Conformational Heterogeneity in the C-terminal Zinc Fingers of Human MTF-1

AN NMR AND ZINC-BINDING STUDY*

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The human metalloregulatory transcription factor, metal-response element (MRE)-binding transcription factor-1 (MTF-1), contains six TFIIA-type Cys2-His2 motifs, each of which was projected to form well-structured ββα domains upon Zn(II) binding. In this report, the structure and backbone dynamics of a fragment containing the unusual C-terminal fingers F4–F6 has been investigated. 15N heteronuclear single quantum coherence (HSQC) spectra of uniformly 15N-labeled hMTF-zf46 show that Zn(II) induces the folding domains. In contrast to fingers F4 and F6, the significantly more tightly than do the other two finger zf46 with Zn(II) reveals that the F4 domain binds Zn(II) to the finger domains in solution. Titration of apo-MTF-zf46 with Zn(II) and Cd(II) reveals that the F4 domain binds Zn(II) significantly more tightly than do the other two finger domains. In contrast to fingers F4 and F6, the ββα fold of finger F5 is unstable and only partially populated at stoichiometric Zn(II); a slight molar excess of zinc results in severe conformational exchange broadening of all F5 NH cross-peaks. Finally, although Cd(II) binds to apo-hMTF-zf46 as revealed by intense S → Cδ(II) absorption, a non-native structure results; addition of stoichiometric Zn(II) to the Cd(II) complex results in quantitative refolding of the ββα structure in F4 and F6. The functional implications of these results are discussed.

Zinc is the second most abundant transition metal ion present in biological systems. Zinc plays essential and ubiquitous catalytic and structural roles, present both at the active sites of many hydrolytic enzymes and as an integral tertiary structural element in many proteins, most prominently exemplified by zinc finger proteins (1, 2). The intracellular concentrations of “bioavailable” essential metals ions, including zinc, appear to be tightly regulated in all cells. Although recent progress in our understanding of zinc toxicity in mammalian cell mitochondria has been made (3), the precise underlying cause for zinc toxicity is not yet known with certainty. However, too much zinc would compete for enzyme active sites requiring other metal ions or alter their homeostatic mechanisms by competing for metal transporter sites. Zinc-specific metalloregulation of gene expression has been documented to occur in essentially all organisms, including mammalian cells (4), plants (5), Saccharomyces cerevisiae (6), and bacteria (7–10). Zinc metalloregulatory transcription factors, which mediate the response of cells to both zinc-deficient and zinc-excess conditions have been identified and partially characterized from many of these sources (10).

In many cell types, including mammalian (mouse, rat, and human) cells (11, 12), fish (13), and Drosophila melanogaster (14), zinc-dependent transcriptional regulation of metallothionein (MT) genes plays an important role in zinc homeostasis and detoxification. This regulation requires the interaction of metal-response element (MRE)-binding transcription factor-1 (MTF-1) (15) with MREs (16, 17) situated in the promoters of zinc-inducible genes (17). MTF-1 is a constitutively expressed protein in mouse and human cells of ~80 kDa that contains six Cys2His2 zinc finger domains and multiple domains for transcriptional activation (18), the latter of which likely play important roles in the signal transduction pathway that leads to a zinc response (19). MTF-1 is an essential protein (20) required for basal and heavy metal-induced expression of metallothionein I and II genes. Other toxic heavy metals, including cadmium (for a review, see ref. 21) and to a lesser extent copper, are also known to induce the expression of metallothioneins in vertebrate and Drosophila cells (22) (copper is a primary inducer of MT expression in Drosophila (14), as do various forms of oxidative stress (23) and hypoxia (24). Some reports suggest that the zinc finger domain of MTF-1 itself is a zinc sensor that exhibits increased DNA binding activity upon zinc treatment in vitro and in vivo. It is becoming increasingly clear, however, that stimulation of MRE-binding activity of MTF-1 by Zn(II) may be necessary but not sufficient for transcriptional activation by zinc and other potent inducers, including Cd(II). Zn(II) and other inducers play a major role in modulating nuclear-cytoplasmic trafficking of MTF-1 (25, 26), whereas Zn(II) and Cd(II) seem to alter the phosphorylation status of MTF-1 (19).

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[The on-line version of this article (available at http://www.jbc.org) contains Table IS.]

1 The abbreviations used are: MT, metallothionein; MRE, metal response element; MTF-1, MRE-binding transcription factor-1; Mops, 4-morpholinepropanesulfonic acid; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; MREd, metal response element d.
The zinc finger domain of MTF-1 from human, mouse, *D. melanogaster*, and Japanese pufferfish is remarkably conserved throughout hundreds of millions of years of evolution (13). Each zinc finger conforms to the classic (F/Y)-Cys-X<sub>3</sub>-Cys-X<sub>2</sub>-H<sub>i</sub>-Cys-X<sub>7</sub>-H<sub>i</sub>-X-H<sub>i</sub>-H<sub>i</sub>-Cys sequence, each of which is connected by nearly consensus TG(E/Q)/K/R/P linkers (27), with the exception of the linker between fingers F3 and F4, which is one residue shorter, TGKT (28). This is likely to alter the conformation of the linker and may well alter the ability of F3 and F4 to bind to successive non-overlapping 3-base pair substrates (for recent reviews on the structures of zinc finger proteins, see Refs. 29, 30). The only deviation to the absolute conservation of the (F/Y)-X-Cys-X<sub>3</sub>-Cys-X<sub>2</sub>-H<sub>i</sub>-Cys-X<sub>7</sub>-H<sub>i</sub>-X-H<sub>i</sub>-H<sub>i</sub>-Cys sequence in MTF-1 is in F4 in *D. melanogaster* MTF-1 where the Cys-X<sub>3</sub>-Cys sequence, predicted to form a β-type II turn, is replaced by the more common Cys-X<sub>2</sub>-Cys sequence, predicted to form a β-type I turn, with otherwise no changes in the structure of the β<sub>α</sub>α domain (30, 31).

