Ratiometric Pulsed Alkylation/Mass Spectrometry of the Cysteine Pairs in Individual Zinc Fingers of MRE-Binding Transcription Factor-1 (MTF-1) as a Probe of Zinc Chelate Stability†

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ABSTRACT: Metal-response element (MRE)-binding transcription factor-1 (MTF-1) is a zinc-regulated transcriptional activator of metallothionein (MT) genes in mammalian cells. The MRE-binding domain of MTF-1 (MTF-zf) has six canonical Cys_2−His_2 zinc finger domains that are distinguished on the basis of their apparent affinities for zinc and their specific roles in MRE-binding. In this paper, pulsed alkylation of the zinc-liganding cysteine thiolate pairs with the sulphydryl-specific alkylation reagent d_5-N-ethylmaleimide (d_5-NEM) is used as a residue-specific probe of the relative stabilities of the individual zinc finger coordination complexes in Zn_6 MTF-zf. A chase with excess H_5-N-ethylmaleimide (H_5-NEM) to fully derivatize MTF-zf concomitant with complete proteolysis, followed by MALDI-TOF mass spectrometry allows quantitation of the mole fraction of d_5,Nd_5,N, d_5,H_5, and H_5,N,N-derivatized peptides corresponding to each individual zinc finger domain as a function of d_5-NEM pulse time. This experiment establishes the hierarchy of cysteine thiolate reactivity in MTF-zf as F_5 > F_6 >> F_1 > F_2 ≈ F_3 ∼ F_4. The apparent second-order rate of reaction of F_1 thiols is comparable to that determined for the DNA binding domain of Sp1, Zn_3 Sp1-zf, under identical solution conditions. The reactivities of all Cys residues in MTF-zf are significantly reduced when bound to an MRE-containing oligonucleotide. An identical experiment carried out with Zn_5 MTF-zf26, an MTF-zf domain lacking the N-terminal F_1 zinc finger, reveals that MTF-zf26 binds to the MREd very weakly, and is characterized by strongly increased reactivity of nonadjacent F_4 thiolates. These findings are discussed in the context of existing models for metalloregulation by MTF-1.

Zinc, an essential trace element found in over 300 proteins and enzymes, plays two primary roles in biology (1, 2). One is a structural role in which a coordinately saturated Zn(II) coordination complex functions to stabilize the native fold of a protein or an intermolecular protein−protein or protein−nucleic acid interface; without bound Zn(II), the conformation is usually altered and often locally unfolded and functionally inactive (3). Zinc finger proteins represent a prominent example of a well-understood structural role for zinc (for a review, see ref 4). Zn(II) can also play a catalytic role, where it typically functions as a Lewis acid in activating an attacking nucleophile, e.g., a water molecule in a hydrolytic enzyme (5). Although Zn(II) is not unique in its ability to perform these biological roles, the bioinorganic chemistry of Zn(II) is ideally suited to carry out these functions (6). In particular, Zn(II) is redox-inert due to its completely filled d-electronic shell and is considered a “borderline” Lewis acid that enables binding to a wide range of biological ligands while adopting a range of coordination numbers (typically 4 to 6) and geometries (7, 8).

All cell types possess regulatory machinery to tightly control the concentration of bioavailable zinc (9−12), although the precise mechanism of zinc toxicity remains unclear. In vertebrate cells, the metalloregulated transcriptional activator protein, metal response element (MRE)−binding transcription factor-1, MTF-1, plays a central role in detoxifying high concentrations of zinc and cadmium as well as protecting cells from oxidative stress (12−14). MTF-1 binds to a cis-acting element(s) found in the promoters of zinc-regulated genes termed a metal-response element (MRE) (15) and is required for both basal and zinc-stimulated transcription of the downstream genes (16, 17). MREs were first characterized in the promoters of genes encoding the abundant intracellular zinc sequestering agent, metallothionein, in mouse, rat, and human cells; more...
recently, functional MREs have been found in the promoters of the gene encoding ZNT1, a widely expressed zinc efflux pump (18, 19), and the gene encoding γ-glutamyl-cysteine synthetase, a highly regulated enzyme in glutathione biosynthesis (16), as well as other target genes (20).

MTF-1 is a canonical TFIIIA-type zinc finger protein that contains six tandemly arrayed Cys2-His2 (CCHH) zinc finger domains, each of which conforms to the Cys-X$_4$-Cys-X$_7$-His-X$_4$-His sequence (21) (Figure 1). The zinc finger domain is necessary and sufficient for high affinity binding to the MRE in vitro (22) and in vivo (23). Limited evidence suggests that flanking domains do stabilize the MTF-1-MRE complex (24) and are required to observe Zn(II)-dependent activation of MRE binding in vitro (cf. ref 25), but the mechanism is poorly understood. Since it is well established that tandemly arranged Cys$_2$-His$_2$ fingers bind to contiguous or overlapping 3–4 base pair subsites (4), the conserved portion of the 12-base pair MRE (26) is not large enough to accommodate sequence-specific recognition by all six zinc fingers in MTF-1. This has fueled speculation that the zinc fingers in MTF-1 play distinct functional roles, with a subset of zinc fingers playing a structural role in MRE-binding, with others required for Zn(II)-dependent activation of MRE binding and transcriptional activation (25). Recent structural and functional findings are largely consistent with this idea (22, 24, 27, 28).

