# Extreme Zinc-Binding Thermodynamics of the Metal Sensor/Regulator Protein, ZntR

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Cells accumulate Zn(II) and employ this metal ion in a wide range of enzyme active sites. Working models for zinc homeostasis, the cumulative processes that maintain the cellular zinc quota within an optimal range, have benefited from recent discoveries of zinc uptake, export, sequestration, and sensing proteins.[1] A key feature of most zinc homeostasis models is the idea that unbound ion accumulates in a so-called "free zinc pool" in the cytosol; however, the magnitude of this pool has proved difficult to evaluate. Here we address this issue by developing a method to directly determine the zinc-binding thermodynamics of ZntR, an intracellular zinc metalloregulatory protein from *Escherichia coli*. While coupled activity assays indicate that ZntR has a femtomolar sensitivity to Zn(II),[2] the direct binding constant is not known. We now show that ZntR exhibits the highest equilibrium Zn(II) binding constant measured for a native zinc-protein to date, although some zinc-finger peptides approach this affinity. The unusual thermodynamic affinity of zinc for this sensor requires a reevaluation of some tenets of cellular zinc homeostasis mechanisms.

ZntR, a homologue of MerR, is a dimeric metalloregulatory protein that functions as a Zn(II)-responsive genetic switch.[3] As zinc concentrations outside the cell rise, ZntR turns on production of proteins that remove any excess zinc ions from the cell. Specifically, the Zn(II) bound form of ZntR, but not the apo-form, stimulates transcription of *zntA*, a gene encoding a zinc efflux pump.[3] ZntR regulates transcription by a metal-induced DNA-distortion mechanism[3c] in which zinc-induced structural changes in the ZntR protein lead to restructuring of the bound DNA.

Metal occupancy of ZntR was monitored by changes in tyrosine fluorescence. ZntR has no tryptophan but five tyrosine residues, one of which is located near the putative zinc-binding site while others are near the helix-turn-helix DNA-binding domain. Excitation of apo-ZntR at 278 nm yields a maximum fluorescence emission at 303 nm, characteristic of tyrosine and not tyrosinate.[4] In the presence of excess zinc, the emission intensity increases by 2.6-fold with no change in the spectral shape, indicating a Zn(II)-induced change in protein conformation that alters the local environment of one or more tyrosines (Figure 1a).

The activity of ZntR is very sensitive to free zinc, therefore a strong zinc chelator is required to see the lowest levels of transcript in the absence of zinc ion.[3c] To directly measure the extremely high zinc affinity under such conditions, we employed a metal-buffered solution method developed for characterization of metal−chelator interactions.[5] *N*,*N*,*N*′,*N*′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) is a zinc chelator with a zinc dissociation constant of $9.2 \times 10^{-14}$ M at pH 7.0,[6] and can be used to buffer the free zinc concentrations between $10^{-16}$ and $10^{-14}$ M. The concentrations of free zinc were calculated using the program SPE based on the published p$K_a$ and log$K_{Zn-TPEN}$ values of TPEN.[6b] The results of a typical fluorescence experiment at pH 7.0 are shown in Figure 1a. Several models for the apparent zinc dissociation constant were fit to the observed binding isotherm. The best fit was obtained for noncooperative 1:1 binding of Zn(II) to the ZntR dimer according to eq 1, with a dissociation constant of log $K_d = -14.8 \pm 0.03$ (Figure 1b):

$$F = (F_{min} K_d + F_{max} Z)/(K_d + Z) \quad (1)$$

where $F$ and $Z$ represent the fluorescence intensity and the free zinc concentration, respectively. Although previous results indicate that the analogous MerR protein binds one Hg(II) per dimer, the ZntR dimer can bind two zinc ions per dimer.[3c] Therefore two other zinc-binding models can be considered which are consistent with the noncooperative binding isotherm (Figure 1b) where the value for a refined Hill coefficient is 1.00: (1) zinc binding to two equivalent sites in the ZntR dimer equally enhances tyrosine fluorescence intensity; or (2) the ZntR dimer has two inequivalent