We have begun to elucidate the biochemical and structural properties of the zinc finger domain of human MTF-1 in an effort to obtain insight into regulatory signaling by MTF-1. Our previous work suggests that the N-terminal four zinc fingers of MTF-1 (F1–F4) adopt canonical β<sub>α</sub>α structures and make large contributions to the specific MRE-binding affinity, with C-terminal fingers F5 and F6 playing an accessory role in stabilizing the protein-DNA complex on an mMT-1 MRE-containing oligonucleotide (32, 33). In addition, a variety of evidence suggested that the apparent affinity of the C-terminal fingers F5 and F6 for Zn(II) was weaker than that for the major DNA-binding fingers of the molecule (33). These findings have led to the hypothesis that the C-terminal zinc fingers of hMTF-1 play a direct role in metalloregulation via reversible Zn(II) binding to the C-terminal fingers F5 and F6 for Zn(II) was weaker than that for the major DNA-binding fingers (36). Previous far-UV-circular dichroism studies of hMTF-zf were interpreted to suggest that the N-terminal four zinc fingers are folded into typical β<sub>α</sub>α motifs (33). In contrast, one or both of the C-terminal fingers were thought to adopt one or more alternative conformations under conditions of saturating Zn(II), although the structural details remained unclear. These structural differences are maintained in the isolated three-finger domain fragments of hMTF-zf [zf13 and zf46 (33), providing justification for investigating the detailed structure of hMTF-zf46 as representative of the structure of this region within intact hMTF-zf and hMTF-1. In this study, we have used heteronuclear NMR spectroscopy to investigate the Zn(II) binding and conformational properties of the C-terminal zinc fingers of hMTF-zf46. We find that F4 and F6 do indeed adopt canonical β<sub>α</sub>α structures, with F6 characterized by a clearly lower affinity for Zn(II). Furthermore, at saturating Zn(II), F5 does not adopt a stable β<sub>α</sub>α structure but is instead in conformational equilibrium with one or more non-native conformations. Finally, titration of apo-hMTF-zf46 with Cd(II) induces a non-native, largely unfolded structure as revealed by 15N HSQC spectroscopy, with a maximum Cd(II)-peptide stoichiometry of ~2:1, with significant S′→Cd(II) thiolate absorption in the complex. This finding is consistent with other results that show that, although Cd(II) is a potent inductor of MT gene expression from the MRE (37), it is unlikely to do so by inducing β<sub>α</sub>α structure into the MRE-binding domain of MTF-1.

**MATERIALS AND METHODS**

**Purification of hMTF-zf46 and hMTF-zf56—PET-derived overexpression plasmids were designed to express zinc finger fragments encompassing zinc fingers F4–F6 (residues Gly<sub>127</sub>–Tyr<sub>132</sub>), denoted pMTF-zf46, or zinc fingers F5–F6 (Gly<sub>110</sub>–Tyr<sub>130</sub>), denoted pMTF-zf56 (see Fig. 1A). In both cases, the C-terminal Tyr was replaced by a Trp, as in all other constructs (32, 33), to facilitate optical spectroscopy studies. Bacterial strains, or T7 expression systems were used to express the desired coding region using pMTF-zf as a template and subcloned between the NcoI and BamHI sites of pET3d, with overexpression in *Escherichia coli* BL21(DE3) essentially as described previously (33). The integrity of the coding sequence was verified by dideoxy sequencing of both DNA strands carried out by the Gene Technologies Laboratory at Texas A&M University. Protein expression was induced with 0.4 mM isopropyl-1-thio-galactopyranoside at a cell culture density of A<sub>600</sub> = 0.5 at 37 °C, and the cells were harvested 3–4 h after induction. hMTF-zf46 and hMTF-zf56 were purified from the low speed supernatant using a chromatographic scheme on a POROS C18 column developed with a linear gradient (40 mM Mops, 5 mM diethanolamine, pH 7.0, 0.2 M NaCl to 1 M NaCl), followed by pooling of the appropriate fractions, an aneucaryal dialysis against 0.1% trifluoroacetic acid, with subsequent repeated lyophilization from double-distilled water. Lyophilized protein was then returned to the aneucaryal globe and resuspended in metal-free 120 mM d<sub>6</sub>-Hepes, pH 7.0, in the absence of exogenously added Zn(II). These samples of apo-hMTF-zf46 and apo-hMTF-zf56 typically contained ≤0.1 mol eq of Zn(II) by atomic absorption spectroscopy on a PE AAnalyst 700 and contained the expected number of reduced cysteines (~6 for apo-hMTF-zf46 and ~4 for apo-hMTF-zf56) by MALDI-TOF mass spectrometry (55). In both cases, the N-terminal Met was removed in vivo to yield a N-terminal native Gly. Uniformly 15N- and 13C-labeled and 15N-labeled proteins were prepared by expressing the proteins on M9 minimal media salts supplemented with 1 g/liter L-[15N]NH<sub>4</sub>SO<sub>4</sub> as the sole nitrogen source, or 1 g/liter L-[13C<sub>6</sub>]NH<sub>4</sub>SO<sub>4</sub> and 3 g/liter [13C<sub>6</sub>]Glucose essentially as described before (39). α-2-2-15N-Lys-labeled hMTF-zf46 was prepared by α-2-2-15N-Lys-labeled pMTF-zf46, transformed into E. coli strain BL21(DE3), and expressed in M9 minimal media salts supplemented with other 19 unlabeled amino acids at the concentrations suggested (39).