In this study, we have determined the rate at which individual cysteine pairs in each zinc finger domain in MTF-zf is derivatized by the sulfhydryl-specific alkylating agent N-ethylmaleimide (NEM) as a probe of zinc chelate stability. Selective chemical modification and mass spectrometry have previously been used to probe protein surface topology (32, 33), protein–protein interactions (34), protein folding (35) and the reactivity of cysteine ligands in metal coordination complexes toward a variety of electrophiles (36), including NEM (37). The underlying principle in these studies is that the reactivity of a specific target residue is due to the net effect of its local environment. Here, we employ selective ratiometric chemical modification, combined with protease digestion and high-resolution mass spectrometry, a method described in detail elsewhere (38), to determine the reactivities of cysteine thiolate pairs in individual zinc finger domains in the uncomplexed and MRE-bound forms of Zn$_6$
FIGURE 2: Schematic representation of the ratiometric pulsed-alkylation mass spectrometry experiment with d5-NEM and H5-NEM used to probe the relative stabilities of individual Cys2–His2 zinc finger-Zn(II) complexes in MTF-zf. Low affinity complexes will be characterized by increased metal–thiolate dissociation, which would make these cysteine thiolates more nucleophilic toward the alkylating reagent d5-NEM, during the pulse time t. In contrast, cysteine thiolates in Zn(II) chelates of high stability will be refractory to modification by d5-NEM and will largely become derivatized during the chase with H5-NEM. The three alkylation products of the pulsed alkylation protocol, d5d4-, d5H-, and H5H5-NEM derivatized peptides, and their molecular masses are shown on the right.

MTF-zf at a resolution not previously possible. The results provide new insight into the solution structure, reactivity, and zinc chelate stability of individual zinc fingers in MTF-1.

MATERIAL AND METHODS

Materials. Dithiothreitol was obtained from Acros-Organics, while DTNB and Mops were from Sigma. d5-NEM was obtained from Medical Isotopes, Inc., while H5-NEM was purchased from ICN. Trifluoroethanol was obtained from Pierce Chemical Co., while HPLC-grade acetonitrile was purchased from EM Science. Sequencing grade trypsin was purchased from Boehringer-Mannheim. Bradykinin was obtained from Promega, while chymotrypsin was purchased from Sigma.

Purification of Zn6 MTF-zf and Zn5 MTF-zf26. The zinc finger domain fragment encoding residues 137–320 of human MTF-1 (14) (Figure 1) denoted MTF-zf (22) was expressed in Escherichia coli BL21(DE3) and purified to homogeneity using nondenaturing conditions essentially as described previously (22). The MTF-zf was then exhaustively dialyzed in an anaerobic Vacuum-Atmospheres glovebox against a zinc- and dithiothreitol-free buffer containing 50 mM Mops, 0.20 M NaCl, pH 7.0. The zinc content of MTF-zf used in these experiments was determined by flame atomic absorption on a Perkin-Elmer AAnalyst 750 atomic absorption spectrometer to be 6.5 mol of Zn(II)/mol of MTF-zf, while the mol equivalents of reduced cysteines was determined to 12.4 (12 expected) by anaerobic DTNB reactivity (38) using ε280 = 16 400 M−1 cm−1 (22). MALDI-TOF revealed a mass of 21 167.3 daltons (21 167.0 daltons calculated from the amino acid sequence) A pET-based overexpression plasmid for MTF-zf26 was prepared (designated pMTF-zf26, designed to express residues 167–320 of hMTF-1, with the MTF-zf26 purified essentially as described above for MTF-zf. Zn6 MTF-zf26 was found to contain 4.7 molar equivalents of Zn(II) and 9.5 (10 expected) reduced thiolates by DTNB reactivity. MALDI-TOF revealed a mass of 17 819.1 daltons (17 819.3 daltons calculated from the amino acid sequence) with an N-terminal sequence of MEYTFv(X), which matches the expected sequence of M16ETYFV (14) (Figure 1).

Purification of Sp1-zf. The bacterial Sp1-zf expression plasmid, pSP1-Zn92 (40), was kindly provided by Professor John Caradonna (Boston University). E. coli BL21(DE3) transformed with pSP1-Zn92 was propagated on rich LB media at 37 °C, with the expression of Sp1-zf induced with 0.4 mM IPTG at an A600 of 0.4 essentially as described. After 4 h, the cells were harvested by low-speed centrifugation. The wet cell paste from 9 L of cell culture was resuspended and sonicated using the methods described above for MTF-zf (vide supra). Solid urea was then added to the supernatant to a final concentration of 5.0 M, and subsequently acidified to pH 2 with HCl, with the resulting suspension centrifuged for 30 min at 12 000 rpm in a Beckman JA-20 rotor. The resulting supernatant was loaded onto a Waters Powerline HPLC system running a POROS reversed-phase C4 column (Perseptive Biosystems) equilibrated with 0.1% trifluoroacetic acid (TFA) and developed with an acetonitrile gradient in 0.1% TFA. Fractions containing Sp1-zf were identified by SDS–PAGE, pooled, and then lyophilized to dryness. The dried acidified apo Sp1-zf sample was then brought into the glovebox, dissolved in a degassed zinc- and dithiothreitol-free buffer containing 50 mM Mops, 0.20 M NaCl, pH 7.0, and subjected to exhaustive anaerobic dialysis against the same buffer to create apo-Sp1-zf. Following dialysis, 3.0 molar equivalents of ZnCl2 was added anaerobically. Zn5 Sp1-zf prepared in this way was found to contain 3.5 (±0.2) mol equivalents of Zn(II) and 6.0 mol equivalents reduced cysteines by DTNB reactivity.