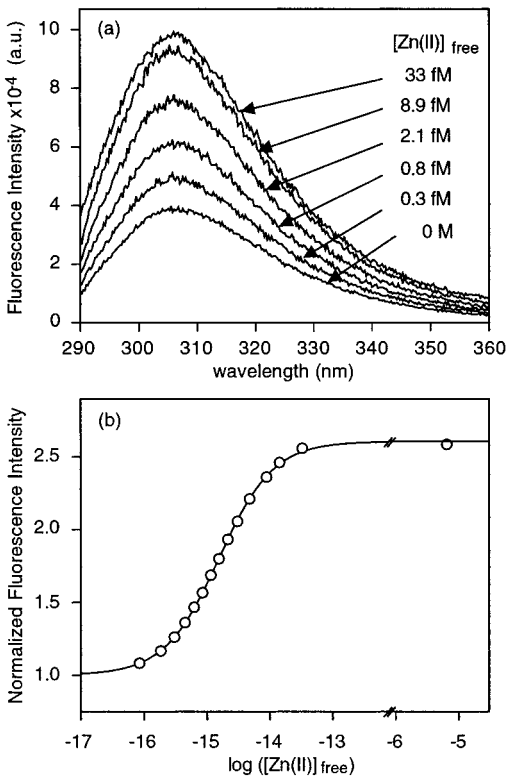


**Figure 1.** Fluorescence emission spectra (a) and normalized fluorescence intensity (b) in the presence of 10.0 $\mu$M ZntR dimer and a range of free zinc ion concentrations, monitered with excitation wavelengths of 278 nm. Conditions: 1.0 mM TPEN, 20 mM Tris-Bis Tris buffer (pH 7.0, $I$ = 0.1 M (NaCl)) at 25 °C. The solid line shows the fitted curve for eq 1 with log $K_d = -14.8$ ($r^2 = 0.999$).

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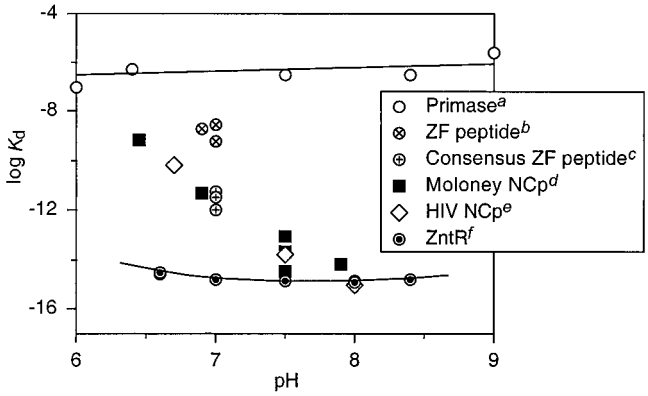


**Figure 2.** pH dependence of zinc dissociation constants for ZntR and selected zinc finger peptides [footnotes: (a) Griep et al. 1997; (b) Witkowski et al. 1998; (c) Krizek et al. 1993 and Berg and Merkle 1989; (d) Mely et al. 1991; (e) Payne et al. 1999, McLendon et al. 1999, and Mely et al. 1996; and (f) this work].

zinc binding sites with one of them having no effect on the fluorescence intensity. In the second model, the two zinc ions must bind to the ZntR protein noncooperatively or with very different affinities given the shape of the observed isotherm (Figure 1b). In either case, the log $K_d$ obtained correlates with the switching event observed in independent transcription assays: the free zinc concentration for half-maximum transcriptional stimulation is $1.1 \times 10^{-15}$ M.[2] We conclude that the functionally relevant zinc binding event gives rise to the fluorescence enhancement.

