Metal Ion Affinities of the Zinc Finger Domains of the Metal Responsive Element-Binding Transcription Factor-1 (MTF1)†

Anthony L. Guerrerio and Jeremy M. Berg*,‡

Department of Biophysics and Biophysical Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received October 12, 2003; Revised Manuscript Received February 8, 2004

ABSTRACT: Metal response element (MRE) binding transcription factor-1 (MTF1) is a six Cys_2His_2 zinc finger-containing transcription factor required for basal and zinc-induced transcription of metallothionein genes. The cobalt(II) and zinc(II) affinities of a protein fragment comprising the six zinc finger domains have been examined to reveal apparent dissociation constants (for the six domains collectively) of 0.5 ± 0.2 μM for cobalt(II) and 31 ± 14 pM for zinc(II). Two approaches have been used to determine the metal ion affinities of the individual domains. First, the six domains have been examined as single domain peptides revealing dissociation constants ranging from 0.3 to 1.7 μM for cobalt(II). The domains fall into two sets with peptides corresponding to domains 2, 3, and 4 showing relatively high affinity (K_d(Co(II)) 0.3−0.5 μM) and peptides corresponding to domains 1, 5, and 6 showing lower affinity (K_d(Co(II)) 1.6−1.7 μM). Second, we examined the affinity of each domain in the context of the six zinc finger domain protein by individually mutating one metal-binding His residue to Cys to allow independent monitoring of the cobalt(II) occupancy of each site. The affinity of each domain was higher in this context than as a single domain peptide with affinities (corrected for the effect of the mutation) ranging from 0.02 to 0.5 μM. The increase in affinity for the individual domains ranged from factors of 1.1 to 20. The order of affinities (from higher to lowest) was observed to be $4 > 2 \approx 5 > 6 \approx 3 \approx 1$. These results reveal that none of the Cys_2His_2 zinc finger domains of MTF1 have dramatically low metal ion affinities, certainly none low enough to respond to changes in free zinc ion concentrations in the micromolar range. Nonetheless, the metal ion affinities of some domains do differ by a factor of 25 with domains at both the amino- and carboxyl-termini showing lower intrinsic affinities for metal ions than the central domains.

Metal ion homeostasis requires that cells maintain the available concentration of a particular ion within a specified range. When the concentration falls below this range, the cell must increase its uptake and/or release the ion from internal stores; when the concentration rises above the homeostatic limit, the cell must eliminate and/or sequester the ions. These processes require mechanisms to sense the concentration of the ion and to transduce this information into an appropriate action. Therefore, it is not surprising that systems allowing for the regulation of gene expression by metal ions exist in organisms ranging from bacteria to mammals (*1*).

One class of proteins that plays an important role in metal ion regulation in eukaryotic cells is the metallothioneins (MTs).[1] Metallothioneins are cysteine-rich, low molecular weight proteins that bind many metal ions, including Zn(II), Cd(II), Cu(II), Hg(II), and Co(II) (*2*−*5*). These proteins function in metal homeostasis, protection from oxidative stress, and metal ion detoxification. MT synthesis is controlled at the level of transcription and can be induced by multiple stimuli including exposure to Zn(II), Cd(II), paraquat, diethylmaleate, and cytokines (*6*−*9*). Transcriptional activation by metal ions is mediated by a cis-acting metal-responsive element (MRE), a 12-base pair loosely conserved sequence present in multiple copies in the promoter region of MT genes (*10*, *11*). The MRE is recognized by a trans-activating factor, termed MRE-binding transcription factor-1 (MTF1). MTF1 was first cloned from mice (mMTF1) and is a 675 amino acid, 72 kDa protein with six TFIIIA-like Cys_2His_2 zinc fingers in the N-terminal half, as shown schematically in Figure 1 (*12*).

MTF1 has also been cloned from humans (*13*, *14*), *Takifugu rubripes* (*15*), and *Drosophila melanogaster* (*16*), and all sequences are similar with the zinc finger domains being the most highly conserved region. A variety of experiments have demonstrated that mMTF1 is essential for basal- and metal-induced gene expression of MRE-containing genes and that treatment of mMTF1 with Zn(II) at appropriate levels enhances its DNA-binding activity (*17*). Through the use of electrophoretic mobility shift assays (EMSA) using nuclear extracts from murine Hepa cells, it has been shown

† This work was supported by a grant from the National Institute of General Medical Sciences to J.M.B. A.L.G. was supported by the Medical Scientist Training Program funded by the National Institutes of Health.

* Corresponding author. Phone: (301) 594-2702; e-mail: bergj@mail.nih.gov.

‡ Present address: National Institute of General Medical Sciences, 45 Center Dr., Bethesda, MD 20892.