Purification of the MREd-Containing Oligonucleotide. 1 μmol synthesizes of crude, complementary mouse MTI-MREd-containing 23-nucleotide oligonucleotides (“top” strand: 5′-GAGCTCTCGACCTCCGAAGAAAN-3′, which matches the expected sequence of 130 MEYTFV(15) with an N-terminal sequence of MEYTFv(X), which matches the expected sequence of M16ETYFV (14) (Figure 1).
to give a final 100:1 molar ratio of H$_2$-NEM:Cys thiolate (the chase contained 10:1 H$_2$-NEM:d$_5$-NEM). Three hours after the last pulse time point was taken, the proteolyzed samples were removed from the glovebox and prepared for elution from an Amika Corp. C4 microtip column. The tip columns were first wet with HPLC-grade acetonitrile, before equilibrating them with 2 mL of 0.1% TFA solution. The sample was loaded and then washed with 2 mL of 0.1% TFA solution before elution with a 0.1% TFA/100% acetonitrile solution. The sample was vacuum-centrifuged to dryness and dissolved in 50 µL deionized, distilled water. A 4 µL aliquot of each sample was then diluted to a final protein concentration of $\cong$0.1 mg/mL in 40 µL of a solution containing 1.5 mg/mL α-cyano-4-hydroxycinnamic acid in 25% aqueous methanol. These samples were then deposited as a series of 500 nL spots on top of an air-dried layer of matrix made by methanol. These samples were then deposited as a series of 500 nL spots on top of an air-dried layer of matrix made by methanol. These samples were then deposited as a series of 500 nL spots on top of an air-dried layer of matrix made by methanol. These samples were then deposited as a series of 500 nL spots on top of an air-dried layer of matrix made by methanol. These samples were then deposited as a series of 500 nL spots on top of an air-dried layer of matrix made by methanol.

An identical experiment was carried out with Zn$_6$ MTF-zf bound to a 23-base pair oligonucleotide containing a single copy of the MReD sequence from the mouse MT-I promoter (27), formed anaerobically by mixing 2.0 mL of 5 µM Zn$_6$ MTF-zf with 28 µL of a 730 µM MReD solution for 15 min (10 µM MReD), prior to addition of d$_5$-NEM as described above. The samples were processed in exactly the same way as free MTF-zf samples. For the experiments that probed the reactivity of Cys thiolates in Zn$_5$ MTF-zf26, 12 µL 0.10 M d$_5$-NEM was added to 2.0 mL 4.76 µM Zn$_5$ MTF-zf26 (0.60 mM d$_5$-NEM final; 12.6-fold excess over cysteine thiolates). At some pulse time, t, 200 µL was withdrawn and added to a solution containing 80 µL of 0.5 M NaCl and 24 µL 0.1 M H$_2$-NEM, for a final concentration of 7.89 mM H$_2$-NEM (13.2:1.0 H$_2$-NEM:d$_5$-NEM). This sample was then divided into two parts, and 10 µL of 0.1 mg/mL solution of trypsin or 0.125 mg/mL chymotrypsin added to each tube. The digestion reactions were allowed to proceed for at least 3 h at ambient temperature, and the resulting peptides prepared for mass spectrometry essentially as described above. For the Zn$_5$ MTF-zf26:MReD complex, an identical experiment was carried out except that 8.76 µM MReD oligonucleotide was present in the pulse-chase.

To measure the reactivity rate profiles of cysteine thiolate pairs in Sp1-zf, 10 µL of 0.10 M d$_5$-NEM was added at time zero to 600 µL of 25 µM Zn$_5$ Sp1-zf (150 µM Cys residues; 1.64 mM d$_5$-NEM), incubated for various lengths of time, whereupon 50 µL aliquots were withdrawn and mixed with a solution containing with 10 µL of 0.10 M H$_2$-NEM, 80 µL of 0.5 M NaCl, and 2 µL of 1 mg/mL sequence grade trypsin (12.1:1.0 H$_2$-NEM:d$_5$-NEM). These samples were then allowed to digest in the glovebox overnight. One-tenth of each sample (6 µL) was then diluted to a final protein concentration of $\cong$0.1 mg/mL in 2 µL of a 1.5 mg/mL ferulic acid in 25% aqueous methanol. These samples were then deposited as a series of 500 nL spots on top of an air-dried layer of matrix made by spotting 5 µL of a 30 mg/mL solution of α-cyano-4-hydroxycinnamic acid in pure methanol. Control experiments were also carried out with apo Sp1-zf, generated by incubating Zn$_5$ Sp1-zf with 300 µM EDTA, or in the presence of 2 mol equivalents of excess Zn(II).

Sp1-zf samples were subjected to mass spectrometry exactly as described above. In all cases, two independent pulsed alkylation/mass spectrometry experiments were carried out for each sample condition, one of which was used to collect data at shorter times (0–60 min d$_5$-NEM pulse) and one at long times (40–250 min) with the combined data simultaneously analyzed as described below.

**MALDI-TOF Mass Spectrometry.** MALDI-TOF mass spectra of all samples were acquired using a Perseptive Biosystem Voyager Elite XL TOF mass spectrometer equipped with a pulsed nitrogen laser emitting at 337 nm manufactured by Laser Science Inc. All spectra were acquired in the positive ion mode using 25 kV acceleration. Each spectrum is the average of 100 laser pulses. The spectra for the tryptic and chymotryptic peptides of MTF-zf were acquired with 200 ns delayed extraction in reflectron mode. The grid and the guide wire voltages were operated at 70 and 0.05% of the acceleration voltage, respectively. Bradykinin [1060.5 Da]$^{15}$ was used as external calibration for the mass spectrometer prior to mass analyses of tryptic and chymotryptic peptides. The mass resolution achieved with this experiment is routinely 0.2 ppm, with typically reproducibility between samples of ± 0.2 ppm.