**NMR Spectroscopy—** All NMR spectra were collected on Varian Unity Inova 500- and 600-MHz spectrometers in the Biomolecular NMR Laboratory at Texas A&M University. For uniformly labeled samples, typical solution conditions were 0.5 and 0.9 mM 15N- and 13C-labeled, and 15N-labeled protein, respectively, pH 7.0, 120 mM d<sub>6</sub>-Hepes, 30 °C. For α-2-2-15N-Lys-labeled hMTF-zf46, 0.6 mM was used. Chemical shift referencing is relative to 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) (41). All spectra were processed and analyzed using NMRPipe (42).

The 1H-15N HSQC spectrum was recorded as a 160° × 480° two-dimensional matrix in the t<sub>1</sub> and t<sub>2</sub> dimensions, respectively, where n<sup>+</sup> represents n complex points. Acquisition times in each dimension were 20 ms (t<sub>1</sub>) and 80 ms (t<sub>2</sub>) as defined by sweep widths of 1600 and 6000 Hz in the t<sub>1</sub> and t<sub>2</sub> dimensions, respectively, for a total experiment time of 1.6 h. The data were apodized along t<sub>2</sub> by using a 90°-shifted squared sine-bell, truncated at 1% (sin<sup>2</sup>) 175°. The t<sub>1</sub> time domain was extended to 256° using mirror-image linear prediction (eight coefficients) (43) and then apodized with an untruncated 90°-shifted squared sine-bell. The data were zero-filled to a digital resolution of 3.1 (F<sub>1</sub>) and 3.9 Hz (F<sub>2</sub>).

The CBCANH spectrum (44) was recorded as a 34° × 55° × 512° three-dimensional matrix with acquisition times of 22.4 (t<sub>1</sub>, 15N), 6.5 (t<sub>2</sub>, 15C<sub>α</sub>), and 73.1 ms (t<sub>3</sub>, H<sup>2</sup>) as defined by sweep widths of 1515.2, 8444.2, and 6999.7 Hz in the t<sub>1</sub>, t<sub>2</sub>, and t<sub>3</sub> dimensions, respectively. The total acquisition time was 39.5 h. Acquired data were apodized along the 90°-shifted squared sine-bell in the t<sub>1</sub> dimension, truncated at 1% at the end of the free induction decay, and an untruncated 90°-shifted squared sine-bell after the data were extended to 58° by using mirror-image linear prediction. Data were zero-filled to digital resolutions of 15.8 (F<sub>1</sub>), 44 (F<sub>2</sub>), and 6.8 Hz (F<sub>3</sub>).

The CBCANH spectrum (45) was recorded as a 33° × 55° × 512° three-dimensional matrix with acquisition times of 21.8 (t<sub>1</sub>, 15N), 844.4 (t<sub>2</sub>, 15C<sub>α</sub>), and 73.1 ms (t<sub>3</sub>, H<sup>2</sup>) as defined by sweep widths of 1515.2, 8444.2, and 6999.7 Hz in the t<sub>1</sub>, t<sub>2</sub>, and t<sub>3</sub> dimensions, respectively. The total acquisition time was 74 h. Acquired data were processed similarly to the CBCA(CO)NH experiment and were zero-filled to yield digital resolutions of 15.8 (F<sub>1</sub>), 44 (F<sub>2</sub>), and 6.8 Hz (F<sub>3</sub>).
The HBBHACO/NH spectrum (46) was recorded as a 32° × 60° × 512° three-dimensional matrix with acquisition times of 21.8 (t₁, 15N), 13.3 (t₂, 1H), and 73.1 ms (t₃, 1H) as defined by sweep widths of 1515.2, 4498.1, and 6999.7 Hz in the t₁, t₂, and t₃ dimensions, respectively. The total acquisition time was 41.1 h. Acquired data were processed similarly to the CBCACO/NH and were zero-filled to yield digital resolutions of 15.8 (F₁), 23.4 (F₂), and 6.8 Hz (F₃).

