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Published on Web 08/17/2006

Comparison of the Specificity of Interaction of Cellular and Viral Zinc-Binding Domains with 2-Mercaptobenzamide Thioesters

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Abstract: The interactions of two 2-mercaptobenzamide thioester compounds with six diverse zinc-binding domains (ZBDs) have been analyzed by UV/visible spectroscopy, NMR spectroscopy, and nucleic acid binding assays. These thioester compounds serve as useful tools for probing the intrinsic chemical stability of ZBDs that exist within a variety of cellular and viral proteins. In our studies, the classical (Cys_2His_2) zinc finger ZBDs, the interleaved RING like ZBDs of protein kinase C δ ($Cys_2HisCys$ and $HisCys_3$), and the carboxyl-terminal ($Cys_2HisCys$) ZBD of Mouse Mammary Tumor Virus nucleocapsid protein (MMTV NCp10) were resistant to reaction with the thioester compounds. In contrast, the thioester compounds were able to efficiently eject zinc from the amino-terminal ($Cys_2HisCys$) ZBD of MMTV NCp10, a $Cys_2HisCys$ ZBD from Friend of GATA-1 (FOG-1), and from both Cys_4 ZBDs of GATA-1. In all cases, zinc ejection led to a loss of protein structure. Interestingly, GATA-1 was resistant to reaction with the thioester compounds when bound to its target DNA sequence. The electronic and steric screening was calculated for select ZBDs to further explore their reactivity. Based on these results, it appears that both first and second zinc-coordination shell interactions within ZBDs, as well as nucleic acid binding, play important roles in determining the chemical stability and reactivity of ZBDs. These studies not only provide information regarding the relative reactivity of cysteine residues within structural ZBDs but also are crucial for the design of future therapeutic agents that selectively target ZBDs, such as those that occur in the HIV-1 nucleocapsid protein.

Introduction

Structural zinc-binding proteins, which use zinc for structure and fold maintenance rather than catalysis, are estimated to be derived from 3% of the human genome, and they are extremely prevalent in DNA-sequence-specific transcription factors.[1] The first zinc binding domain (ZBD) to be described was the "classical zinc finger" from the *Xenopus* transcription factor TFIIIA.[2] TFIIIA contains nine ZBDs, each of which coordinates a zinc ion using two cysteine and two histidine residues.[2] Structural ZBDs are often classified according to the distribution of cysteine and histidine residues that coordinate the zinc ion (Cys_2His_2, Cys_3His, and Cys_4)[3] or first coordination shell, here defined as the zinc-binding domain "core".

Numerous structural studies indicate that the bound zinc ion serves to stabilize the three-dimensional fold of the ZBD by participating in the formation of local secondary structure elements.[4,5] Conversely, the chemical stability of the zinc-binding core can be enhanced through steric and electronic couplings to the surrounding protein, often through cysteine−amide interactions, that define a second "coordination" shell.[6,7] Various protein structural motifs have now been associated with the common classes of ZBDs, many with a conserved number of amino acids between the zinc-coordinated residues.[3,4,8,9] ZBDs are involved in a wide range of functions: regulation of apoptosis, transcriptional regulation, binding of nucleic acid,

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(1) International Human Genome Sequencing Consortium. *Nature* **2001**, *409*, 860−921.
(2) Miller, J.; McLachlan, A. D.; Klug, A. *EMBO J.* **1985**, *4*, 1609−14.
(3) Harrison, S. C. *Nature* **1991**, *353*, 715−9.
(4) Laity, J. H.; Lee, B. M.; Wright, P. E. *Curr. Opin. Struct. Biol.* **2001**, *11*, 39−46.
(5) South, T. L.; Summers, M. F. *Adv. Inorg. Biochem.* **1990**, *8*, 199−248.
(6) Konrat, R.; Weiskirchen, R.; Bister, K.; Kräutler, B. *J. Am. Chem. Soc.* **1998**, *120*, 7127−30.
(7) Smith, J. N.; Hoffman, J. T.; Shirin, Z.; Carrano, C. J. *Inorg. Chem.* **2005**, *44*, 2012−17.
(8) Berg, J. M. *Curr. Opin. Struct. Biol.* **1993**, *3*, 11−16.

10.1021/ja063329e CCC: $33.50

nuclear hormone receptors, protein−protein interaction, and protein−lipid interactions (for a review see ref 4). Though the residues that make up a structural zinc-binding core do not play an enzymatic role, they are not chemically inert either. Rather, the zinc-binding core is chemically active and susceptible to oxidation, which can be induced by oxidative stress conditions.[10] The degree of stability of each ZBD varies as a function of the zinc-coordinating ligands and the surrounding amino acid residues.[11] Experimental studies have shown that coordination of zinc by cysteine activates the cysteine thiol, making it more nucleophilic compared with the free thiol.[12] The potential reactivity of ZBDs has begun to be exploited for the development of new drug therapies for cancer and human immunodeficiency virus type-1 (HIV-1) infections by targeting a human estrogen receptor[13] or the HIV-1 nucleocapsid protein (HIV-1 NCp7), respectively.

Several different classes of electrophilic compounds have been reported to directly function as antiviral agents through their ability to inhibit the activities of HIV-1 NCp7.[14−25] Most of the compounds function by interacting with cysteine residues in the ZBDs and ejecting zinc from HIV-1 NCp7 (zinc ejectors).[19] Their activity is highly dependent on the electrophilic strength of the compounds,[26] and the ensuing zinc ejection leads to a loss of both protein structure and function.[16,19,27] Several chemotypes have been studied, which inhibit HIV-1 replication and appear to selectively eject zinc from the carboxyl-terminal ZBD (ZD2)[17−19,26] without inhibiting the specific nucleic acid binding activity of some cellular proteins containing structural ZBDs.[20] Recently, thioester compounds that selectively target the HIV-1 NCp7 protein have been developed that also display low cellular toxicity and potent antiviral activity.[22,28,29] We have recently shown that two highly active thioester compounds, compounds **1** and **2** (Figure 1A), selectively eject zinc from NCp7 by covalently modifying Cys_{39} in ZD2 of HIV-1 NCp7 via an acyl transfer mechanism (Figure 1B).[30] This modification leads to loss of protein structure and loss of sequence-specific nucleic acid binding by HIV-1 NCp7.