[1] Abbreviations: MTF1, metal responsive element-binding transcription factor-1; MT, metallothionein; mMTF1, murine metal responsive element-binding transcription factor-1; hMTF1, human metal responsive element-binding transcription factor-1; HPLC, high performance liquid chromatography; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

10.1021/bi0358418 CCC: $27.50
Published on Web 04/16/2004

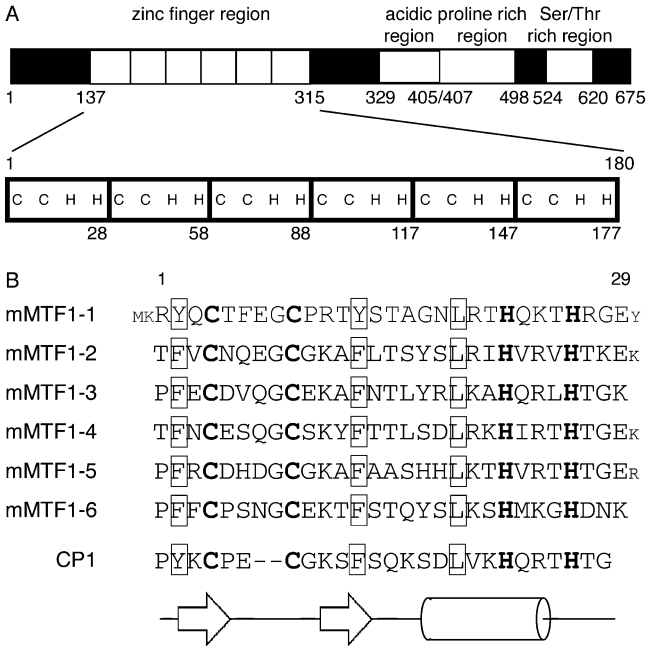

FIGURE 1: Structure of mMTF1. (A) Schematic of mMTF1 showing the position of the zinc finger domains. (B) Sequence of the six zinc finger domain construct mMTF1-ZF corresponding to residues 137−315 of mMTF1 plus the initiating methionine. Amino acids are renumbered 1−180. The sequences that correspond to the six single domain zinc finger peptides are shown in the larger font. Amino acids at the start of the protein or in the linker regions that are not included in synthetic peptides are in a smaller font. Metal binding residues are in bold. Hydrophobic residues characteristic of this class of zinc finger domains are boxed. The sequence of the prototypical zinc finger peptide CP1 is also shown as is the schematic secondary structure characteristic of this class of zinc finger domains.

that mMTF1 binding of the MRE increases 10-fold after incubating the cells in media plus 100 μM zinc for 30 min (*18*). Binding was also evident in whole cell extracts from cultures treated with 90 μM zinc. Incubation of this extract in low zinc buffer for 40 min at 37 °C resulted in loss of MRE binding; binding was restored, however, after the addition of zinc to 30 μM and incubation at 37 °C. Incubation of whole cell extracts with 0.5 mM EDTA abolished binding after 40 min. MRE binding of mMTF1 synthesized in vitro in a coupled transcription and translation assay (buffer approximately 20 μM total zinc) was negligible until total zinc concentration was adjusted to 30 μM. MRE binding of recombinant mMTF1 recapitulated the binding properties of native mMTF1 in that no binding was observed after incubation at 37 °C in 0.6 μM zinc solution, but significant binding was restored after incubation with zinc at 30 μM. On the basis of these experimental results, the DNA-binding activation of mMTF1 was estimated to be half-maximal at a total zinc concentration of 2−3 μM (*19*). Similar results could be obtained with deletion mutants of mMTF1 containing the N-terminus through the zinc fingers, or the zinc fingers through the C-terminus (*18*), implicating the zinc finger domains themselves in zinc responsiveness.

Two models to explain zinc regulation by MTF1 have been proposed. According to the first model, proposed by Westin and Schaffner (*20*), all the Cys_2His_2 zinc finger domains of MTF1 have an intrinsically low affinity for zinc. The protein, therefore, binds zinc and folds into an appropriate structure only at high zinc concentrations. A second related model suggests that the individual fingers of MTF1 have different affinities for metal ions, with the high affinity sites serving a structural role and the lower affinity sites serving a regulatory role (*21*).

To address these models and to identify candidates for the regulatory zinc finger domains, several studies have probed the properties of the individual zinc fingers of MTF1. A form of human MTF1 (hMTF1-ZF) consisting of only the six zinc fingers was purified and found to contain 5.5 Zn(II) per protein or, if dialyzed against mag-fura-2 (K_a(Zn(II)) = 5×10^7 M^{-1}), 3.5 Zn(II)/protein (*21*). In both cases, there were approximately 12 reactive thiols, suggesting that cysteine oxidation was not responsible for loss of zinc binding. Treatment with EDTA (K_a(Zn(II)) $\approx$ 10^{12} M^{-1}) stripped away all zinc ions leading to an apoprotein with low solubility. hMTF1-ZF showed high affinity MRE binding whether there were 3.5 Zn(II) per protein, 5.5 Zn(II) per protein, or 3.5 Zn(II) per protein plus 3 equiv of zinc(II) (*21*). Additionally, the kinetics of air oxidation of the thiols in the 5.5 Zn(II) protein preparation exhibited two distinct phases that differed in rate by a factor of approximately 50 and possessed amplitudes of approximately six thiols each. Limited trypsinolysis of hMTF1-ZF in the presence of an MRE-containing double-stranded oligonucleotide produced a single stable proteolytic fragment consisting of zinc finger domains 1−4 (*22*). Investigation of the DNA-binding properties of this fragment showed approximately 40-fold weaker binding to DNA than intact hMTF1-ZF, while a fragment consisting of zinc finger domains 1−3 showed approximately 700-fold weaker binding. The reactivity of cysteines in hMTF1-ZF in response to pulsed alkylation was found to be F5 > F6 $\gg$ F1 > F2 $\approx$ F3 $\approx$ F4 (*23*). Binding of hMTF1 to an MRE-containing double-stranded oligonucleotide led to increased protection of all thiols to within the same order of magnitude, although domains 5 and 6 were still most reactive. Interestingly, zinc finger domain 1 became the least reactive. Deletion of zinc finger domain 1 from the hMTF1-ZF protein led to poor MRE binding and increased reactivity of the thiols of zinc finger domain 4 (*23*). NMR studies of a peptide consisting of fingers 4−6 from hMTF1 revealed that finger 5 demonstrated exchange broadening of its resonances at saturating zinc levels (*24*). These result have led some authors to conclude that zinc finger domains 5 and 6 bind zinc weakly and are, therefore, responsible for zinc-induced activation of DNA binding (*22*).