The HBBHANN spectrum (47) was recorded as a 33° × 60° × 512° three-dimensional matrix with acquisition times of 21.8 (t₁, 15N), 13.3 (t₂, 1H), and 73.1 ms (t₃, 1H) as defined by sweep widths of 1515.2, 4498.1, and 6999.7 Hz in the t₁, t₂, and t₃ dimensions, respectively. The total acquisition time was 88.1 h. Acquired data were processed similarly to the CBCACO/NH and were zero-filled to yield digital resolutions of 15.8 (F₁), 23.4 (F₂), and 6.8 Hz (F₃).

The HNHA spectrum (48) for measurement of J(spacer) was recorded as a 60° × 78° × 512° three-dimensional matrix with acquisition times of 1600, 5000, and 6000 Hz in the t₁, t₂, and t₃ dimensions, respectively. Acquired data were apodized with a 90°-shifted squared sine-bell in the t₁ dimension, truncated at 0% at the end of the free induction decay, and with a 90°-shifted sine-bell in the t₁ dimension, truncated at 16%. The t₂ dimension was apodized with an untruncated 90°-shifted squared sine-bell. Data were zero-filled such that the digital resolutions were 25 (F₁), 19.5 (F₂), and 5.86 Hz (F₃). Three-bond HN-Hz coupling constants (J₁₂₋₋) were estimated from the ratio of the intensities of cross-peaks on (J₂₁₋) and off (J₂₁₋) the diagonal according the following expression (49),

\[ \frac{1}{J_{12 busted}} = \frac{1.11 \times \tan^{-1} (\text{signal}(J_{12 busted})/2\Pi \delta)}{(2\Pi \delta)} \]  

where \( \delta \) is 13.2 ms.

The three-dimensional 15N-separated NOESY spectrum (50) with a 100-ms mixing time was recorded as a 32° × 128° × 512° three-dimensional matrix with acquisition times of 20 (t₁, 15N), 25.6 (t₂, 1H), and 85.3 ms (t₃, 1H) as defined by sweep widths of 1600, 5000, and 6000 Hz in the t₁, t₂, and t₃ dimensions, respectively. The total acquisition time was 91.3 h. Acquired data were apodized with a 90°-shifted squared sine-bell in the t₁ dimension, truncated at 0%, and with a 90°-shifted sine-bell in the t₁ dimension, truncated at 16%. The t₂ dimension was apodized with a 90°-shifted squared sine-bell, truncated at 16%. Data were zero-filled to yield digital resolutions of 16.7 (F₁), 19.5 (F₂), and 5.86 Hz (F₃).

The three-dimensional 15N-separated total correlation spectroscopy spectrum (50) with a mixing time of 120 ms was recorded as a 32° × 128° × 512° three-dimensional matrix with acquisition times of 20 (t₁, 15N), 25.6 (t₂, 1H), and 85.3 ms (t₃, 1H) as defined by sweep widths of 1600, 5000, and 6000 Hz in the t₁, t₂, and t₃ dimensions, respectively. Acquired data were processed similarly to the 15N-separated NOESY experiment and were zero-filled such that the digital resolutions were 16.7 (F₁), 19.5 (F₂), and 5.86 Hz (F₃).

Relaxation Experiments—The pulse sequences used to determine the 15N R₁ (1/τ₁), R₂ (1/τ₂), and 1H-15N NOE values were derived from Fig. 10 of (51), which incorporates gradient selection and were recorded at 30 °C. All spectra were collected as 160° × 512° data matrices in the t₁ and t₂ dimensions, with acquisition times of 83.3 (t₁) and 73.1 ms (t₃) as defined by sweep widths of 1920 (t₁) and 6999.7 Hz (t₃). The 15N T₁ data were collected using 15N delays of 21, 61, 121, 151, 261, 521, 761, 1001, and 1251 ms. The T₂ experiments were collected using 15N delays of 32, 48, 64, 80, 96, 128, 144, 160, 192, and 240 ms. Data sets were collected in duplicate to access the reproducibility of the experiments. To evaluate steady-state 1H,15N NOES, two-dimensional spectra were recorded with (NOE experiment) and without (NONOE experiments) application of 1H saturation applied during the delay between successive transients. In the NONOE experiment, a relaxation delay of 5 s was used between transients, whereas in the NOE experiment, a 2-s delay followed by a 3-s proton presaturation achieved with the use of 120° 1H pulses applied every 5 s. 15N T₁, T₂, and 1H-15N NOE data sets were collected twice, several days apart. The time points for the T₁ and T₂ data sets were randomized differently for each set and interleaved such that all 10 15N delays were sampled at each t₁ period, to minimize spectrometer drift effects. Acquisition times were 22.8, 18.0, and 29.4 h for each T₁, T₂, and NOE data set, respectively. All spectra were apodized in the t₁ dimension with a 90°-shifted sine-bell, truncated at 1%. The t₂ dimension was apodized with a 90°-shifted sine-bell, truncated at 6%. The spectra were zero-filled to 3.7 (t₁) and 4.6 Hz (t₂). Peak intensities were fitted to a monoexponential decay function in Kaleidagraph 3.09 (Abelbeck Software). Uncertainties in the (1H-15N) NOE values were estimated from individual data sets collected in duplicate.

Absorption Spectroscopy—Anaerobic optical titrations of apo-hMTF-zf46 or apo-hMTF-zf56 were carried out at ambient temperature with absorption spectra recorded on an HP8453 diode array spectrophotometer essentially as described previously (52, 53). After each addition of Co(II) or Cd(II), the spectra were recorded after 5 min to ensure that complex formation had come to equilibrium.