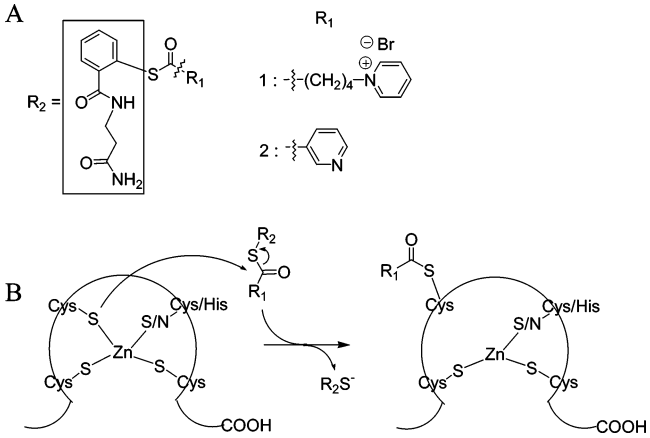

Figure 1. (A) Chemical structure of thioester compounds used in this study. (B) Proposed mechanism for the covalent modification of a cysteine residue in ZBDs by thioester antiviral compounds **1** and **2**. Note that the identity of the first modified cysteine can vary and that the other cysteines of the ZBD can be subsequently modified.

As the thioester compounds are able to react with HIV-1 NCp7, they can be used as a tool to probe the reactivity of other structural ZBDs. Theoretical studies have attempted to predict the relative reactivity of different classes of ZBDs toward electrophilic compounds, including the thioester compounds.[22,26,31] Unfortunately, very few experimental studies have been conducted to either support or refute the theoretical predictions. Most of the experimental studies have examined the interactions of electrophilic compounds with various classes of structural ZBDs only in the presence of nucleic acids.[20,22] Some studies have analyzed the effect of electrophilic compounds on only a single ZBD-containing protein, such as the estrogen receptor or metal response element-binding transcription factor-1,[13,32,33] or on synthetic mimics of the zinc−thiolate interaction.[7] Additional experimental studies are required to better understand the two determinants of ZBD reactivity: the different zinc-coordination motifs (e.g., Cys_2His_2, Cys_3His, Cys_4) that define the first coordination shell (zinc-binding core) and the interaction of the zinc-binding core with the secondary shell of residues provided by the surrounding protein. ZBDs should further be compared in both the free and nucleic-acid-bound states to more thoroughly evaluate the effect of nucleic acid binding on the stability of structural ZBDs. The thioester compounds can, thus,

(9) Klug, A.; Schwabe, J. W. R. *FASEB J.* **1995**, *9*, 597−604.
(10) Baldwin, M. A.; Benz, C. C. *Methods Enzymol.* **2002**, *353*, 54−69.
(11) Maynard, A. T.; Covell, D. G. *J. Am. Chem. Soc.* **2001**, *123*, 1047−58.
(12) Boerzel, H.; Koeckert, M.; Bu, W.; Spingler, B.; Lippard, S. J. *Inorg. Chem.* **2003**, *42*, 1604−15.
(13) Wang, L. H.; Yang, X. Y.; Zhang, X.; Mihalic, K.; Fan, Y. X.; Xiao, W.; Howard, O. M. Z.; Appella, E.; Maynard, A. T.; Farrar, W. L. *Nat. Med.* **2004**, *10*, 40−7.
(14) Rice, W. G.; Baker, D. C.; Schaeffer, C. A.; Graham, L.; Bu, M.; Terpening, S.; Clanton, D.; Schultz, R.; Bader, J. P.; Buckheit, R. W., Jr.; Field, L.; Singh, P. K.; Turpin, J. A. *Antimicrob. Agents Chemother.* **1997**, *41*, 419−26.
(15) Rice, W. G.; Schaeffer, C. A.; Graham, L.; Bu, M.; McDougal, J. S.; Orloff, S. L.; Villinger, F.; Young, M.; Oroszlan, S.; Fesen, M. R.; Pommier, Y.; Mendeleyev, J.; Kun, E. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 9721−4.
(16) Rice, W. G.; Schaeffer, C. A.; Harten, B.; Villinger, F.; South, T. L.; Summers, M. F.; Henderson, L. E.; Bess, J. W., Jr.; Arthur, L. O.; McDougal, J. S.; Orloff, S. L.; Mendeleyev, J.; Kun, E. *Nature* **1993**, *361*, 473−5.
(17) Rice, W. G. et al. *Science* **1995**, *270*, 1194−7.
(18) Rice, W. G.; Turpin, J. A.; Huang, M.; Clanton, D.; Buckheit, R. W., Jr.; Covell, D. G.; Wallqvist, A.; McDonnell, N. B.; DeGuzman, R. N.; Summers, M. F.; Zalkow, L.; Bader, J. P.; Haugwitz, R. D.; Sausville, E. A. *Nat. Med.* **1997**, *3*, 341−5.
(19) Loo, J. A.; Holler, T. P.; Sanchez, J.; Gogliotti, R.; Maloney, L.; Reily, M. D. *J. Med. Chem.* **1996**, *39*, 4313−20.
(20) Huang, M.; Maynard, A.; Turpin, J. A.; Graham, L.; Janini, G. M.; Covell, D. G.; Rice, W. G. *J. Med. Chem.* **1998**, *41*, 1371−81.
(21) Tummino, P. J.; Harvey, P. J.; McQuade, T.; Domagala, J.; Gogliotti, R.; Sanchez, J.; Song, Y.; Hupe, D. *Antimicrob. Agents Chemother.* **1997**, *41*, 394−400.
(22) Turpin, J. A.; Song, Y.; Inman, J. K.; Huang, M.; Wallqvist, A.; Maynard, A.; Covell, D. G.; Rice, W. G.; Appella, E. *J. Med. Chem.* **1999**, *42*, 67−86.
(23) Turpin, J. A.; Terpening, S. J.; Schaeffer, C. A.; Yu, G.; Glover, C. J.; Felsted, R. L.; Sausville, E. A.; Rice, W. G. *J. Virol.* **1996**, *70*, 6180−9.
(24) Goel, A.; Mazur, S. J.; Fattah, R. J.; Hartman, T. L.; Turpin, J. A.; Huang, M.; Rice, W. G.; Appella, E.; Inman, J. K. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 767−70.
(25) Basrur, V.; Song, Y.; Mazur, S. J.; Higashimoto, Y.; Turpin, J. A.; Rice, W. G.; Inman, J. K.; Appella, E. *J. Biol. Chem.* **2000**, *275*, 14890−7.
(26) Maynard, A. T.; Huang, M.; Rice, W. G.; Covell, D. G. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 11578−83.
(27) Topol, I. A.; Nemukhin, A. V.; Dobrogorskaya, Y. I.; Burt, S. K. *J. Phys. Chem. B* **2001**, *105*, 11341−50.
(28) Schito, M. L.; Goel, A.; Song, Y.; Inman, J. K.; Fattah, R. J.; Rice, W. G.; Turpin, J. A.; Sher, A.; Appella, E. *AIDS Res. Hum. Retroviruses* **2003**, *19*, 91−101.
(29) Srivastava, P.; Schito, M.; Fattah, R. J.; Hara, T.; Hartman, T.; Buckheit, R. W., Jr.; Turpin, J. A.; Inman, J. K.; Appella, E. *Bioorg. Med. Chem.* **2004**, *12*, 6437−50.
(30) Miller Jenkins, L. M.; Byrd, J. C.; Hara, T.; Srivastava, P.; Mazur, S.; Stahl, S. J.; Inman, J. K.; Appella, E.; Omichinski, J. G.; Legault, P. *J. Med. Chem.* **2005**, *48*, 2847−58.
(31) Khandogin, J.; Musier-Forsyth, K.; York, D. M. Insights into the regioselectivity and RNA-binding affinity of HIV-1 nucleocapsid protein from linear-scaling quantum methods. *J. Mol. Biol.* **2003**, *330*, 993−1004.
(32) Atsriku, C.; Scott, G. K.; Benz, C. C.; Baldwin, M. A. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 2017−26.
(33) Apuy, J. L.; Chen, X.; Russell, D. H.; Baldwin, T. O.; Giedroc, D. P. *Biochemistry* **2001**, *40*, 15164−75.