A single MALDI-TOF mass spectrum was acquired for each d$_5$-NEM time pulse-H$_2$-NEM chase experiment and processed using GRAMS 32 for peak localization and integration. Each mass spectrum contains a complete set of tryptic (or chymotryptic) peptides except that all cysteine-containing peptides are fully alkylated at each of the two cysteine residues by NEM, which are resolved as either H$_2$H$_5$-NEM derivatized peptides (mass = expected peptide mass + 250 mu), d$_5$H$_5$-NEM derivatized peptides (mass = expected peptide mass + 255 mu), or as d$_5$d$_5$-NEM derivatized peptides (mass = expected peptide mass + 260 mu) by mass spectrometry (Figure 2). The sequences of the tryptic and chymotryptic peptides used for this purpose are listed in Table 1. The mol fraction ($\Theta$) of each of the three doubly derivatized peptides in a mixture is simply given as the ratio of the peak area integration (A) of the ith NEM-derivatized species to the sum of the integrated areas of all (H$_5$H$_5$ + H$_5$d$_5$ + d$_5$d$_5$) alkylated species. For example, the mol fraction of the d$_5$d$_5$-derivatized peptide, $\Theta$(d$_5$d$_5$), is defined as

$$\Theta(d_5d_5) = A(d_5d_5)/[A(H_5H_5) + A(H_5d_5) + A(d_5d_5)]$$

As the pulse time of d$_5$-NEM reactivity increases, $\Theta(d_5d_5)$ will increase with other species correspondingly decreasing. Any cysteine thiolate that survives the d$_5$-NEM pulse is then fully alkylated in the chase by an NEM solution containing ≈10:1 molar ratio of H$_2$-NEM to d$_5$-NEM. A chase designed in this way will derivatize all unmodified two-Cys containing peptides that survive the d$_5$-NEM with an isotopic distribution of H$_5$H$_5$:d$_5$:d$_5$ of $\approx$10:1:0.1. Thus, the mole fraction of reactive zinc finger cysteine pairs for the jth zinc finger domain peptide (ZF)$^j$ which are alkylated in the d$_5$-NEM pulse, $\Theta$(reactive ZF)$^j$, will not be substantially perturbed by this chase composition. Therefore,

$$\Theta(\text{reactive } ZF)^j = \Theta(d_5d_5)$$

The mole fraction of unmodified zinc finger domain peptides,
by problems associated with differentiated peptides, the quantitation is not complicated satisfactorily fit to a first-order exponential decay, as exactly as described previously (22). L-format (40 mM Mops, pH 7.0, 0.20 M NaCl, 25 °C) steady-state anisotropy of coumarin-labeled MREd duplex with the proteases.

**RESULTS**

Cysteine Thiolate Alkylation Profiles with the Model Zinc Finger Protein, Sp1-zf. A stack plot of representative sections of MALDI-TOF mass spectra derived from different d5-NEM pulse times selected to show the isotopic distribution of the doubly-NEM derivatized tryptic peptides which correspond to zinc finger F2 (Table 1) of Zn3 Sp1-zf is shown in Figure 3A. Analogous sections selected to show reactivity profiles for F1 and F3 are not shown due to space considerations. Note that H5,H5-, d5,H5-, and d5,d5-NEM derivatized peptides are cleanly separated in the mass spectrum in a manner consistent with their expected isotopic distributions, with sufficient signal-to-noise for quantitation of individual peptide species. Second, as expected, as the pulse time with d5-NEM increases, there is a greater fraction of doubly alkylated F2 peptide containing two d5,d5-NEM adducts, with a corresponding decrease in the fraction of peptides recovered as H5,H5-derivatized peptides (not shown). In no case, under these conditions, does the mixed derivatized H5,d5-peptide accumulate to appreciable fraction of the total alkylated species. Since under all conditions, NEM is in great molar excess over cysteine thiolates, the rates of decay of H5,H5-, peptide and appearance of d5,d5-peptide each follow a pseudo-first-order process (Figure 3C) characterized by a rate constant of reactivity, k_app, of 5.4 (± 0.3) x 10⁻³ min⁻¹. If k_app is expressed as a function of the total d5,d5-NEM concentration (1.64 mM), an apparent second-order rate constant of 3.26 (± 0.23) M⁻¹min⁻¹ is obtained (Table 2).

