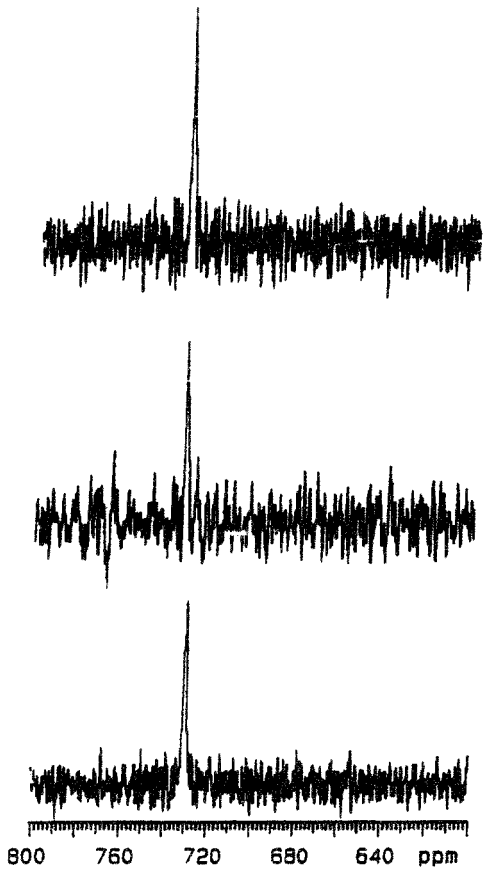

Figure 5. ^{113}Cd NMR (110.9 MHz) spectra of (top) 0.8 mM I22V COM at pH 4.3, (middle) 1.0 mM E23Q,C39S COM at pH 4.2, and (bottom) 2.0 mM wild-type COM at pH 4.3. In all, 120 000, 57 000, and 46 000 transients were acquired with spectral widths of 22.3 kHz, 31.0 kHz, and 31.0 kHz, respectively. Samples contained stoichiometric amounts of Cd(II).

Zn(II), log $K_{Cd}^{app} \approx 7$. This result comes somewhat as a surprise in comparison to a ligand variation study of other zinc finger peptides.[5] In the work by Krizek et al.,[5] the histidine residues of the Cys_2His_2 coordination site were replaced with cysteine residues to produce Cys_3His and Cys_4 metal sites. The zinc finger peptides showed an increased affinity for Cd(II) with increasing cysteinate content. Consistent with hard−soft acid−base theory, the Cys_4 zinc finger peptide displayed a preference for Cd(II) over Zn(II), with log K_{metal}^{app} of 13 and 12, respectively. This trend was not observed for COM. Although COM has a Cys4 metal site, nonetheless, COM shows a 10-fold preference for binding Zn(II) over Cd(II). The preference for Zn(II) may be a manifestation of the metal ion size, i.e., the larger ion size of Cd(II) could perturb the tertiary structure of COM, resulting in a lower affinity, following the thermodynamic coupling discussed above.

In contrast to that of Zn(II), the addition of more than 5 equiv of Cd(II) resulted in the denaturation of COM. Cd(II)-induced denaturation is shown quantitatively in Figure 7 for COM C39S,E23Q. In excess of 5 equiv of Cd(II), ^{1}H chemical shift dispersion is consistent with COM unfolding. Metal-induced denaturation was not observed when zinc was in excess. This suggests that Cd(II) binding at secondary sites disrupts the tertiary structure of COM. This may be a common mechanism for heavy-metal-induced protein unfolding.