Several other studies have failed to support a role for zinc finger domains 5 and 6 as zinc sensors. Expression of mMTF1 in yeast led to zinc-induced expression of an MRE driven reporter, but deletion of zinc finger domains 5 and 6 did not diminish the in vivo zinc induction (*25*). Furthermore, mutation of the second cysteine to a tyrosine in zinc finger domains 1, 2, 3, or 4 resulted in decreased transcription form a reporter construct as well as decreased DNA binding. This same cysteine to tyrosine mutation in fingers 5 or 6 had no effect, suggesting these two fingers are not primarily responsible for the metal regulation of mMTF1 binding (*26*).

Other groups have suggested that zinc finger domain 1 is responsible for the metal regulation of MTF1 (*27*). These studies utilized chimeric proteins and have provided further support to the hypothesis that the zinc finger domains are responsible for the zinc-induced binding of MTF1 to the

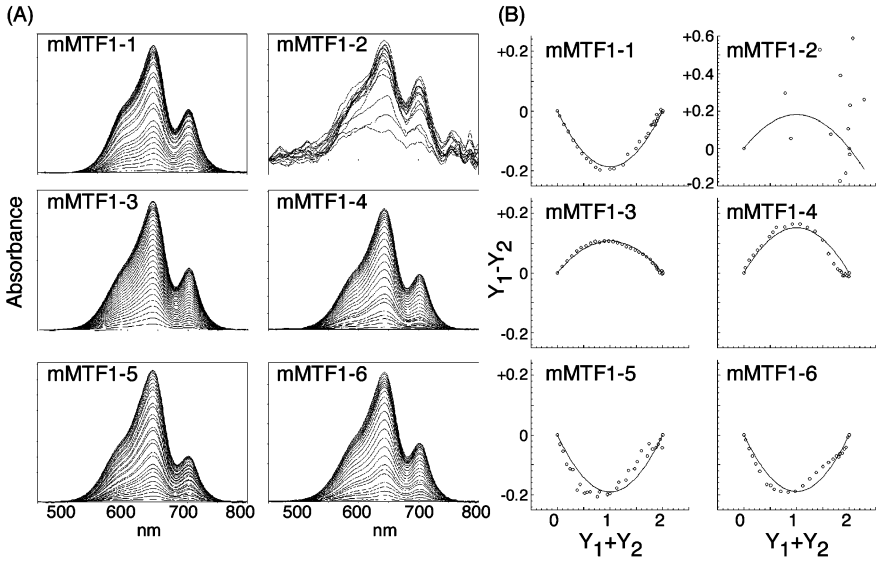

FIGURE 2: Analysis of cobalt(II) binding by single zinc finger peptides. (A) Spectra from cotitrations of single zinc finger peptides with mMTF1−3(H26C) as a common internal standard. (B) Fractional saturation analysis plots of the data from panel A. Upward curvature in this plot indicates that the peptide under study binds cobalt(II) more tightly than does the internal standard.

MREs. Swapping all the zinc finger domains of Sp1 and mMTF1 led to an Sp1-mMTF1−Sp1 chimeric protein that appeared to have zinc-inducible binding to the MRE in an in vitro EMSA assay, although an mMTF1−Sp1-mMTF1 chimeric protein did not. The Sp1-mMTF1−Sp1 protein, however, did not show zinc-inducible MRE-dependent transcription from a reporter construct when transfected into cells. Deletion mutant analysis showed that a form of mMTF1 missing the fifth and sixth zinc finger domains had DNA binding similar to full length mMTF1, while a form lacking finger 1 from mMTF1 demonstrated constitutive DNA binding. Deletion of zinc finger domains 1 and 2 from mMTF1 led to poor DNA binding, and deletion of fingers 1−3 abolished DNA binding. Most surprisingly, placing zinc finger domain 1 from mMTF1 in front of the three zinc finger domains in Sp1 led to zinc-inducible DNA binding of the Sp1 target. Again, however, this chimeric protein also did not produce zinc-inducible expression from a reporter construct in vivo.

Taken together, these studies support the notion that the zinc fingers themselves are involved in the zinc-sensing role of the MTF1 proteins. However, it is not clear which domain or domains function as zinc sensors. An additional complication relates to the concentration range over which MTF1 responds to zinc. As noted previously, several studies have shown responsiveness when total zinc concentrations are in the micromolar range. This value is surprising in that previous work on Cys_2His_2 zinc finger domains has shown these fingers typically have dissociation constants for zinc in the subnanomolar range (10^{-9}−10^{-11} M) (*28*, *29*). The studies of MTF1 have all been performed in cells or extracts with unknown zinc-binding capacity. Thus, it is difficult to know the relationship between the total zinc concentration and the zinc concentration available to a zinc-binding domain with a particular zinc affinity.