be used as effective chemical tools to probe the reactivity of a variety of zinc-binding motifs.

In this study, we have analyzed the reactivity of thioester compounds **1** and **2** with structural ZBDs from six different proteins. These six proteins represent three different classes (Cys_2His_2, Cys_3His, Cys_4) of ZBDs with five distinct zinc-coordination motifs. We have used UV/visible spectroscopy and NMR spectroscopy to evaluate the ability of the thioester compounds to eject metal from the various classes of ZBDs and to alter their structure. In addition, we have determined the ability of these compounds to disrupt the sequence-specific nucleic acid binding properties of selected ZBDs. Computational calculation of the electrostatic and steric screening of selected ZBDs has also been performed. The results demonstrate that only specific classes of structural ZBDs react with the thioester compounds. The relative reactivity of various ZBDs with the thioester compounds is determined by the intrinsic reactivity of the cores of the zinc-binding domains,[34] as well as by the secondary shell of interactions that electronically stabilize the zinc-binding core complex, primarily through cysteine−amide interactions.[6,7,11] In addition, binding to nucleic acids greatly reduces ZBD reactivity toward the thioester compounds by enhancing the steric and electrostatic screening of the potentially reactive cores. These results help explain the concomitant excellent antiviral and low cytotoxicity properties observed for compounds **1** and **2**,[28] reflecting the range of intrinsic chemical stability found in ZBDs from viral and cellular proteins. This information can also be incorporated into the design of future antiviral agents specifically directed toward HIV-1 NCp7.

Experimental Procedures

Protein Subcloning. The coding sequences for the *Drosophila* GAGA ZBD ($GAGA_{310-372}$ - amino acids 310−372),[35] the double ZBD of human GATA-1 protein ($hGATA\text{-}1_{200-317}$ - amino acids 200−317),[36] the carboxyl-terminal ZBD of chicken GATA-1 protein ($cGATA\text{-}1_{158-223}$ - amino acids 158−223), and a glutathione-*S*-transferase (GST) fusion of the second cysteine-rich region of human protein kinase C delta protein ($PKC\delta_{231-280}$ - amino acids 231−280)[37] were transformed and expressed in host strain BL21(DE3) (Novagen, WI), as previously described. The coding sequence for the double ZBD of the major histocompatibility binding protein-1 ($MBP\text{-}1_{2085-2142}$ - amino acids 2085−2142) was PCR amplified from pL-EB(F12).[38] The amplified coding sequence was subcloned into pET3a (Novagen, WI) using the *Nde*I−*Bam*HI restriction sites and expressed in BL21(DE3)pLysS (Novagen, WI). The coding sequence for Mouse Mammary Tumor Virus NCp10 (C3H strain) was PCR amplified from the tetracycline-resistant plasmid p202 containing the partial gag-pol region,[39] obtained from the American Type Culture Collection (no. 45005). The MMTV NCp10 sequence was subcloned into the *Bam*HI and *Nco*I sites of pET11a (Novagen, WI)[40] and expressed in *Escherichia coli* host strain Rosetta (DE3) (Novagen, WI). The coding sequence for the ninth ZBD of mouse Friend of GATA-1 protein ($FOG\text{-}1_{950-995}$ - amino acids 950−995) was kindly provided by Dr. Edward Morrissey (University of Pennsylvania).

Protein Expression and Purification. Cells expressing $GAGA_{310-372}$,[41] $hGATA\text{-}1_{200-317}$,[36] $cGATA\text{-}1_{158-223}$,[42] or MMTV NCp10 were grown in Luria Broth at 37 °C. Uniformly (>98%) ^{15}N-labeled $cGATA\text{-}1_{158-223}$, ^{15}N-labeled $hGATA\text{-}1_{200-317}$, and ^{15}N-labeled MMTV NCp10 were obtained by growing the cells in modified minimal media containing ^{15}N-labeled ammonium chloride as the sole nitrogen source (Figure 2). Protein expression was induced for 4 h with 0.66 mM or 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for cultures grown in minimal media or Luria Broth, respectively. The cells were resuspended in 25 mM Tris pH 8.0, 1 mM EDTA, 2 mM dithiothreitol (DTT), and 6 mM benzamidine. The cells were then lysed by French press and centrifuged at 100 000 × *g* for 45 min. The supernatant was applied to a DEAE-Sepharose Fast Flow (Amersham Biosciences, NJ) column (300 mL bed volume), equilibrated with Buffer A (25 mM Tris pH 8.0, 1 mM EDTA, and 2 mM DTT), and eluted using a gradient (0−100% B over 1500 mL) of Buffer B (25 mM Tris pH 8.0, 1 mM EDTA, 2 mM DTT, and 1 M NaCl). The pooled protein fractions were then applied to a SP-Sepharose Fast Flow (Amersham Biosciences, NJ) column (300 mL bed volume), equilibrated with Buffer A and eluted using a gradient (0−100% B over 1500 mL) of Buffer B. The fractions containing the desired protein were pooled and subsequently purified on a C-4 (for $GAGA_{310-372}$, $hGATA\text{-}1_{200-317}$, and $cGATA\text{-}1_{158-223}$) or on a C-8 (for MMTV NCp10) reversed phase (Vydac) high performance liquid chromatography (HPLC) column. The following acetonitrile gradients in 0.05% aqueous trifluoroacetic acid (TFA) were used: 20−40% for $GAGA_{310-372}$, 20−40% for $cGATA\text{-}1_{158-223}$, 15−35% for $hGATA\text{-}1_{200-317}$, and 15−35% for MMTV NCp10. The eluted proteins were subsequently flash frozen and lyophilized.