Figure 3B shows that coordination to Zn(II) by F2 results in a substantial protection against cysteine thiolate reactivity as expected (30), since all cysteine thiols are fully derivatized during the d5-NEM pulse time when the reaction is run in the presence of excess EDTA. Although the rate of reaction of Zn(II)-free thiolates was too fast to measure from this experiment, a lower limit for the apparent second-order rate constant is >5000 M⁻¹min⁻¹, or greater than 250 times faster than in the presence of Zn(II). This rate constant is of the same order of magnitude as the true second-order rate constant measured for the alkylation of the sulfhydryl group of glutathione (k = 38 000 M⁻¹min⁻¹) (41) and faster than the solvent-exposed Cys106 in the α-subunit of bacterial luciferase (k = 1670 M⁻¹min⁻¹) under similar
solution conditions \( \text{pH} = 7.0, 25 \, ^\circ\text{C} \) \((42)\). The kinetics of pulsed alkylation of the F1 thiolates in Zn\(_3\) Sp1 are indistinguishable from that of the F2 cysteine pair, with \( k_{\text{app}} = 5.3 \pm 0.3 \times 10^{-3} \text{ min}^{-1} \) (Table 2). In contrast to F1 and F2, the alkylation profile for F3 thiolates in Zn\(_3\) Sp1-zf is best described by a sum of two exponentials of approximately equal amplitude, with the second slower phase \( (A_2 = 0.54) \) indistinguishable from that of F1 and F2, with \( k_{\text{app}} = 4.9 \pm 1.2 \times 10^{-3} \text{ min}^{-1} \) (Table 2). The faster phase \( (A_1 = 0.46) \) is \( \approx 21 \)-fold faster, with \( k_{\text{app}} = 0.11 \pm 0.03 \text{ min}^{-1} \). Repeating this experiment in excess EDTA has no effect (data not shown). The structural origin for the two distinct phases of pulsed alkylation for F3 thiolates is unknown but may be reporting on two (or more) distinct conformations of F3 in Zn\(_3\) Sp1-zf which coexist in solution. F3 of Sp1-zf differs from F1 and F2 in that it is characterized by Cys–Pro–Glu–Cys–Pro (Cys–Pro–Glu–Cys–Pro) sequence, while F1 and F2 have Cys–Xel–Cys sequences \((43)\).

These control experiments with Zn(II)-saturated Sp1-zf show that the reactivity profiles of cysteine thiolate pairs within the three-finger domain fragment are nearly identical and follow the expected pseudo-first-order kinetics of alkylation, at least with F1 and F2 thiolates. Furthermore, the data suggest that the distinct characteristic of F3 that leads to two resolvable kinetic phases has no effect on the neighboring zinc finger domain F2, since the reactivity profile of F1 is indistinguishable from that of F2. This result suggests that the intrinsic reactivities of individual zinc finger domains may not be strongly influenced by alkylation at neighboring finger domains. This is the expected behavior of three independently folded, noninteracting zinc finger domains that are connected by flexible tethers, which likely characterizes uncomplexed Sp1-zf well \((43, 44)\).

**Table 2: Apparent Second Order Rates of Reactivity of Cysteine Pairs (\( k_{\text{app}} \)) in Individual Zinc Fingers in MTF-zf, MTF-zf26, and Sp1-zf in the Presence and Absence of Bound DNA\(^*\)**

<table>
<thead>
<tr>
<th>protein</th>
<th>( k_{\text{app}} ) (M(^{-1})NEM min(^{-1}))</th>
<th>fold-protection(^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTF-zf:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−MREd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>5.66 (± 0.35)</td>
<td>0.30 (± 0.01)</td>
</tr>
<tr>
<td>F2</td>
<td>1.42 (± 0.55)</td>
<td>0.34 (± 0.16)</td>
</tr>
<tr>
<td>F3</td>
<td>3.80 (± 0.40)</td>
<td>0.72 (± 0.14)</td>
</tr>
<tr>
<td>F4</td>
<td>2.50 (± 0.33)</td>
<td>0.71 (± 0.21)</td>
</tr>
<tr>
<td>F5</td>
<td>43.8 (± 3.3)</td>
<td>1.40 (± 0.21)</td>
</tr>
<tr>
<td>F6</td>
<td>23.0 (± 5.5)</td>
<td>2.51 (± 0.37)</td>
</tr>
<tr>
<td>MTF-zf26:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>2.02 (± 0.14)</td>
<td>0.92 (± 0.14)</td>
</tr>
<tr>
<td>F3</td>
<td>3.39 (± 0.23)</td>
<td>1.61 (± 0.10)</td>
</tr>
<tr>
<td>F4</td>
<td>12.8 (± 0.4)</td>
<td>5.94 (± 0.18)</td>
</tr>
<tr>
<td>F5</td>
<td>22.3 (± 1.2)</td>
<td>13.1 (± 0.9)</td>
</tr>
<tr>
<td>F6</td>
<td>24.7 (± 1.8)</td>
<td>15.0 (± 0.8)</td>
</tr>
<tr>
<td>Sp1-zf:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>3.25 (± 0.20)</td>
<td>N.D.</td>
</tr>
<tr>
<td>F2</td>
<td>3.26 (± 0.23)</td>
<td>N.D.</td>
</tr>
<tr>
<td>F3</td>
<td>2.99 (± 0.74) ((A_2 = 0.54)^*)</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

\( ^* \) Determined as described in Materials and Methods (see Figures 4, 5, and 7). \(^{1}\) \( k_{\text{app}} \)\(^{-1}\text{M}^{-1}\text{NEM min}^{-1} \)\(^{-1}\); \(^{2}\) Not determined. \(^{2}\) Two rate constants were resolved from these data (see text for details).
in Figure 5 in the absence of DNA. As can be seen, F6 thiolates are considerably more reactive than are F1 thiolates, with an apparent second-order rate constant of 5.7 (± 0.4) M<sub>NEM</sub> min<sup>-1</sup> for F1 cysteines versus 23.0 (± 5.5) M<sub>NEM</sub> min<sup>-1</sup> for F6 thiolates, or approximately 5-fold faster under these conditions (Table 2). For comparison, the same analysis for F3 thiolates gives $k_{app}$ = 3.8 (± 0.4) M<sub>NEM</sub> min<sup>-1</sup> or about 8-fold slower that F6 cysteines.