Among the information that has been lacking to evaluate the mechanism of zinc sensing by the zinc finger domains of MTF1 is a direct measure of the metal ion affinities of the protein and its individual domains. To this end, we have measured the metal affinity of each of the six zinc fingers in isolation as well as in the context of a fragment of mMTF1 consisting of the six zinc fingers. In addition to its potential implications for zinc sensing by MTF1, this appears to be the first comprehensive analysis of the binding affinities of individual zinc finger domains in the context of a natural multidomain zinc finger protein.

RESULTS

Peptides corresponding to each of the six Cys_2His_2 zinc finger domains of mMTF1 were synthesized. These are termed mMTF1-x, where x refers to the particular zinc finger from which it is derived. The sequences of these peptides are found in Figure 1. All of these peptides were soluble and well-behaved with the exception of mMTF1−2, which proved to be rather insoluble, limiting the precision of the data obtained. The metal ion affinities of these peptides were determined through the use of cobalt(II) as a zinc(II) substitute since cobalt(II) binding is readily monitored using absorption spectroscopy. Two approaches were used to examine the relative affinities of these peptides. First, direct cobalt(II) titrations (*28*, *29*) were performed using peptides corresponding to domains 1 and 5 as these domains have attracted the most attention as possible sites of zinc-mediated regulation. For comparison, a titration of mMTF-3 was also performed. Fits of binding curves based on the absorption at 640 nm due to formation of the peptide−cobalt(II) complexes (data not shown) yielded dissociation constants of 2.9 ± 0.4 μM for mMTF1−1, 0.2 ± 0.1 μM for mMTF1−3, and 1.3 ± 0.4 μM for mMTF1−5. To define more precisely the relative affinities of the various peptides for cobalt(II), cotitrations of the individual fingers with a common internal standard were performed (*30*). mMTF1−3(H26C), a peptide corresponding to domain 3 with the second His residue of the peptide changed to Cys, was used as this standard. The His to Cys substitution allows the absorption spectra of cobalt(II) complexes of the peptide under study and that of the internal peptide to be deconvoluted (*29*). Spectra from the cotitrations and fractional saturation analysis graphs are shown in Figure 2. Relative

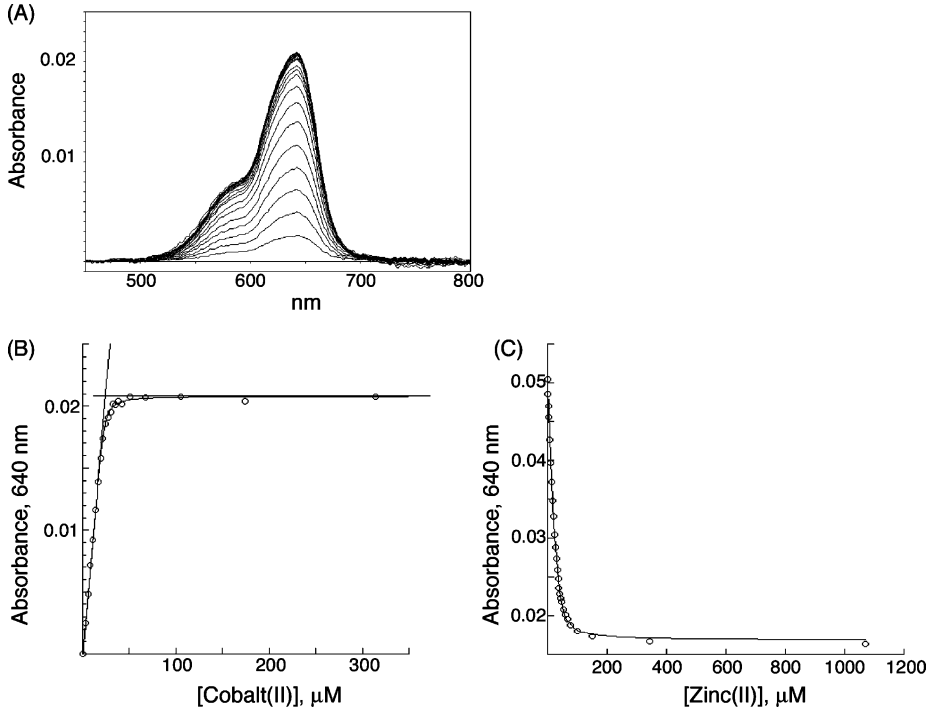

FIGURE 3: Metal binding by the six zinc finger fragment mMTF1-ZF. (A) Spectral analysis of cobalt(II) binding to mMTF1-ZF monitored in the visible region. (B) A plot of the absorbance at 640 nm as a function of added cobalt(II) concentration and a fit to a single site binding model with six equivalent sites per protein with a dissociation constant of 0.5 ± 0.2 μM. (C) Results of a back-titration of mMTF1-ZF in the presence of a 2000-fold excess of cobalt(II) with zinc(II) fit to a single ratio of zinc(II) affinity to cobalt(II) affinity.