^{15}N-Labeled $MBP\text{-}1_{2085-2142}$ was obtained by growing the cells in modified minimal media containing ^{15}N-labeled (>98%) ammonium chloride as the sole nitrogen source. Protein expression was induced for 4 h with 0.66 mM IPTG. The cells were resuspended in 25 mM sodium phosphate pH 7.0, 1 mM EDTA, and 2 mM DTT. The cells were then lysed by French press and centrifuged at 19 500 × *g* for 30 min. ^{15}N-Labeled $MBP\text{-}1_{2085-2142}$ was purified from an inclusion body by resuspending the pellet in 25 mM sodium phosphate pH 7.0, 0.5% Triton X-100, 2 M urea, and 2 mM DTT and centrifuging at 19 500 × *g* for 30 min. The pellet was resuspended in 6 M guanidine hydrochloride and incubated at 50 °C for 10 min. This suspension was centrifuged at 100 000 × *g* for 45 min, and the resulting supernatant dialyzed into 5% aqueous acetic acid containing 5 mM DTT. The ^{15}N-labeled $MBP\text{-}1_{2085-2142}$ was further purified from the dialysate on a C-4 reversed phase (Vydac) HPLC column using a 20−40% gradient of acetonitrile in 0.05% aqueous TFA. The eluted protein was flash frozen and lyophilized.

$PKC\delta_{231-280}$ and $FOG\text{-}1_{950-995}$ were prepared as GST-fusion proteins. Cells expressing $GST\text{-}PKC\delta_{231-280}$ or $GST\text{-}FOG\text{-}1_{950-995}$ were grown in Luria Broth at 37 °C. Protein expression was induced for 4 h with 1 mM IPTG at 30 °C. The cells were resuspended in EBC Buffer (50 mM Tris pH 8.0, 120 mM NaCl, 0.5% NP-40, and 2 mM DTT), then lysed by French press, and centrifuged at 100 000 × *g* for 45 min. The supernatant was incubated for 1 h at 4 °C with glutathione sepharose (Amersham Biosciences, NJ) equilibrated in EBC Buffer. The bound $GST\text{-}PKC\delta_{231-280}$ was equilibrated in phosphate-buffered saline and digested with 100 U thrombin (Calbiochem, CA) for 2 h at 25 °C to cleave $PKC\delta_{231-280}$ from the GST tag. The bound $GST\text{-}FOG1_{950-995}$ was equilibrated in 50 mM Tris pH 8.0, 100 mM NaCl, and 1 mM $CaCl_2$ and digested with Factor Xa (Novagen, WI) for 16 h at 25 °C to cleave $FOG1_{950-995}$ from the GST tag. The cleaved $PKC\delta_{231-280}$ or $FOG\text{-}1_{950-995}$ was dialyzed into 10% acetic acid and subsequently purified on a C-4 reversed phase (Vydac) HPLC column using a 25−

(34) Wilker, J. J.; Lippard, S. J. *Inorg. Chem.* **1997**, *36*, 969−78.
(35) Omichinski, J. G.; Pedone, P. V.; Felsenfeld, G.; Gronenborn, A. M.; Clore, G. M. *Nat. Struct. Biol.* **1997**, *4*, 122−32.
(36) Trainor, C. D.; Omichinski, J. G.; Vandergon, T. L.; Gronenborn, A. M.; Clore, G. M.; Felsenfeld, G. *Mol. Cell. Biol.* **1996**, *16*, 2238−47.
(37) Kazanietz, M. G.; Barchi, J. J., Jr.; Omichinski, J. G.; Blumberg, P. M. *J. Biol. Chem.* **1995**, *270*, 14679−84.
(38) Scharf, S. J.; Horn, G. T.; Erlich, H. A. *Science* **1986**, *233*, 1076−8.
(39) Majors, J. E.; Varmus, H. E. *Nature* **1981**, *289*, 253−8.
(40) Klein, D. J.; Johnson, P. E.; Zollars, E. S.; De Guzman, R. N.; Summers, M. F. *Biochemistry* **2000**, *39*, 1604−12.
(41) Pedone, P. V.; Ghirlando, R.; Clore, G. M.; Gronenborn, A. M.; Felsenfeld, G.; Omichinski, J. G. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2822−6.
(42) Omichinski, J. G.; Clore, G. M.; Schaad, O.; Felsenfeld, G.; Trainor, C.; Appella, E.; Stahl, S. J.; Gronenborn, A. M. *Science* **1993**, *261*, 438−46.