RESULTS

Fig. 1A shows the primary structure of the C-terminal zinc finger fragment of hMTF-1, hMTF-zf46 (residues 226–320) used in these studies. Apo-hMTF-zf46 was purified to homogeneity in a form with a residual Zn(II) content of 0.1 g mol⁻¹ Zn(II), with nearly all the Cys in their reduced form by 5,5'-dithiobis(nitrobenzoic acid) analysis (see "Materials and Methods"). Metal binding studies of metal-free apo-MTF-zf46 revealed that, as expected, this peptide fragment binds ~3 mol eq of Co(II) (Fig. 2). The metal binding capacity of the Co(II)-saturated complex is identical to those previously published for canonical TFIIIA-type Cys-2His-zinc finger peptides and proteins (31, 54). Additions of stoichiometric Zn(II) results in complete bleaching of the optical spectrum (data not shown).

An 15N HSQC spectrum is shown in Fig. 3 for a sample of
apo- and fully reduced hMTF-zf46 to which was added 3 mol eq of Zn(II). As can be seen, the most intense cross-peaks in the spectrum are of a relatively uniform intensity, and of narrow linewidths, giving rise to excellent spectral dispersion, indicative of a well-folded protein. As expected, the apoprotein 15N HSQC spectrum is essentially that expected for an unfolded protein (data not shown), revealing that Zn(II) binding folds at least a subset of the C-terminal fingers into 55. Interestingly, only about 65 or so of 91 expected backbone 1H-15N correlations are actually cleanly observed in this spectrum of the zinc-saturated protein. Consistent with this, in addition to these sharp resonances, there are cross-peaks clustered in the middle of the spectrum of very weak intensity, suggestive of chemical exchange broadening in at least one region of the hMTF-zf46 molecule.

To determine which regions of MTF-zf46 are characterized by chemical exchange broadening, 1H, 13C, 15N sequential resonance assignments were obtained for the regions of the zinc-saturated protein that could be connected by implementation of a backbone walk obtained upon sequential application of CBCA(CO)NH (44), CBCANH (45), HBHA(CO)NH (46), and HBHANH (47) triple resonance experiments. A representative run through F4 residues Cys231-Leu243 is shown in Fig. 4, with nearly complete 15N, 13C, 13Cβ, HN, Ha, and Hβ resonance assignments shown in Table IS (see supplemental material). It was trivial to link all resonances from the residues Lys227 to Lys257 in F4 and Ser293 to the C-terminal Trp320; these assignments are superimposed on the 15N HSQC spectrum shown in Fig. 3. As can be seen, these assignments identify the vast majority of intense peaks that are well-resolved in the spectrum, with the remainder of the cross-peaks poorly resolved and of low intensity. These data reveal that, under these conditions of saturating Zn(II), F5 resonances as a group experience severe chemical exchange broadening, with essentially no evidence for stably folded ββα structure.

Secondary Structure Analysis of F4 and F6 Zinc Finger Domains in MTF-zf46—Shown in Fig. 5 are the results of the...
residue-specific secondary structural analysis of F4, F6, and C-terminal tail residues in hMTF-zf46 as inferred from chemical shift indexing with the most reliable indicator, the $^{13}$Ca chemical shift (56), and the magnitude of the $^{3}J_{He-HN}$ coupling constant. A contiguous run of at least four residues in which the $^{13}$Ca chemical shifts are $>0.8$ ppm or greater than the random-coil values (i.e. $+^{13}$Ca $\Delta \delta_{col}$) is a strong indication of $\alpha$-helical secondary structure (57). On the other hand, three or more residues with $^{13}$Ca chemical shifts $<0.5$ ppm or greater than the random coil value (i.e. $-^{13}$Ca $\Delta \delta_{col}$) have a $\beta$-strand conformation. Residues 227, 228, and 239 in F4 and the corresponding residue in F6 (299) are representative of this class of residues. To obtain further support for this secondary structure assignment, the magnitude of $^{3}J_{He-HN}$ was measured with a three-dimensional HNHA experiment (48). $\alpha$-Helical regions are characterized by a continuous stretch of $^{3}J_{He-HN} < 5$ Hz, whereas residues in $\beta$-strand conformations have $^{3}J_{He-HN} > 8$ Hz. Residues in random coil or other structured regions are typically characterized by $^{3}J_{He-HN}$ between these two extrema, typically $^{3}J_{He-HN} \sim 7.0$ Hz.