Table 1: Cobalt(II) Binding Affinities for Individual Zinc Finger Domains of MMTF1 as Single Domain Peptides

peptide	binding ratio[a]	K_d(Co(II), μM
mMTF1-1	1.9 ± 0.3	1.6 ± 0.3
mMTF1-2	0.4 ± 0.1	0.3 ± 0.1
mMTF1-3	0.6 ± 0.1	0.5 ± 0.1
mMTF1-4	0.5 ± 0.1	0.4 ± 0.1
mMTF1-5	2.1 ± 0.1	1.7 ± 0.3
mMTF1-6	2.1 ± 0.1	1.7 ± 0.3

[a] The binding ratio is the dissociation constant for peptide under study divided by the dissociation constant for the internal standard peptide mMTF1−3(H26C).

and absolute dissociation constants are found in Table 1. The absolute dissociation constants are in reasonable agreement with those derived from the direct titration, and the relative dissociation constants are likely to be more accurate since the cotitration approach minimizes the effects of errors in peptide and cobalt(II) concentration. These experiments reveal that the order of binding from tightest to weakest for individual zinc fingers is mMTF1$-2 \approx -4 \approx -3 > -1 \approx -5 \approx -6$. The overall range of affinity for Co(II) is less than 5-fold, and all dissociation constants are in the micro- to submicromolar range. Previous studies of other zinc finger peptides, as well as studies of a fragment of mMTF1 including all six zinc finger domains, strongly suggest that the zinc(II) affinities of the mMTF1 peptides are in the subnanomolar range.

The previous studies involve peptides corresponding to single zinc finger domains. To determine if the binding affinities of the zinc finger domains were affected by the context of the protein, a fragment of mMTF1 corresponding to the six zinc fingers, termed mMTF1-ZF (see Figure 1), was expressed and purified. After purification, mMTF1-ZF, was shown to have 12.2 ± 1.3 thiols per protein. A cobalt titration was carried out to 25 mM cobalt(II), revealing that mMTF1-ZF bound 6.1 ± 0.4 Co^{2+} per protein with an apparent dissociation constant of 0.5 ± 0.2 μM (Figure 3). No new features appeared in the spectra at high cobalt concentrations. The results of a back-titration (*28*) of mMTF1-ZF with zinc(II) in the presence of an approximately 2000-fold excess cobalt(II) could be fit to a ratio of K_d(Co(II))/ K_d(Zn(II)) of $1.6 \pm 0.2 \times 10^4$. This corresponds to an apparent dissociation constant of 31 ± 14 pM for zinc(II). No evidence for domains with marked differences in affinity for cobalt(II) or zinc(II) were observed in these experiments.

To assess the cobalt(II) binding of the individual zinc finger domains in the context of mMTF1-ZF, six mutated proteins were produced. In each mutated protein, the last histidine of the zinc finger domain was changed to cysteine. This substitution allows the binding of cobalt(II) to each domain to be monitored in the presence of the remaining, nonmutated domains. Cobalt(II) titrations were then performed with each mutant, and fractional saturation analysis was done using the spectra of cobalt(II) complexes of mMTF1-ZF and of the His to Cys peptide corresponding to the particular domain mutated as basis spectra.

The spectra from the cobalt cotitrations and fractional saturation analysis graphs are shown in Figure 4. Since mMTF1−2(H26C) proved quite insoluble, mMTF1−1(H26C) was used as a basis vector for deconvolution of mMTF1-ZF(H58C). The fitting parameter from the fractional saturation analysis for these titrations gives a ratio of the product of the apparent dissociation constant for the unmutated domains to the dissociation constant for the mutated domain. If we assume that the apparent dissociation constant of the unmutated domains is equal to the dissociation constant for unmutated mMTF1-ZF, (0.5 ± 0.2 μM), we can calculate

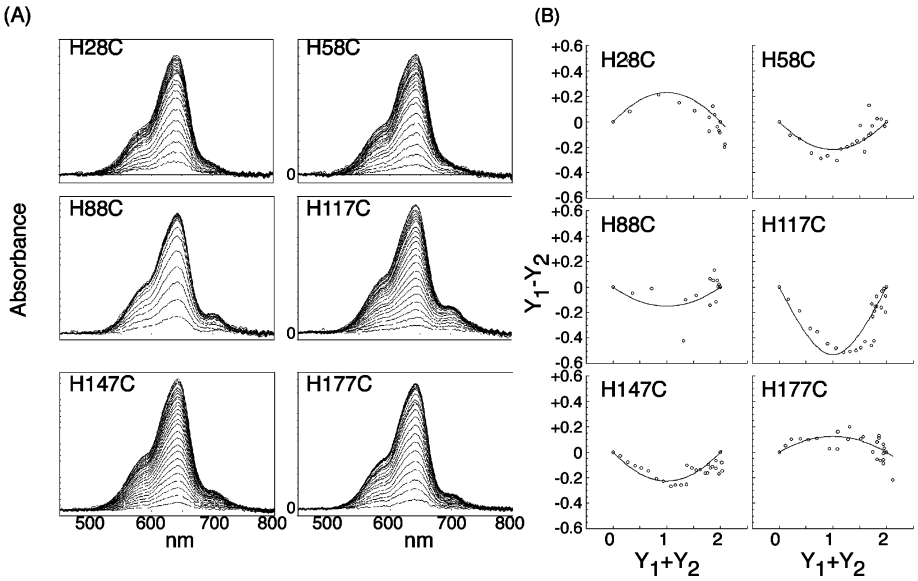

FIGURE 4: Analysis of cobalt(II) binding to individual zinc finger domains with mMTF1-ZF monitored through His to Cys substitutions. (A) Spectra from titrations of six His to Cys mutated proteins with cobalt(II). (B) Fractional saturation analysis plots of the data from panel A. Upward curvature in this plot indicates that the domain under study binds cobalt(II) more tightly than do the remaining, unmutated domains.