Figure 6A shows a bar plot of the apparent second-order rate constants obtained for the kinetics of the appearance of Cys pairs in MTF-zf in the presence and absence of bound MRE<sub>d</sub> oligonucleotide. These data are also summarized in Table 2. In contrast to Sp1-zf, the rate constants for pulsed alkylation are remarkably nonuniform. The data suggest that uncomplexed Zn<sub>6</sub> MTF-zf contains roughly two zinc sites of relatively lower affinity and 3–4 of high affinity. These pulsed alkylation experiments provide direct evidence that F5 and F6 are indeed the weak zinc-binding fingers of MTF-zf (27), with finger domains F1–F4 forming very stable complexes with the metal.

The reactivities of all finger domains in MTF-zf are strongly reduced when bound to the high affinity MRE<sub>d</sub> oligonucleotide, with F6 becoming the most reactive finger of MTF-zf (27), with finger domains F1–F4 forming very stable complexes with the metal.

with 2–3 zinc sites of relatively lower affinity and 3–4 of high affinity. These pulsed alkylation experiments provide direct evidence that F5 and F6 are indeed the weak zinc-binding fingers of MTF-zf (27), with finger domains F1–F4 forming very stable complexes with the metal.
Cysteine Thiolate Reactivities in Individual Zinc Fingers in the Uncomplexed and the MREd-Bound Forms of Zn5 MTF-zf26, a ΔF1 Deletion Mutant. A recently proposed functional model of MTF-1 activity invokes a special role for zinc finger F1 in reversible activation of high affinity MRE binding by low affinity Zn(II) binding to this zinc finger domain (28). This model makes the prediction that F1 would have a lower affinity for Zn(II) than the other finger domains. Furthermore, functional characterization of ΔF1 MTF-1 in vivo suggested that this form of intact MTF-1 is a constitutive activator of MRE-driven reporter gene expression, a finding that makes the prediction that ΔF1 MTF-zf would have high affinity for the MREd, whose binding activity could not be modulated by exogenous Zn(II). It was therefore of interest to characterize Zn5 MTF-zf26 in our pulsed alkylation experiments.

The apparent second-order rate constants measured for alkylation of cysteine pairs in each of the five zinc fingers for Zn5 MTF-zf26 in the absence (gray bars) and presence (black bars) of bound MREd oligonucleotide. The same data are shown for Zn3 Sp1-zf on the right side of the figure for comparison.

FIGURE 7: Binding isotherms of Zn6 MTF-zf (●) and Zn5 MTF-zf26 (○) to a coumarin-labeled 23-base pair MRE-containing oligonucleotide duplex of the same sequence as used panel A (81 nM duplex, 40 mM Mops, 0.20 M NaCl, pH 7.0, 25 °C). The solid curve in each case defines a fit to a 1:1 binding model. For MTF-zf, $K_a = 1.05 (± 0.17) \times 10^4$ M$^{-1}$; $r_{max} = 0.273$. For MTF-zf26, $K_a = 1.03 (± 0.15) \times 10^6$ M$^{-1}$; $r_{max}$ fixed at 0.268 (or comparable to the change in $r$ with MTF-zf). The residuals are shown in the top portion of the figure. MTF-zf (●); MTF-zf26 (○).

The F4 domain thiolates in MTF-zf26 are highly reactive toward d5-NEM, with $k_{app}$ within a factor of 2 of the cysteine pairs in the C-terminal finger domains F5 and F6. Furthermore, the protection afforded by MREd binding is far less than that observed for cysteines in the MTF-zf-MRE complex and is never larger than a factor of approximately two (Figure 6B; Table 2), a result that suggests a significantly lower equilibrium binding affinity for the MREd. Consistent with this, a fluorescence anisotropy-based DNA binding experiment reveals that Zn5 MTF-zf26 binds to a coumarin-labeled MREd oligonucleotide $\approx$100-fold more weakly than MTF-zf (Figure 7). In fact, the binding isotherm for MTF-zf26 is not well modeled by a simple 1:1 binding model and may be indicative of essentially nonspecific binding or two or more MTF-zf26 monomers. Interestingly, the addition of 50 $\mu$M Zn(II) to these binding assays increases the apparent affinity of MTF-zf26 for the MREd sequence by a factor of approximately 10 (data not shown). We conclude that deletion of F1 from MTF-zf is markedly destabilizing, both toward the structure of the uncomplexed zinc finger domain and toward MTF-zf-MREd complex formation.

Evidence against Independent Reactivity of Cysteines in Internal Zinc Fingers in MTF-zf. If all the zinc fingers of MTF-zf behave in solution as independent folded domains connected by flexible tethers, the kinetics of reaction at each thiolate pair should obey pseudo-first-order kinetics under these conditions. More detailed analysis of the kinetics of alkylation of cysteine thiolates in MTF-zf F4, in particular (Figure 8A), reveals that these progress curves are modeled better by a sequential two-step mechanism

A $\rightarrow$ B $\rightarrow$ C

defined by the rate constants $k_{app}^{1}$ and $k_{app}^{2}$, with C corresponding to the doubly alkylated F4 peptide. If the value of one of these rate constants is constrained to $k_{app}$ for the
addition of 12 and 6 NEM groups per peptide chain, respectively. The method we outline here is perfectly general, i.e., any residue-specific reagent that results in a satisfactory increase in the molecular mass of the protein can be used and, when carried out in a ratiometric mode is particularly powerful, since the quantitation of the kinetics of reactivity relies only the relative, rather than absolute, amounts of deuterated and protiated peptides resolved by the mass spectrometer. This mass spectrometry-based method is exactly analogous to previously described radioisotope-based ratiometric $^{[3]H}/[^{14}C]$ acetylation used to probe the intrinsic reactivities of lysine residues toward $^{[3]H}$- vs $^{[14}C$-acetic anhydride (45).