To test whether the free energy of metal binding to ZntR is coupled to the free energy of DNA binding, the zinc affinity of ZntR was determined in the presence of a 29 base pair double-stranded DNA fragment from the *zntA* promoter region.[7] At pH 8.0, the Zn(II) dissociation constant for the ZntR/DNA complex is log $K_d = -15.2$. The similarity of this value in the presence and absence of DNA ($-15.2$ vs $-14.9$) is consistent with the broader mechanism seen with other MerR family members, all of which bind tightly to DNA regardless of the presence of a bound metal ion.[3c]

The high affinity of ZntR for Zn(II) is not significantly affected by pH changes within a physiological range from 6.6 to 8.4 (Figure 2).[8] In contrast, the Zn(II) affinity of zinc finger peptides generally shows a large pH dependence in this range. Zinc binding of these zinc finger peptides is coupled with folding of the peptides, which are typically 20−30 amino acids.[9] ZntR and primase conserve the zinc-binding sites in the folded protein interior without the zinc ion.[8d]

The zinc affinity of ZntR is significantly higher than that reported for other native zinc proteins and most peptides characterized to date (Table 1).[10] ZntR protein can successfully compete thermodynamically with endogenous zinc-binding apo-proteins as well as other metal-binding apo-proteins for the free zinc ions within the *E. coli* cell. In terms of current zinc homeostasis models, an ensemble of cellular zinc-export systems maintains the intracellular free zinc concentration at a femtomolar level. Considering the small volume of the cell (ca. $2 \times 10^{-15}$ L) and the extraordinary affinity of this Zn(II) sensor, the hypothetical "free zinc pool" invoked in most models for zinc homeostasis leads to a physiological paradox: the concentration of free Zn(II) that turns on the efflux system is 6 orders of magnitude lower than the concentration of one free zinc in the cell.[2] Furthermore, if ZntR is saturated at $[Zn(II)]_{free} < 10^{-13}$ M and then turns on expression of zinc efflux pumps, the question of how lower affinity proteins are "loaded" with Zn(II) becomes an issue. These paradoxes are resolved if ZntR monitors the zinc quota of the cell not by sensing changes in intracellular $[Zn(II)]_{free}$ but rather by undergoing rapid ligand exchange reactions with a series of Zn-binding factors, including putative Zn(II) chaperones. In contrast, most zinc enzymes exhibit very slow off rates for Zn(II). Thus, a corollary of this model is that Zn(II) transfer, insertion, and occupancy processes are under kinetic control within the cell. A reevaluation of the zinc affinity of SmtB (Table 1), a zinc metalloregulatory protein in *Synechococcus* PCC7942,[10i] supports our hypothesis that cells do not operate with any significant pool of free zinc and that zinc homeostasis in the cell is under kinetic control.

**Table 1.** Comparative Zinc Affinities of ZntR and Other Native Zinc Proteins

| protein | log $K_d$ | pH | ref |
|---|---|---|---|
| ZntR | −14.8 | 7.0 | this work |
| | −14.9 | 8.0 | this work |
| ZntR/dsDNA | −15.2 | 8.0 | this work |
| metallothionein | −11.3 | 7.4 | 10h |
| SmtB | <−11 | 7.4 | 10i |
| phosphatase | −7.5 | 6.5 | 10b |
| ATCase | −12.1 | 7.0 | 10f |
| aldolase | −6.1 | 7.5 | 10g |
| glycoxalase | −10.9 | 8.5 | 10c |
| carboxypeptidase A | −7.3 | 8.0 | 10d |
| thermolysin | −12.6 | 7.5 | 10e |
| carbonic anhydrase | −11.4 | 7.0 | 10a |

In conclusion, this TPEN-based Zn(II) buffering method is general and can be used with proteins that bind Zn(II) more tightly than EDTA, i.e., when log $K_d$ is as low as $-14$ to $-16$. This approach also uses changes in fluorescence of intrinsic Tyr as opposed to the less abundant Trp. The nature of the coordination environment in ZntR that allows both a high affinity and apparently rapid kinetics is currently under study.

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