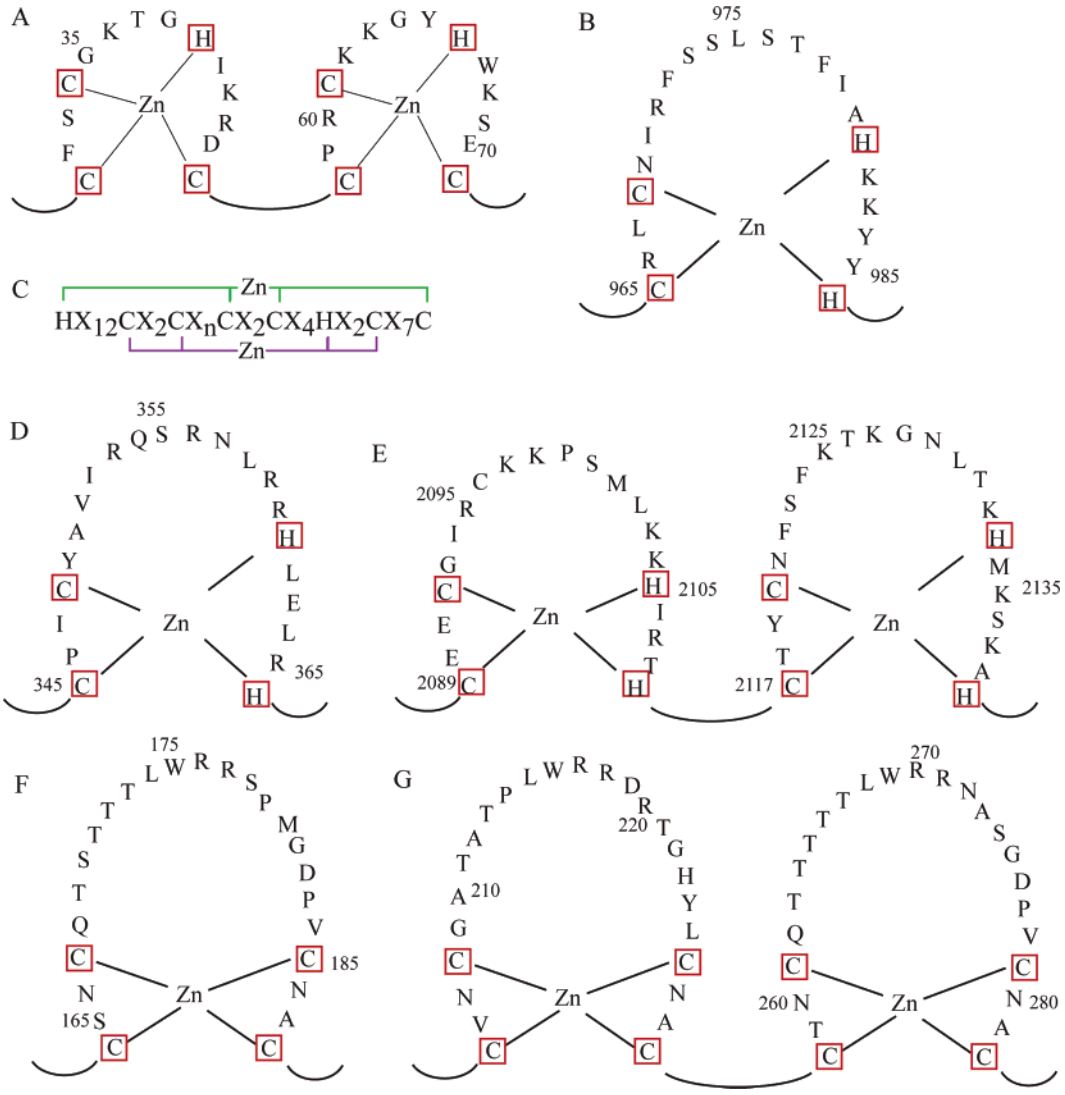

Figure 2. Primary sequence of zinc-binding domains used in this study: (A) MMTV NCp10; (B) FOG-$1_{950-995}$; (C) PKC$\delta_{231-280}$; (D) $GAGA_{310-372}$; (E) MBP-$1_{2085-2142}$; (F) cGATA-$1_{158-223}$; (G) hGATA-$1_{200-317}$. Zinc-coordinating residues are boxed.

45% or 15−35% gradient of acetonitrile in 0.05% aqueous TFA, respectively. The eluted protein was subsequently flash frozen and lyophilized.

Sample Preparation. Purified proteins were refolded using the following procedure: first, the lyophilized material was resuspended in 0.05% TFA. $ZnCl_2$ (zinc-refolded protein, 5 equiv) or $CoCl_2$ (cobalt-refolded protein, 5 equiv) was then added to the protein solution as a 50 mM metal solution in 0.05% TFA. The pH of the solution was titrated to pH 6.0 with 0.2 M NaOH. The zinc-refolded MMTV NCp10 and MBP-$1_{2085-2142}$ were then flash frozen and lyophilized. Prior to use, zinc-refolded MMTV NCp10 or MBP-$1_{2085-2142}$ (1 mM) was resuspended in NMR Buffer A (20 mM sodium phosphate pH 7.0) in 90% H_2O/10% D_2O. The zinc-refolded PKC$\delta_{231-280}$, cGATA-$1_{158-223}$, and hGATA-$1_{200-317}$ were concentrated with an Amicon Centricon-3 concentrator (Millipore, MA) and exchanged into NMR Buffer A in 90% H_2O/10% D_2O. The buffer of the cobalt-refolded proteins was adjusted to 20 mM sodium phosphate pH 7.0. The cobalt-refolded proteins were then used immediately after refolding.

The complex of ^{15}N-labeled cGATA-$1_{158-223}$ (1 mM) with AGATAA DNA (1 mM) was prepared by titrating ^{15}N-labeled cGATA$_{158-223}$ into double-stranded AGATAA DNA (Midland Certified Reagents, TX) in NMR buffer A in 90% H_2O/10% D_2O. The complex was concentrated to 500 μL using an Amicon Centricon-3 concentrator (Millipore, Bedford, MA). The sequence of the top strand of the double-stranded AGATAA DNA used for NMR studies was 5′ GTTGCAGATAAA-CATT 3′.

Synthesis of Compounds. Compound **1** (*N*-[2-(5-pyridinovaleroylthio)benzoyl]-β-alaninamide bromide) and compound **2** (*N*-[2-nicotinoylthiobenzoyl]-β-alaninamide hydrochloride) were synthesized as described.[22,29] The thioester compounds were lyophilized in aliquots such that the concentration was 1 mM in a volume of 500 μL and stored at −20 °C.

UV/visible Spectroscopy. For UV/visible spectroscopy studies, cobalt-refolded proteins (150 μM) were incubated with compound **2** (150 μM) at 25 °C. The UV/visible spectrum was recorded from 220 to 800 nm every 0.5 h for 3 h using a Shimadzu UV-1601 spectrophotometer equipped with a PC control via the UVPC software (Shimadzu Scientific Instruments, MD). The wavelengths of maximum absorbance (λ_{max}) of the tetrahedrally coordinated cobalt ions of each protein are listed in Table 1. The absorbance at λ_{max} was specifically monitored and compared against a control sample in which no thioester compound was added. Experiments were repeated a minimum of 3 times. Net absorbance values were calculated by subtracting the absorbance of the control sample from that of the thioester-treated samples. The net absorbance at λ_{max} was plotted versus time, and the initial rate of absorbance loss (0−3 h) was obtained from the slope of the linear regression analysis.

Table 1. Rate of UV/visible Absorbance Loss of Cobalt-Refolded Proteins when Incubated with Compound **2**

protein	type of ZBD	λ max (nm)	rate (AU/h)
$GAGA_{310-372}$	CCHH	634	1.0 ± 0.7
HIV-1 NCp7[a]	2 CCHC	642, 698	36.0 ± 3.7
MMTV NCp10	2 CCHC	642, 698	12.1 ± 0.5
$FOG\text{-}1_{950-995}$	CCHC	642, 698	17.9 ± 0.5
$PKC\delta_{231-280}$	HCCC, CCHC	665	2.8 ± 0.6
$cGATA_{158-223}$	2 CCCC	741, 705	15.2 ± 0.2

[a] Reference 30.