Both experiments lead to essentially the same conclusion, i.e. there is excellent correspondence of secondary structure in F4 and F6 and strong evidence that the F4 and F6 domains adopt the canonical $\beta\beta\alpha$ fold (Fig. 5). These structural features for hMTF-zf F4 and F6 bear a detailed similarity to the N-terminal three finger domains of TFIIIA (zf1–3) and the four zinc fingers of the Wilms tumor suppressor protein (wt1–4) (27, 58). The two anticipated short stretches of classic $\beta$-structure are reasonably well-defined by the data, with the N-terminal start of the $\alpha$-helix (residue +1; Thr$^{243}$ in F4 and Thr$^{303}$ in F6) in exactly the anticipated position. Furthermore, the amino acids in the $-1$ position relative to the start of the $\alpha$-helix, Thr$^{242}$ and Thr$^{302}$ in F4 an F6, respectively, exhibit strongly upfield-shifted $^{13}$Ca and downfield-shifted $^{13}$Cb chemical shifts, consistent with this residue serving as the N-cap on the $\alpha$-helix in both cases, especially as previously found in TFIIIA zf1–3 and wt1–4 (27). Interestingly, the run of $\alpha$-helical residues in each finger domain appears to terminate at the second residue of the His-X$_4$-His motif (of residues Arg$^{251}$ and Lys$^{311}$, respectively), as evidenced by the relatively small positive values of $^{13}$Ca $\Delta \delta_{col}$ and $^{3}J_{He-HN}$ values of $\sim 8$ Hz (Fig. 5). This is consistent with a distortion in the $\alpha$-helical geometry to $\beta$$_o$-helical geometry as observed in other His-X$_4$-His motifs in classic fingers, particularly when not bound to DNA (27). This is required to accommodate bis-imidazole coordination of the Zn(II) in this structure.

$^{15}$N Heteronuclear Relaxation Behavior of F4 and F6 Zinc Finger Domains in MTF-zf46—Fig. 6 shows a presentation of the $^{15}$N heteronuclear relaxation data acquired at 600-MHz $^{1}H$ frequency for F4, F6, and C-terminal tail residues. The magnitude and uniformity of $T_{1}/T_{2}$ ($R_{2}/R_{1}$) ratio when considered along with the $(^{1}H)-^{15}$N heteronuclear NOE can provide insight into backbone conformational dynamics on the picosecond to nanosecond, as well as microsecond, time scales (51). The $(^{1}H)-^{15}$N NOE values reveal that, by and large, F4 residues from Phe$^{229}$ in the $\beta$$_1$ strand to His$^{313}$ encompassing the entire $\beta\beta\alpha$-structure tumble as an independently folded globular domain, with the mean ($\pm$ S.D.) value of the NOE of 0.61 $\pm$ 0.07 averaged over these 24 residues. This is comparable to the magnitude of the mean $(^{1}H)-^{15}$N NOE determined in other $\beta\beta\alpha$ zinc fingers under similar solution conditions (59–61). The same is true of residues Ser$^{293}$ to His$^{313}$ in F6, with the mean ($\pm$ S.D.) value of the $(^{1}H)-^{15}$N NOE of 0.65 $\pm$ 0.06 averaged over these 19 residues. As expected, the N-terminal residues Gly$^{226}$ and Lys$^{317}$, as well as the F6 C-terminal tail residues Lys$^{318}$ to Trp$^{320}$ are characterized by weakly positive or strongly negative NOE values, consistent with these residues reorienting rapidly in the picosecond to nanosecond time scale and are otherwise unstructured.

The mean $T_{1}$ value for 24 F4 (229–253) and 19 F6 residues (292–313) are essentially indistinguishable, with $T_{1}(F4) = 0.518 \pm 0.020$ s and $T_{1}(F6) = 0.529 \pm 0.022$ s. The $T_{1}$ values are significantly different, which makes the $T_{1}/T_{2}$ ratio significantly larger for the C-terminal F6 domains relative to F4. For F4, $T_{2} = 0.140 \pm 0.028$ s to give $T_{1}/T_{2} = 3.84 \pm 0.09$, whereas for F6 residues, $T_{2} = 0.126 \pm 0.022$ s to give $T_{1}/T_{2} = 4.33 \pm 0.09$. Assuming isotropic tumbling for individual zinc finger domains, the rotational molecular correlation times, $\tau_{m}$, calculated for the F4 and F6 finger domains, are 5.3 ($\pm 0.8$) ns and 5.8 ($\pm 0.8$) ns, respectively. These $\tau_{m}$ values are comparable to that previously measured for folded zinc finger domains within multizinc finger proteins, TFIIIA zf1–3 (60) and yeast ADR1 (59) when not bound to the DNA; thus, F4 and F6 domains are clearly characterized by essentially independent tumbling in solution. However, although the effect is small and not statistically outside of the standard deviation of the mean $T_{2}$ or $T_{1}/T_{2}$.
values observed for the residues within individual finger domains, the C-terminal F6 zinc finger domain seems to behave, relaxation-wise, as though it were a slightly larger globular particle than the F4 ββα domain. A similar observation was made for one the zinc finger domains of the yeast ADR1 DNA-binding domain (59). The structural origin of this effect is unknown, but systematic shortening of $T_2$ throughout an entire globular domain is likely indicative of some other relaxation process operating in the μs time scale superimposed on local short-time N–H bond vector reorientation and globular tumbling of that domain (cf. Ref. 39).

**Zinc Binding Studies of Apo-MTF-zf46**—The data presented above reveal that both F4 and F6 domains fold into the canonical ββα-structural module in the presence of saturating Zn(II), with zinc finger F5 specifically characterized by unusual structural properties. In addition, previous data suggest that the C-terminal zinc finger domains form weaker zinc coordination structures than do N-terminal finger domains F1–F4 (33, 36). Therefore, a series of Zn(II) binding titrations were carried out in an attempt to gain additional insight into these two processes.