**DISCUSSION**

In this paper, we present a new application of ratiometric pulsed alkylation mass spectrometry (38) to probe the residue-specific kinetics of reaction of cysteines in zinc finger proteins with a sulfhydryl-specific alkylation reagent, $N$-ethylmaleimide. Although NEM will potentially react with other nucleophiles, we could find no evidence for derivatization of other amino acid residues under these solution conditions, with fully alkylated MTF-zf or Sp1-zf each characterized by a molecular mass consistent with the
remarkably uniform (Figure 6). Inspection of the average solution structures of the F2 and F3 finger peptides of Sp1-zf reveals that in both domains, the S' atom of the second Cys in the Cys−X3−Cys sequence is largely exposed to solvent, while the most N-terminal Cys is largely buried (43). Interestingly, the S' atom of the second Cys in the Cys−X3−Cys in F3 appears somewhat more exposed to solvent relative to that in the Cys−X4−Cys F2 domain; this of course depends to some degree on the nature and side chain conformations of residues on the surface of the domain. In any case, we see no evidence for the accumulation of d3-H2-derivatized peptide over the time course (hours) of the experiment (Figure 5); this would be expected if the singly alkylated zinc finger domain was a stable intermediate that was slowly converted to the fully d4-d4-derivatized adduct. This suggests that once one of the cysteines in a zinc finger structure is alkylated by NEM, the other cysteine is largely buried (15173) and is rapidly alkylated. This behavior contrasts with what was found previously with CysSz−Hisz retroviral-type nucleocapsid protein zinc finger domains, where a singly alkylated zinc finger peptide accumulated as an intermediate to a fully derivatized peptide (37).

Structural Conclusions for the Zinc Finger Domain of MTF-1. In contrast to that which was observed for Sp1-zf, the alkylation profiles for the each of the zinc finger domains in the zinc finger fragment of MTF-1 are remarkably nonuniform (Figures 4–6). The C-terminal fingers are far more reactive than the other N-terminal zinc finger domains of the fragment, the latter of which are characterized by alkylation rate constants quite similar to Sp1−zf. This finding is consistent with the idea that there is clear structural heterogeneity among the zinc fingers of MTF-zf, and that this heterogeneity can be detected at the level of Zn(II)-S' coordination bonds. Cysteine thiolates in zinc fingers F5 and F6 are far more reactive, which suggests the stabilities of these zinc chelates may well be lower. These domains correspond to the two zinc domains from which bound Zn(II) can be lost upon extensive dialysis (22). One possible interpretation of these findings is that the F5 and F6 do not actually adopt stable folded βα-structures in the presence of saturating Zn(II) under these conditions. In fact, previous far-UV CD studies were interpreted to suggest that one or more of the C-terminal fingers adopts an alternative conformation under conditions of saturating Zn(II) (27). Heteronuclear NMR studies of a peptide fragment encompassing the C-terminal F4, F5, and F6 finger domains, MTF-zf46, show that F4 and F6 do indeed adopt βα-structures, with F6 characterized by a lower affinity as determined by in a zinc titration experiment with apo-MTF-zf46 (50). Furthermore, at saturating Zn(II), F5 apparently does not adopt a stable βα-structure but is instead in equilibrium with an alternative, as yet undefined, conformation (50). This might explain its enhanced reactivity relative to F6. Regardless of the structural details, these data suggest that the stability of F5 is even less than that of F6 with both domains far less stable than that of the N-terminal fingers; this would give rise to the observed strongly enhanced reactivities of F5 and F6 thiolates toward NEM.

More detailed inspection of the alkylation rate profiles of free Zn6 MTF-zf reveals that the N-terminal finger F1 is characterized by a reactivity that is slightly enhanced relative to F2, F3, and F4. In contrast, under all conditions, F2 and F4 thiolates are extremely slow to react, with the kinetics of reactivity of F4 cysteines, in particular, better modeled by a sequential mechanism (Figure 8A). Characterization of ΔF1 MTF-zf26 suggests that the N-terminal F1 domain plays some role in mediating this protection of F4 thiolates since deletion of the F1 domain results in conversion of F4 thiolates to an alkylation profile that obeys pseudo-first-order kinetics (Figure 8B), but reflective of a zinc complex that is kinetically quite labile (Figure 6). Although the interpretation of this experiment must be viewed with caution, one scenario that would give rise to this behavior is one where nonadjacent fingers F1 and F4 interact directly with one another, or alternatively, the presence of the F1 domain in MTF-zf stabilizes an intramolecular interaction between F4 and adjacent zinc domains. Only upon disruption of the F1 domain by alkylation do the F4 domain thiolates become susceptible to alkylation. There is at least one example of a Cysz−Hisz zinc finger protein, mouse GLI, where it has been shown that adjacent fingers (F1 and F2) pack against one another, at least in the GLI-DNA complex (51). More detailed structural studies are required to substantiate this proposal.