NMR Spectroscopy. Zinc-refolded ^{15}N-labeled $PKC\delta_{231-280}$, ^{15}N-labeled $hGATA\text{-}1_{200-317}$, ^{15}N-labeled $cGATA\text{-}1_{158-223}$, ^{15}N-labeled $cGATA\text{-}1_{158-223}$/AGATAA DNA complex, ^{15}N-labeled MMTV NCp10, or ^{15}N-labeled $MBP\text{-}1_{2085-2142}$ (1 mM) was incubated with an equimolar amount (1 mM) of compound **1** at 25 °C for 48 h. The samples were analyzed before and at various times after addition of compound **1** on Varian ^{UNITY}Inova 500 and 600 MHz spectrometers equipped with an HCN triple resonance probe with an actively shielded *z*-gradient. Three experiments were recorded at each time point: a 1D ^{15}N-decoupled watergate,[43] a 1D difference water-sLED,[44] and a 2D ^{1}H−^{15}N HSQC.[45] 1D difference water-sLED spectra[44] were recorded as previously described.[30] The difference water-sLED experiment is run such that the difference spectrum shows resonances only from the more quickly diffusing part of the sample, i.e., the thioester compound. If the thioester compound covalently modifies a thiol residue in the protein, it will diffuse more slowly with the protein, and the resonances for the thioester will no longer be observed. The 2D ^{1}H−^{15}N HSQC spectra were processed with the NMRPipe package[46] and analyzed with PiPP.[47] The 2D peak volume integration was plotted against time (0−48 h), and the initial rate of signal intensity loss was obtained by linear regression. ^{1}H and ^{15}N chemical shift assignments for MMTV NCp10[40], $hGATA\text{-}1_{200-317}$ (JGO, unpublished data), and $cGATA\text{-}1_{158-223}$ complexed with AGATAA DNA (JGO, unpublished data), and the ^{1}H chemical shift assignments for $MBP\text{-}1_{2085-2142}$[48] were obtained from previous studies.

Gel Mobility Shift Assay. Zinc-refolded $cGATA\text{-}1_{158-223}$ (100 nM) was incubated with either compound **1** (500 nM) or **2** (500 nM) in 50 mM Tris pH 7.0, 0.125% NP-40, 2 mM EDTA, 20 μg/mL poly(dI-dC), and 10% glycerol at 25 °C for 0 min, 4 h, and 24 h. After each incubation period, an aliquot of $cGATA\text{-}1_{158-223}$ (final $cGATA\text{-}1_{158-223}$ concentration 5 nM) was removed and incubated with 1 nM 5′-^{32}P-labeled double-stranded AGATAA DNA (Integrated DNA Technologies, Inc, IA) for 30 min at 25 °C. The sequence of the AGATAA DNA used for gel-binding studies was 5′ AGCTTCGGTTGCA-GATAAACATTGAATTCA 3′. The top strand of the double-stranded DNA was 5′-end-labeled with γ-(^{32}P)ATP using T4 polynucleotide kinase (New England Biolabs, MA) according to the manufacturer's instructions.

The $cGATA\text{-}1_{158-223}$/DNA binding reactions were analyzed by 8% native gel electrophoresis in 10 mM Tris, 10 mM HEPES pH 7.5, and 1 mM EDTA at 150 V and 4 °C. The 5′-^{32}P-labeled DNA was detected using a Storm PhosphorImager (Amersham Biosciences, NJ). The density of the bands of the free and bound DNA was determined with the ImageQuant program (Molecular Dynamics). The percent free DNA was calculated as $D_f/(D_b + D_f)$, where D_f is the normalized density of the free DNA and D_b is the normalized density of the bound DNA. Normalized densities were calculated by subtracting out the measured densities of a region of background density in each lane.

Computational Analysis of ZBD Stability. Using experimental structures, the relative reactivity of each zinc-binding domain was qualitatively predicted by the calculation of two structural metrics: the steric (ρ_s) and electrostatic (V_s) screening of the corresponding zinc-binding core within each domain. The rationale for these metrics is that stabilization of a zinc-binding core (i.e., cysteine thiolates) by the surrounding protein structure, through local steric or electrostatic interactions, determines the susceptibility of a zinc-binding domain to undergo an oxidation, as in the case of electrophilic inhibition. These metrics are intimately related to the observation that zinc-binding cores are coupled to a surrounding shell of protein amide interactions that stabilize the ZBD.[6] The detailed definition and calculation of ρ_s and V_s have been previously described,[11] and only the minor differences in calculation, where applicable, are presented here.

The atomic coordinates of the proteins were obtained from the following files of the Protein Data Bank (PDB)[49] (http://www.pdb.org/): 1DSQ, N-terminal zinc finger of MMTV NCp10; 1DSV, C-terminal zinc finger of MMTV NCp10; 1MFS, N- and C-terminal zinc fingers of HIV NCp7; 1GNF, N-terminal zinc finger of GATA-1; 3GAT, C-terminal zinc finger of GATA-1; and 1FU9, ninth zinc finger of the U-shaped Friend-Of-GATA transcription factor. As all of these structures were determined by NMR, ρ_s and V_s were calculated and averaged over all of the models in each structure file. This ranged from 20 models for 1DSQ, 1DSV, and 1FU9 to 34 models for 3GAT. For the initial setup, the molecular mechanics program CHARMM[50] was used to standardize all the hydrogen and heavy atom names to those contained in the "top_all22_prot" amino acid topology file.[51]

As described previously,[11] ρ_s was calculated by integration of the radial packing profile of each zinc-binding core using a simple trapezoidal quadrature rule. While the step size was kept the same at 0.5 Å, the upper bound, R_{max} value, was increased to 8.0 Å. V_s was calculated with the newer version of DelPhi, v. 4.1.1, in standalone mode (Accelrys Software Inc.). All calculations were done with atomic charges from the "top_all22_prot" topology set of CHARMM, except for the atoms of the zinc-binding domain, which were set as previously described.[11] Rather than the focusing procedure, a single, larger grid of 201 points along each side was used. In each case, the longest dimension of the protein was set to take-up 50% of a side, which led to scales ranging from 1.5 to 4.2 grids/Å, with equal mean and median values of 2.6 grids/Å. In addition to calculating V_s from the difference between $V_{protein}$ (all protein and ZBD atoms) and V_{core} (only atoms from the zinc-binding core), it was also calculated from simply charging all atoms except those of the zinc-binding core. Since the ionic strength was always set at zero, both methods should yield the same result, thus serving as an internal test of quality. Using the first model of the C-terminal domain of GATA-1 in the 3GAT.pdb protein data bank file as a benchmark: calculation of the protein screening profile to 16 Å in 0.5 Å steps took 1.5 s on an Intel Xeon processor clocked at 2.4 GHz, and calculation of the all-atom-charged potential energy field took 76 s on an Intel Pentium 4 processor clocked at 3.0 GHz.