Fig. 7A shows selected representative $^{15}$N HSQC spectra of
apo-α-¹⁵N-Lys-labeled hMTF-zf46 acquired over the course of a Zn(II) titration, with the normalized intensities of individual cross-peaks found in Zn₃ hMTF-zf46 plotted as a function of added Zn(II) shown in Fig. 7B. hMTF-zf46 contains 10 lysine residues, distributed relatively evenly throughout the primary structure, some in identical positions in different fingers, e.g. Lys238, Lys268, and Lys298 in the predicted β strand in fingers F4, F5, and F6, respectively (Fig. 1A). Given the heteronuclear ¹⁵N relaxation results described above, lysines in individual finger domains will report on Zn(II) binding to that finger only. The apoprotein spectrum (Fig. 7A, upper left) shows the expected ten resolvable cross-peaks, although one is of relatively low intensity. The integrated cross-peak intensities (Fig. 7B) derived from individual ¹⁵N HSQC spectra are normalized to that of Lys316 in the unstructured C-terminal tail (Fig. 6), whose chemical shift does not change as a function of bound Zn(II).

Simple inspection of the spectrum obtained upon addition of ~3 mol eq of Zn(II) reveals spectral dispersion indicative of a folded structure in all three zinc finger domains. However, cross-peaks assigned to F5 lysines are of very low intensity, even in this spectrum (see below). Fig. 7B reveals that the cross-peak intensities of lysines in MTF-zf46 cluster into essentially three groups as a function of added zinc. The cross-peaks of Lys238 and Lys248 reach maximum intensity at ~1 mol eq of Zn(II), with the other five cross-peaks not achieving maximum intensity until ~2 to 3 mol eq of metal is added. Finger F6 resonances (Lys298, Lys307, and Lys311) remain fully developed even at excess Zn(II). A third pair of resonances, which by elimination correspond to Lys268 and Lys277 in F5, actually disappear completely at excess Zn(II), concomitant with the appearance of at least one new cross-peak just outside of this spectral window (data not shown). Lys227 near the N terminus is just outside this spectral window; the intensity of Lys257 in the F4–F5 linker could not be easily monitored due to low intensity and spectral overlap.

These data reveal that the apo-F4 domain is preferentially loaded with Zn(II) in the presence of stoichiometric metal. After the binding of Zn(II) to apo-F4, the F5 and F6 domains compete about equally well for subsequent additions of Zn(II). Under conditions of excess Zn(II), the F6 domain is stably folded; in strong contrast, F5 resonances become exchanged broadened beyond detection (Fig. 7B).

Zinc Binding Studies of Apo-MTF-zf46—The conformational heterogeneity in F5 was further explored within the context of C-terminal two-finger domain fragment of hMTF-1, hMTF-
zf56, in an analogous Zn(II) titration. This experiment was carried out to determine if F4 influenced in any way the unusual structural properties of F5. Representative spectral regions of complete $^{15}$N HSQC spectra (the upfield $\alpha$-helical region is shown) for uniformly $^{15}$N-labeled hMTF-zf56 as a function of added Zn(II) are presented in Fig. 8 with normalized peak intensities plotted in Fig. 9. Tentative resonance assignments for F5 cross-peaks were obtained by analogy with F4 and F6 cross-peaks of the corresponding residues.

These data are in qualitative accord with the Zn(II) titration of $\alpha$-$^{15}$N-Lys-MTF-zf46 shown above (Fig. 7B). However, the surprising conclusion is that the entire F5 finger domain, in contrast to that of F6 (and F4), is only metastable under these conditions during the course of a zinc titration. In fact, at 2.0 mol eq of Zn(II), the F5 cross-peaks which derive from the $\beta\beta\alpha$-fold are already significantly broadened; at $\approx$ 3.0 mol eq of Zn(II), these peaks have effectively disappeared (Fig. 9). These data suggest that the canonical $\beta\beta\alpha$ structure of F5 is reorganizing into some other structure(s), which is in intermediate conformational exchange with the canonical structure, a structural change largely complete upon the addition of approximately one stoichiometric equivalent of excess Zn(II).
Cd(II) Binding to Apo-hMTF-zf46

Although numerous reports suggest that cadmium is a potent inducer of MRE-mediated MTF-1-dependent activation of gene expression in mammalian cells (4), the mechanism of the induction remains poorly defined. Furthermore, Cd(II) does not activate the DNA-binding activity of MTF-1, like that which can be demonstrated to occur with Zn(II). Thus, if folding of βα zinc fingers upon addition of Zn(II) lies as the molecular basis for Zn(II)-dependent stimulation of the MRE-binding activity, the prediction can be made that Cd(II) would not be capable of inducing the folding of the finger domains F4 and F6, for example.

An anaerobic Cd(II) titration of apo-MTF-zf46 as monitored by UV spectroscopy is shown in Fig. 10. This titration indicates that Cd(II) forms metal complexes with apo-MTF-zf46 involving at least some Cd(II)-cysteine thiolate coordination bonds. The intensity of the ligand-to-metal charge transfer absorption at 250 nm is apparently maximal at about 1.5–2.0 mol eq, which falls off slightly upon addition of one additional mol eq of the metal. The molar absorptivity, depending on how many Cd(II) ions are actually bound in each case, is consistent with no more than 1–2 cysteine thiolates per bound Cd(II) ion, if one assumes 5000 M⁻¹ cm⁻¹ per Cd-S bond at 250 nm (62).