In contrast to the results for Zn(II) and Cd(II), COM does not fold in the presence of 10^{-3} M Co(II) or Fe(II) at pH 4. This negative result requires a Co(II) binding constant of $<10^3$ M^{-1} at pH 4. However, COM does bind and fold around Co(II) at pH 5.4 with an affinity of 10^6 M^{-1}. At this pH, the pH-corrected zinc binding constant is $K_{Zn} \approx 10^{11}$ M^{-1}. The low affinity for Co(II) is consistent with that of other zinc finger peptides as explained by Berg.[5]

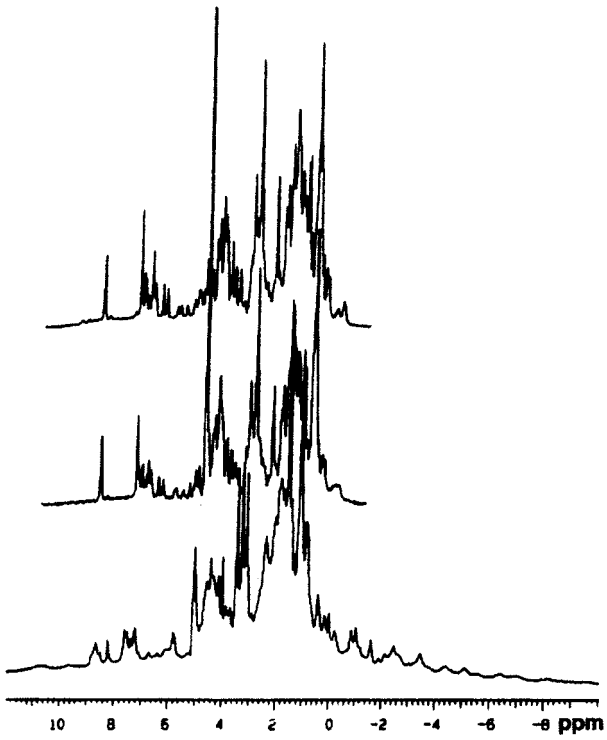

Figure 6. ^{1}H NMR spectra of COM substituted (top) with Zn(II) at pH 4.2, (middle) with Cd(II) at pH 4.5, and (bottom) with Co(II) at pH 5.4 in ^2H_2O at 293 K.

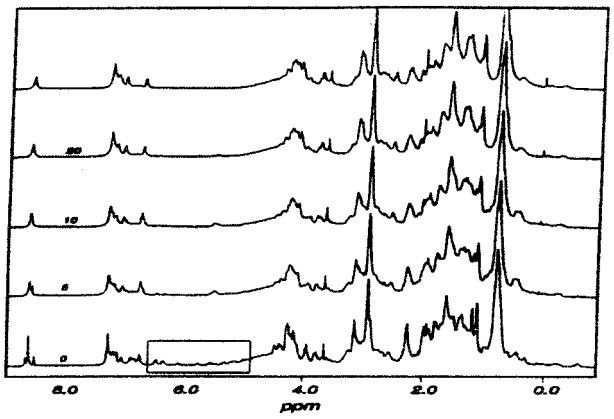

Figure 7. ^{1}H NMR spectra of 1.0 mM E23Q,C39S COM titrated with Cd(II) at pH 4.2 in ^2H_2O at 293 K. The boxed region shows the downfield shifted Cα protons that are characteristic of the formation of secondary structure that is induced by metal ion binding.

Thus, the metal specificity of the COM protein (Zn(II) ≥ Cd(II) ≫ Co(II), Fe(II)) is quite similar to that of classical zinc finger peptides. COM, like any ligand, follows ligand field stabilization energy characteristics and clearly shows a preference for zinc over open shell transition metals. However, the unusually high differential discrimination of COM for Zn(II) over other metals is more surprising.

In summary, the zinc-dependent translational activator protein, COM, shows a uniquely high binding affinity for Zn(II) ions. Thus, strong binding is retained even at pH 4, where Zn binding is abolished for the zinc finger binding domains. We suggest

that such high binding allows the phage to compete for a limited pool of "free" zinc in a critical stage during the phage development cycle. The relative affinity (Zn > Cd ≫ Co) is qualitatively similar to that of other tetrahedral metal binding sites, but shows an unusually high relative affinity for Zn:K(Zn)/K(Co) > 100 000.

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