Results

$Cys_2HisCys$ ZBDs interact with thioester compounds. Thioester compounds **1** and **2** have been shown to selectively eject tetrahedrally coordinated zinc from the $Cys_2HisCys$ ZD2 of HIV-1 NCp7 by covalently modifying Cys_{39} (Figure 1).[30] Here, we analyze the effect of compounds **1** and **2** on a variety of structural ZBDs from cellular and retroviral proteins. The

(43) Piotto, M.; Saudek, V.; Sklenar, V. *J. Biomol. NMR* **1992**, *2*, 661−5.
(44) Altieri, A. S.; Hinton, D. P.; Byrd, R. A. *J. Am. Chem. Soc.* **1995**, *117*, 7566−67.
(45) Kay, L. E.; Keifer, P.; Saarinen, T. Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. *J. Am. Chem. Soc.* **1992**, *114*, 10663.
(46) Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. *J. Biomol. NMR* **1995**, *6*, 277−93.
(47) Garrett, D. S.; Powers, R.; Gronenborn, A. M.; Clore, G. M. *J. Magn. Reson.* **1991**, *95*, 214−20.
(48) Omichinski, J. G.; Clore, G. M.; Robien, M.; Sakaguchi, K.; Appella, E.; Gronenborn, A. M. *Biochemistry* **1992**, *31*, 3907−17.
(49) Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. *Nucleic Acids Res.* **2000**, *28*, 235−242.
(50) Brooks, B. R.; Bruccoleri, R. E.; Olafson, B. D.; States, D. J.; Swaminathan, S.; Karplus, M. *J. Comput. Chem.* **1983**, *4*, 187−217.
(51) MacKerell, A. D., Jr., et al. *J. Phys. Chem. B* **1998**, *102*, 3586−3616.

metal ejecting properties of compound **2** toward several types of ZBDs were initially determined by UV/visible spectroscopy using cobalt-refolded ZBDs (Table 1). Unlike zinc, cobalt is a spectroscopically active metal that will give rise to absorption maxima in the visible region of the spectrum as a consequence of $d-d$ ligand field transitions when it is tetrahedrally coordinated.[52] The wavelengths of the absorption maxima are highly dependent upon the specific makeup of the four cobalt bound ligands, in our case a variable mixture of sulfurs (cysteine) and/or nitrogens (histidine) depending on the ZBD. In each case, the wavelength of the absorbance maxima (λ_{max}) varies with the type of ZBD that is binding the cobalt (Table 1).

We first analyzed the interaction of compound **2** on Cys_2-HisCys ZBDs from two proteins, one cellular (FOG-$1_{950-995}$) and one retroviral (MMTV NCp10). The two ZBDs use the same combination of cysteine and histidine ligands for metal binding as HIV-1 NCp7. MMTV NCp10 is the nucleocapsid protein from Mouse Mammary Tumor Virus (MMTV), and like HIV-1 NCp7, it contains two retroviral $Cys_2HisCys$ domains (Figure 2A). The two ZBDs in MMTV NCp10 have the same amino acid spacing between the metal-binding ligands as those found in HIV-1 NCp7.[40] The two ZBDs of MMTV NCp10 are structurally very similar, but not identical to, the two ZBDs of HIV-1 NCp7. FOG-$1_{950-995}$ is a $Cys_2HisCys$ domain from the mouse Friend of GATA-1 (FOG-1) protein.[53] The composition of the FOG-$1_{950-995}$ metal-binding ligands is the same as that of the HIV-1 NCp7 ligands, but the amino acid spacing between the binding residues is quite different from that in HIV-1 NCp7 (Figure 2B). In addition, the $Cys_2HisCys$ domain of FOG-1 adopts a $\beta\beta\alpha$ fold in the presence of zinc ions.[53] The three-dimensional structure of this ZBD is closely related to the structures of the Cys_2His_2 classical zinc fingers and bears little resemblance to the structure of the known retroviral ZBDs.

Compound **2** was incubated with cobalt-refolded MMTV NCp10 and cobalt-refolded FOG-$1_{950-995}$. As was previously observed for cobalt-refolded HIV-1 NCp7, there was a significant loss of absorbance in the visible region of the UV/visible spectra with both cobalt-refolded proteins over a period of 3 h (Table 1). The λ_{max} are consistent with previously reported values.[54] The loss of absorbance is due to ejection of the tetrahedrally coordinated cobalt by compound **2**. The rate of absorbance loss for MMTV NCp10 was ~3 times lower and that of FOG-$1_{950-995}$ was ~2 times lower than what had been observed with HIV-1 NCp7[30] (Table 1). These results clearly indicate that compound **2** is capable of ejecting metal from the $Cys_2HisCys$ ZBDs of both MMTV NCp10 and FOG-$1_{950-995}$, although the rate of metal ejection appears slightly reduced when compared to NCp7.

Though it was clear that the thioester compounds were able to eject tetrahedrally coordinated cobalt from both MMTV NCp10 and FOG-$1_{950-995}$, the UV/visible spectroscopy experiments failed to provide detailed information on the mechanism of metal ejection. Therefore, NMR spectroscopy experiments were used to obtain a more detailed understanding of the mechanism of metal ejection by the thioesters. Zinc-refolded ^{15}N-labeled MMTV NCp10 was incubated with compound **1** for 48 h at 25 °C. The 2D $^1H-^{15}N$ HSQC spectrum collected immediately after addition of compound **1** to the protein was identical to that of free MMTV NCp10 (data not shown). Thus, there were no changes in the protein amide signals, indicating that the MMTV NCp10 did not undergo a significant structural change upon addition of compound **1**. This result also suggests that no covalent modification of the protein thiols occurred immediately upon addition of the thioester compound to the protein. Indeed, chemical shift changes would have been expected if a stable interaction between MMTV and compounds **1** was forming. This result is similar to what we previously observed when HIV-1 NCp7 was mixed with either compound **1** or compound **2**.[30]