Inspection of the 15N HSQC spectrum of -15N-Lys-MTF-zf46 reveals that the addition of 2 mol eq of Cd(II) fails to properly fold the structure, and that 2.0 mol eq of Zn(II) readily displaces the bound Cd(II) to give rise to spectrum indistinguishable from that recorded when 2.0 mol eq of Zn(II) are added directly to the apoprotein (Fig. 7A). These data reveal that apo-MTF-zf46 binds Cd(II) at cysteine-thiolates to form a weak, non-native complex, which are readily displaced upon addition of Zn(II). The same spectra are obtained when greater than two equivalents of metal are added.

**DISCUSSION**

An amino acid sequence alignment of MTF-1 from human, mouse, Japanese pufferfish, and *D. melanogaster* reveals that the zinc finger domain is the most highly evolutionarily conserved region of the molecule (13, 28). Finger F6 is the most divergent among all of the zinc fingers, but even these sequences share ~70% sequence similarity with most of the differences in the Cys-X₄-Cys loop. A sequence alignment of finger F5 domains (Fig. 1B) reveals that F5 is as highly conserved as the major MRE-binding zinc fingers of the molecule (F1–F4), despite the fact that it plays an ancillary role in DNA binding as revealed by limited proteolysis and biophysical char-
structure of Zn(II)-saturated hMTF-zf46 and hMTF-zf56 by far-UV-circular dichroism suggests the F5 domain does not adopt the predicted ββα fold under these conditions (32, 33) (data not shown). This is consistent with the $^{15}$N HSQC spectrum shown in Fig. 3 and, therefore, suggests that a slight excess of Zn(II) stabilizes one or more alternate conformations of the finger domain. The structure of this alternate conformation cannot be determined using NMR methods but would appear to have little or no stable secondary structure.

Although the structural details are unclear, the sequence conservation of both F5 among different MTF-1s is striking and unusual (Fig. 1B). A BLAST search reveals that no other Cys$_2$-His$_2$ zinc finger domain conserves the combination of potential zinc-liganding amino acids in the Cys-X$_4$-Cys loop, the His pair in the α-helix, and the strongly helix-promoting AFAA sequence in what is supposed to be the finger-tip region of the ββα structure (Fig. 1B). One or more of these conserved features could significantly alter the conformational equilibria of this finger when presented with a slight excess of Zn(II). Zn(II) titration experiments reveal that the ββα fold does become populated at low concentrations of the metal; thus, this sequence is intrinsically capable of forming a canonical zinc finger. However, as the Zn(II) concentration is raised, this structure is in equilibrium with one or more competing conformations, with chemical exchange broadening largely complete with the addition of a second equivalent of metal (Fig. 9). This situation is reversed in certain designed zinc finger peptides, which form a 2:1 peptide:metal complex at limiting metal, which then rearrange to the ββα fold upon addition of stoichiometric or greater metal (54). The simplest interpretation of our data is that the ββα conformation of F5 is unstable and readily binds a second mol eq of Zn(II), likely mediated by one or more the zinc-chelating residues situated in the Cys-X$_4$-Cys β-turn and/or the solvent-exposed His pair in the α-helix (Fig. 1B). Puzzling, however, is the finding that Co(II) appears to stabilize the ββα fold, as determined by Co(II) optical spectroscopy, even at excess metal (Fig. 2). Thus, the effects of Co(II) and Zn(II) on hMTF-zf structure are easily distinguished from one another. Co(II) is not a strong inducer of the MT gene expression in vivo. Unlike Co(II), the other strong inducer, Cd(II), binds to at least a subset of cysteine thiolates, but clearly induces the formation of an alternate structure(s).
rather than the βα fold under any conditions (Figs. 6 and 9). These findings are in agreement with recent proton NMR experiments carried out with Sp1-like peptides (65).

The functional impact of this structural heterogeneity in F5 in full-length MTF-1 is unknown and currently under investigation. However, it may well be important in zinc metallolregulation in this system, particularly so if large-scale structural changes occur in MTF-1 as a result. It is tempting to speculate that this additional bound Zn(II) ion might recruit additional Zn(II)-coordinating residues in the immediately adjacent acidic activation domain or the conserved Cys-rich metallothionein-like domain at the C terminus; such a structural change could functionally couple the DNA binding domain with the transcriptional activation domains and/or sequences responsible for nuclear export of MTF-1 (25) and therefore play an important role in signal transduction in this system.

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... and hypoxia (71, 72); and (iii) IHCA of the zinc finger domain is enhanced in patients with specific diseases, such as cancer (27, 28), Alzheimer’s disease (29, 30), and idiopathic pulmonary fibrosis (21). This study provides a detailed understanding of the structural and functional properties of the zinc finger domain in hypoxic conditions, which may have implications for the development of novel therapeutic strategies for these diseases.
Conformational Heterogeneity in the C-terminal Zinc Fingers of Human MTF-1: AN NMR AND ZINC-BINDING STUDY
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