The MTF-zf:MREd Complex. Previous limited trypsinolysis experiments revealed that the N-terminal fingers of MTF-zf are bound to the MREd in way in which these finger domains are protected from proteolysis (27). In contrast, the C-terminal finger domains F5 and F6 (C-terminal to Arg260; see Figure 1) are readily digested to smaller fragments by trypsin. This suggested that finger domains F5 and F6 may not be as intimately associated with the MREd as the N-terminal fingers. On the other hand, near-UV CD, FRET, and fluorescence anisotropy experiments suggested that C-terminal fingers made a detectable contribution to the structural changes induced on MTF-zf binding to the MREd oligonucleotide, as well as the affinity and specificity of the protein-DNA complex, perhaps through sequence nonspecific interactions (27). Our pulsed alkylation experiments reveal that the reactivity of all finger domains, including F5 and F6, are strongly protected from alkylation in the high affinity MTF-zf:MREd complex. Strikingly, F5 thiolates go from the most reactive in the uncomplexed molecule to within a factor of ≤3 of F1−F4 cysteines (Table 2). On the other hand, F6 shows the lowest fold-protection upon MREd binding and is 5−10-fold more reactive than F1−F4 thiolates toward alkylation by NEM in the complex. These results are fully compatible with previous studies and strongly suggest that F5, and to a lesser extent, F6 zinc finger domains interact directly with the DNA. A recent proposal for structure of the MTF-zf:MREd complex places these finger domains downstream of the highly conserved 5'-TGCRGCNC core sequence element, overlapping the flanking GC-rich subdomain of the high affinity mMREd (25).

Although the differences are small, the N-terminal finger domain F1 becomes the least reactive in the protein-MREd complex and is also characterized by the second largest fold-reduction in alkylation rate constant, of nearly ~19-fold (Table 2). This suggests that F1 is intimately associated with the DNA and may play a key role, directly or indirectly, in organizing the MTF-zf on the MREd in such a way that high affinity binding results. Consistent with this idea is the
finding that the ΔF1 MTF-zf26 binds quite weakly to the MREd, as measured directly by fluorescence anisotropy binding experiments (Figure 7) and the extent of protection of the cysteine thiolates from alkylation by NEM in the protein–DNA complex (Figure 6B; Table 2). Thus, F1 appears to play a critical role in stabilizing the overall structure of zinc finger fragment of the MTF-1, as well as in MTF-zf:MREd complex formation, either directly or indirectly. Recent studies of “finger-swap” mutants of Sp1-zf suggest that the C-terminal F3 zinc finger plays a primary role in maximizing the complementarity of the interactions between the nonadjacent F1 zinc finger and DNA major groove (52). A primary role of F1 in MRE-binding by MTF-1 is in contrast to a recent report that suggested that deletion of F1 from intact MTF-1 results in a protein that binds the MREd constitutively, at least as measured by gel mobility shift assays in crude cell extracts (28).

Implications for the Mechanism of Metalloregulation by MTF-1. Our findings establish that F5 and F6 zinc finger domains form less stable zinc chelates at equilibrium or are characterized by enhanced rates of microscopic dissociation of Zn(II)-S coordination bonds, relative to other domains of the molecule. If the mechanism of zinc-dependent activation of gene expression lies principally at the level of activation of DNA binding by reversible binding of Zn(II) to weak binding fingers of the molecule, these findings implicate zinc finger domains F5 and/or F6 as playing a functionally important roles in transcriptional activation in vivo. However, recent mammalian cell transfection experiments in which “missing-finger” and “broken-finger” mutants of F5 and F6 in mMTF-1 were characterized showed essentially no diminution of the ability of zinc to activate MREd binding in gel mobility shift assays, and in one case, appeared to have no effect on the zinc-induced transcriptional activation (24, 28). Thus, the functional roles played by F5 and F6, under conditions of overexpression in mammalian cells, therefore remains unclear. In contrast, F1 (like F2, F3, and F4) have been shown to play important roles in functional activity of MTF-1, since broken-finger mutations deposited in F1, F2, F3, or F4 appeared to block MRE binding in vitro. A more recent report suggested that apo-F1 plays a negative regulatory role in modulating the MREd binding affinity of MTF-1; under conditions of low Zn(II), F1 contains no bound Zn(II) and physically occludes DNA binding by DNA-binding fingers F2, F3, and F4. When F1 is loaded with Zn(II), this negative repression is removed, and high affinity binding results (28). Deletion of F1 removes this negative regulation and creates a zinc-independent constitutive activator. These results make the prediction that F1 binds Zn(II) weakly, and its deletion would not be detrimental to high affinity MREd binding. Our pulsed alkylation experiments are not strongly supportive of either prediction. Although our results do suggest that reactivity of F1 can be distinguished from F2, F3, and F4, the effect is small; in fact, the alkylation rate constant for F1 thiolates is within a factor of 2 of that of the Sp1-zf zinc finger domains. This does not rule out a regulatory role for F1 based solely on differential affinity for Zn(II) since, in particular, it is not yet known how flanking regions might influence the Zn(II)-binding properties of individual zinc fingers. Furthermore, if recent studies from prokaryotic systems can be extended to mammalian cells, zinc homeostasis is not necessarily dictated by thermodynamic stability of metallo-regulatory zinc complexes and may well be under kinetic control (53). In any case, Zn(II)-loaded F1 clearly plays a critical role in stabilizing the protein–DNA complex. In fact, the affinity of MTF-zf26 is comparable to that of MTF-zf13 under the same solution conditions and may be indicative of essentially nonspecific binding (27). The implication of these results is that F1 and F4 play key roles in maintaining high affinity and specificity of binding by the zinc finger fragment of MTF-1. Further studies are required to better understand how and to what extent the zinc finger domain of intact MTF-1 mediates the zinc metalloregulation of MRE binding in vitro and transcriptional activation in mammalian cells in vivo.

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