Table 2: Cobalt(II) Binding Affinities for Individual Zinc Finger Domains of MMTF1 in the Context of the Six-Zinc Finger Protein Fragment mMTF1-ZF

domain	binding ratio[a]	K_d(Co(II), μM, corrected[b]
mMTF1-ZF(H28C)	0.5 ± 0.3	0.4 ± 0.2
mMTF1-ZF(H58C)	4.6 ± 2.4	0.07 ± 0.04
mMTF1-ZF(H88C)	1.2 ± 0.6	0.4 ± 0.2
mMTF1-ZF(H117C)	13.5 ± 2.5	0.02 ± 0.01
mMTF1-ZF(H147C)	2.6 ± 0.4	0.11 ± 0.02
mMTF1-ZF(H177C)	0.8 ± 0.2	0.4 ± 0.1

[a] The binding ratio is the apparent dissociation constant for the five remaining Cys_2His_2 zinc finger domains divided by the dissociation constant for the particular $Cys_2HisCys$ zinc finger domain present in the mutated protein under study. [b] The dissociation constant is calculated by dividing the apparent dissociation constant for the Cys_2His_2 zinc finger domains of mMTF1-ZF (0.5 μM) by the binding ratio and multiplying by the correction factor for the anticipated effect of the His to Cys change (factor 0.4 for domain 1; 0.6 for domain 3; and 0.5 for remaining domains).

the dissociation constants for each $Cys_2HisCys$ domain in the context of the mMTF1-ZF protein. The substitution of the final His for Cys results in a modest decrease in cobalt(II) affinity at pH 7.0. To confirm this for MTF-1 domains, cotitrations were performed for mMTF1−1 versus mMTF1−1(H26C) and for mMTF1−3 versus mMTF1−3(H26C). Dissociation ratios of 0.4 ± 1 and 0.6 ± 0.1 were found. On the basis of these values and results with other zinc finger peptides (*29*), a ratio of 0.5 was assumed for the remaining domains. With these assumptions, dissociation constants for each of the six unmutated zinc finger domains in the context of MTF1 can be estimated, and these values are shown in Table 2. On the basis of this analysis, the dissociation constants for the Co(II) complexes of the domains range from 0.02 to 0.5 μM. The affinity of each domain determined in the context of MTF1-ZF is higher than determined for the free peptide by factors ranging from 1.1 to 20. The overall range of affinities is 25-fold. The order of affinities for the fingers when joined in the mMTF1-ZF peptide is (tightest to weakest): $4 > 2 \approx 5 > 6 \approx 3 \approx 1$.

DISCUSSION

mMTF1 is a six Cys_2His_2 zinc finger-containing protein that regulates gene expression in response to elevated zinc concentrations. We have examined the metal-binding properties of a fragment of mMTF1 that comprises these six zinc finger domains. Furthermore, we have investigated the metal affinity of the Cys_2His_2 zinc fingers of mMTF1 both as isolated peptides and in the context of the six-domain protein.

The six finger domain protein, mMTF1-ZF, binds approximately 6.1 ± 0.4 cobalt ions with an apparent dissociation constant of 0.5 ± 0.2 μM. A back-titration with zinc yielded an apparent dissociation constant of 31 ± 14 pM. These observations indicate that all of the six zinc finger domains of MTF1 have metal ion affinities that are comparable to other Cys_2His_2 zinc finger domains that have been characterized. The affinities for zinc are substantially higher than the values in the micromolar range that have been determined for the zinc responsiveness of MTF1 in vivo and in extracts. This difference can have at least two explanations. First, and most likely, the concentration of zinc(II) ions available to MTF1 under the conditions used in previous experiments are likely to be much lower than the micromolar total zinc(II) concentrations due to zinc(II) binding to other components of the cells or extracts. Second, regulatory zinc(II)-binding sites may be present in MTF1-ZF that are distinct from the Cys_2His_2 zinc-binding sites within the zinc finger domains. The cobalt(II) binding assay that we have utilized depends on the relatively large extinction coefficients for Co(II) ions bound in tetrahedral sites. No spectral features suggestive of such sites were observed for cobalt(II) concentrations up to 25 mM.

Further experiments were designed to probe the metal ion affinities of the six Cys_2His_2 binding sites individually. When examined as single zinc finger peptides, the six zinc finger domains of mMTF1 bind cobalt(II) with affinities ranging from 0.3 to 1.7 μM, a range of less than 6-fold in affinity. The affinities (from tightest to weakest) are mMTF1−2 ≈

$-4 \approx -3 > -1 \approx -5 \approx -6$. This order is similar to that observed for these six domains of MTF1 observed from pulsed alkylation experiments (*23*).

We then examined the metal-binding properties of these zinc finger domains in the context of the mMTF1-ZF protein. This was accomplished by mutating the final histidine of each zinc finger domain to cysteine in turn to provide a unique spectroscopic signature for cobalt(II) binding to each domain. The results of cobalt(II) titration experiments of these mMTF1-ZF mutants were deconvoluted, and the data were fit to yield affinities for each individual $Cys_2HisCys$ domain relative to the remaining five domains of the protein. These affinities could be corrected for the effects of the His to Cys substitution to yield apparent affinities of each individual domain in the context of the protein. For each of the domains, the affinity for cobalt(II) increased from that observed for the corresponding single domain peptide. The affinities of domains 4 and 5 increased approximately 20-fold; those for domains 1, 2, and 6 increased 3−5-fold; and that for domain 3 increased only slightly. Because of the differential effects, the order of affinities for the domains in the context of mMTF1-ZF is (from highest to lowest) $4 > 2 \approx 5 > 6 \approx 3 \approx 1$. While the present studies cannot distinguish between differences in intrinsic affinity from cooperative interactions between domains, it has been previously demonstrated that such cooperativity is not a general property of arrays of zinc finger domains of this class (*31*).

The range of affinities observed is 25-fold. The results suggest that domains 1, 3, and 6 are the most likely candidates for zinc concentration-sensing domains, if the Cys_2His_2 sites are truly responsible for zinc responsiveness. Examination of the amino acid sequences of these zinc finger domains of MTF1 do not reveal any obvious features that account for the observed differences in metal ion affinity. However, since domain folding is largely or completely coupled to metal binding for this class of domains, sequence features throughout the domain can directly influence metal ion affinity.

These studies have determined, for the first time, the metal ion affinities of the six Cys_2His_2 zinc finger domains from MTF1. The results reveal that the metal ion affinities of these domains are not dramatically lower than those observed for other zinc finger domains and are completely inconsistent with these sites being responsible for zinc responsiveness to changes in free zinc concentration in the micromolar range. Even if none of the MTF1 zinc finger domains were found to have dramatically low metal ion affinities, a 25-fold range of metal ion affinities was observed; domains 1, 3, and 6 showed lower affinities than the remaining domains in the context of a six zinc finger protein fragment. Thus, these domains, in particular domains 1 and 6, remain as the most likely candidates if the Cys_2His_2 metal ion-binding sites of MTF1 are, indeed, responsible for zinc sensing in vivo.

An additional factor that we have not considered is the metal ion affinities of the MTF1 zinc finger domains in the presence of MRE-containing or other DNA molecules. The zinc finger domain affinities could be significantly and differentially perturbed by the presence of DNA molecules to which the protein binds. Future studies will be required to address this possibility.

MATERIALS AND METHODS

Preparation of Single Domain Peptides. Single domain peptides were either synthesized on a Milligen/Biosearch 9050 peptide synthesizer using 9-fluorenylmethoxycarbonyl chemistry as described previously (*29*) or purchased from Bio-Synthesis (Lewisville, TX) or from Syn-pep (Dublin, CA). The peptides were purified by reversed phase HPLC and then resuspended in 400 mM Tris, 100 mM tris-(carboxyethyl)phosphine, 5% acetonitrile to reduce all cysteine residues. The reduced peptides were then purified on a Vydac diphenyl reversed phase HPLC column and dried under a 95% nitrogen/5% hydrogen atmosphere in a Savant SpeedVac concentrator. All peptide manipulations from this point on were performed in this atmosphere to prevent cysteine oxidation, and all solvents were degassed with helium prior to use. The masses of all peptides were confirmed using MALDI mass spectrometry.

Cloning and Mutagenesis of mMTF1-ZF. Murine retinal cDNA was a generous gift of Dr. Jeremy Nathans. The DNA encoding mMTF1-ZF, the zinc finger region (residues 137−315 plus an initiating methionine) of mMTF1, was PCR-amplified using primers 5′-ggaattccatatgaagcggtaccagtg-caccttgag-3′ and 5′-acgcgtcgactcactatttgttatcatgacctttcat-gtgacttt-3′ and cloned into pG5 (a pT7−7- and pGX2627-derived plasmid) using NdeI and SalI restriction sites. The six histidine to cysteine mutants of mMTF1-ZF were created using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). All mutations were confirmed by sequencing.

Expression and Purification of mMTF1-ZF and Associated Mutants. Plasmids were transfected into *Escherichia coli* (BL21)DE3. A resulting colony was grown in baffled flasks of LB supplemented with 100 μM $ZnCl_2$ and 100 μg/mL ampicillin. The culture was grown to OD_{600} 0.4−0.8 at 37 °C. At this point, an additional 100 μg/mL ampicillin was added, and the culture shifted to 20 °C for 1 h, after which expression was induced with 0.5 mM IPTG. After 12−14 h at 20 °C, the culture was centrifuged at 6500*g* in a Beckman centrifuge at 4 °C. The resulting pellet was resuspended in 25 mM Tris pH 8.0, 300 mM NaCl, 100 μM $ZnCl_2$ and processed through a French Press at 1250 psi using an Aminco French Pressure Cell at 4 °C. The solution was centrifuged at 35 000*g* for 45 min at 4 °C. The supernatant was centrifuged a second time at 48 000*g* for 30 min at 4 °C. The resulting supernatant was purified over a heparin sepharose column at room temperature. The column was washed with 4 column volumes of 25 mM Tris, 400 mM NaCl, 100 μM $ZnCl_2$, and the protein eluted with 25 mM Tris pH 8.0, 1 M NaCl, 100 μM $ZnCl_2$. After elution, samples were placed on ice. The elutant was concentrated, and the buffer was changed to 25 mM Tris pH 8.0, 200 mM NaCl, 100 μM $ZnCl_2$ using an Amicon Centricon spin dialysis column with a 5000 Da molecular weight cutoff at 20 °C, except for mMTF1-ZF(H58C), which appeared to degrade if concentrated. This solution was then purified on a Vydac C4 reversed phase HPLC column with a 1%/min acetonitrile gradient containing 0.1% TFA. The peptide eluted from the column at 22% acetonitrile. Collected fractions were dried in a Savant SpeedVac concentrator. The peptide was then resuspended in 0.5 mL of 100 mM TCEP, 0.5 mM EDTA, and 5% acetonitrile. Tris pH 8.0 was added dropwise to a concentration of 400−500 mM with swirling. The precipi-

tated protein was spun at 13 000*g*, and the supernatant was discarded. The pellet was resuspended in 5% acetonitrile, 0.1% TFA, 100 mM TCEP and purified on a Vydac diphenyl reversed phase HPLC column with a 0.5%/min acetonitrile gradient containing 0.1% TFA. Collected fractions were dried under a 95% nitrogen/5% hydrogen atmosphere in a Savant SpeedVac concentrator. All peptide manipulations from this point on were performed in this atmosphere to prevent cysteine oxidation, and all solvents were degassed with helium prior to use. The mass of the peptide was confirmed using MALDI mass spectrometry.

*Assay for Thiol*s. Assay using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was performed as in Riddles et al. (*32*) using the 6.4 M guanidinium chloride-containing phosphate buffer. Assays were performed under a 95% nitrogen/5% hydrogen atmosphere using solvents degassed with helium.

Determination of Extinction Coefficients. Absorbance of a sample of mMTF1−1, mMTF1−3, and mMTF1-ZF was determined at 275 nm. Absorbance of a sample of mMTF1−5 was determined at 257 nm. An equivalent amount of peptide/protein was sent to Bio-synthesis Inc. (Lewisville, TX) for amino acid analysis. mMTF1-ZF was also sent to the Protein and Carbohydrate Structure Facility at the University of Michigan. The extinction coefficient used for mMTF1-ZF was an average of the two coefficients obtained from the two facilities (ϵ_{275} = 11 955 and 11 572 M^{-1} cm^{-1}).

Metal Ion Titrations. Cobalt(II) binding was measured by spectrophotometrically monitoring the titration of a solution of the peptide(s) with $CoCl_2$. Cobalt(II) concentrations were determined with the use of an ϵ_{512} of 4.8 M^{-1} cm^{-1}. Zinc concentrations were measured by atomic absorption. The experiments were performed in 100 mM HEPES (Sigma Ultra), 50 mM NaCl (Sigma Ultra), pH = 7.0 that had been treated with Chlex-100 beads (Sigma) and degassed with helium. Titrations were performed at 25 °C under anaerobic conditions (5% hydrogen/95% nitrogen) using a Perkin-Elmer Lambda 9 spectrophotometer. Spectra were collected from 450 to 800 nm. Cotitrations of single domain peptides were done with approximately equimolar amounts of the peptide under study and the internal standard peptide. Single finger titrations were done in triplicate; peptide cotitrations were done in duplicate; the cobalt(II) titration of mMTF1-ZF was repeated 9 times; titrations of the mMTF1-ZF mutants were performed 3−5 times.

Analysis of Cobalt(II) Binding Data. For the direct single domain peptide titrations, the absorbance at 640 nM as a function of total added cobalt(II) concentration was fitted to a 1:1 binding model using Kalidagraph software (Synergy) to determine the dissociation constants for the peptide−cobalt(II) complexes. Analysis of cotitrations and titrations of proteins containing His to Cys mutated domains fingers followed methods described previously (*30*). For the single domain peptides, the basis spectra used in the deconvolution were taken from the corresponding single domain peptides. For the His to Cys mutants of the six zinc finger domain protein, the absorption spectrum of the unmutated protein was used for the Cys_2His_{2-}cobalt(II) complex spectrum, while the spectra of the cobalt(II) complexes of the individual His to Cys single domain peptide variant were used except for domain 2 where this peptide proved to be too insoluble. The spectrum from mMTF1−1(H26C) was used as a surrogate.

The fractional saturation data from this analysis were used to determine the ratio of dissociation constants of the two sites for cobalt(II). The ratio of dissociation constants K_{d1}/K_{d2} can be expressed in terms of the fractional saturation values as $c = K_{d1}/K_{d2} = (Y_2(1 - Y_1))/(Y_1(1 - Y_2))$. This can be solved for the difference in fractional saturation $Y_1 - Y_2 = ((1 + c) - ((x - 1)^2(1 - c)^2 + 4c))^{1/2}/(1 - c)$ where $x = Y_1 + Y_2$. The data in the form $Y_1 - Y_2$ versus $Y_1 + Y_2$ were fit with use of the program Kaleidagraph (Synergy). The results were corrected for the anticipated effect of the His to Cys substitution by a factor of 0.4 (domain 1), 0.6 (domain 3), or 0.5 (remaining domains).

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BI0358418