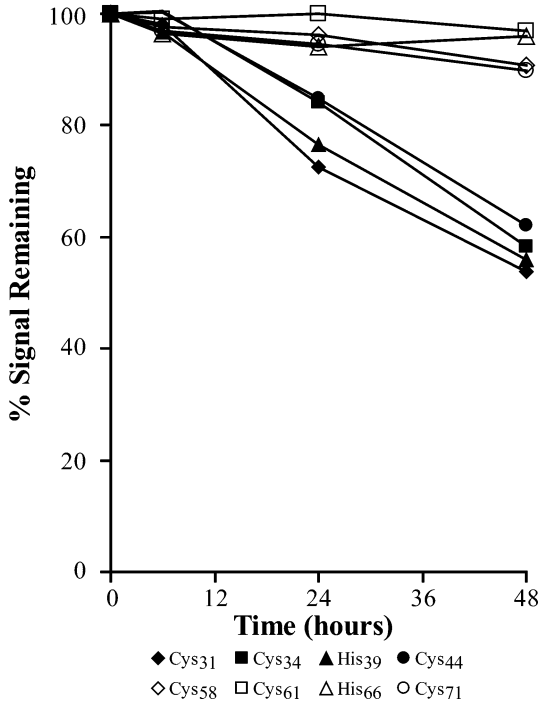

Figure 3. Changes in 2D $^1H-^{15}N$ HSQC signal intensity of MMTV NCp10 zinc-coordinating residues after addition of compound **1**.

During the 48 h incubation with compound **1**, no change in chemical shifts was observed in the 2D $^1H-^{15}N$ HSQC spectra of MMTV NCp10. The primary changes observed in the 2D $^1H-^{15}N$ HSQC spectra were loss of signal intensity for several amide signals (Supporting Information Figure 1). Concurrently, new signals appear in the spectra at chemical shifts indicative of an unstructured protein (Supporting Information Figure 1). At the pH used in the NMR studies, most amide signals for the unstructured protein are unobservable due to solvent exchange. The loss of MMTV NCp10 amide signal intensity in the 2D $^1H-^{15}N$ HSQC spectra was seen specifically for residues in the amino-terminal zinc-binding domain (ZD1). After 24 h, the signals for Cys_{31} and His_{39} had only 75% of their original signal intensity, whereas the equivalent zinc-coordinating residues in ZD2 (Cys_{58} and His_{66}) retained greater than 95% of their original signal intensity (Figure 2). In general, the zinc-coordinating residues from ZD2 (Cys_{58}, Cys_{61}, His_{66}, and Cys_{71}) showed only a small overall change in amide signal intensity over the 48 h incubation period (<5%), whereas the zinc-coordinating residues from ZD1 (Cys_{31}, Cys_{34}, His_{39}, Cys_{44}) lost >55% of their original amide signal intensity over the same period (Figure 3). The average rate of signal intensity loss for the zinc-coordinating residues of ZD1 was 7.5 times greater than that

(52) Maret, W.; Vallee, B. L. *Methods Enzymol.* **1993**, *226*, 52−71.
(53) Tsang, A. P.; Visvader, J. E.; Turner, C. A.; Fujiwara, Y.; Yu, C. N.; Weiss, M. J.; Crossley, M.; Orkin, S. H. *Cell* **1997**, *90*, 109−19.
(54) Chen, X.; Chu, M.; Giedroc, D. P. *J. Biol. Inorg. Chem.* **2000**, *5*, 93−101.

Table 2. Average Rate of Loss of 2D $^1H-^{15}N$ HSQC Signal Intensity for Zinc-Coordinating Residues during Incubation with Compound **1**

protein	ZBD	protein state	rate (IU/h) ZD1	rate (IU/h) ZD2
MBP-$1_{2085-2142}$	CCHH	free	0.04 ± 0.04	0.09 ± 0.04
HIV-1 NCp7[a]	CCHC	free	0.11 ± 0.18	1.20 ± 0.10
MMTV NCp10	CCHC	free	0.93 ± 0.06	0.12 ± 0.06
hGATA-$1_{200-317}$	CCCC	free	1.90 ± 0.30	2.40 ± 0.30
$cGATA_{158-223}$	CCCC	complex	N.D.	0.10 ± 0.02

[a] Reference 30.

observed for ZD2 (Table 2). This disparity in the signal intensity changes between ZD1 and ZD2 indicates that compound **1** preferentially interacts with ZD1 over ZD2. This result is very similar to what was observed with HIV-1 NCp7 and compound **1**, except that for the HIV-1 NCp7 compound **1** preferentially interacted with ZD2.[30]

The interleaved ZBD of PKCδ does not interact with thioester compounds. The next zinc-binding domains to be studied were the interleaved ZBDs from PKCδ (Figure 2C). In PKC$\delta_{231-280}$, the interleaved ZBDs can actually be divided into two ZBDs formed by alternating pairs of Cys and His ligands. The metal-binding ligands are arranged as His-Cys_2-Cys_2-His/Cys-Cys.[37] The first Cys_2 pair chelates a zinc ion with the His/Cys pair to form a Cys_2HisCys ZBD. The second zinc ion is bound by the first His residue, the second Cys_2 pair, and the final Cys residue to form a $HisCys_3$ ZBD.[55] The two ZBDs of PKC$\delta_{231-280}$ are thus a Cys_2HisCys domain and a $HisCys_3$ domain. These domains have an identical combination of first shell ligands as the ZBDs in HIV-1 NCp7, MMTV NCp10, and FOG-$1_{950-995}$. We first incubated cobalt-refolded PKC$\delta_{231-280}$ with compound **2** and monitored changes in the UV/visible absorbance of the tetrahedrally coordinated cobalt. Over the course of the 3 h incubation, very small changes in absorbance were observed (Table 1). The rate of absorbance loss of PKC$\delta_{231-280}$ was ~6 times lower than that observed for FOG-$1_{950-995}$ and ~4 times lower than that observed for MMTV NCp10 (Table 1). This indicates that compound **2** was able to eject only a minimal amount of metal from the interleaved ZBDs of PKC$\delta_{231-280}$. This result is interesting, as the interleaved zinc-binding domains of PKC$\delta_{231-280}$ have the same distribution of Cys and His zinc-coordinating ligands as FOG-$1_{950-995}$, MMTV NCp10, and HIV-1 NCp7.

NMR spectroscopy was used to examine the interactions between PKC$\delta_{231-280}$ and the thioester compounds in more detail. Zinc-refolded ^{15}N-labeled PKC$\delta_{231-280}$ was incubated with compound **1** for 48 h at 25 °C. The 2D $^1H-^{15}N$ HSQC spectrum recorded immediately after addition of compound **1** showed no changes to the PKC$\delta_{231-280}$ chemical shifts (Figure 3). This indicates that the conformation of PKC$\delta_{231-280}$ remained intact after addition of compound **1** and that the thioester compound did not covalently modifiy the thiols of PKC$\delta_{231-280}$.

The 2D $^1H-^{15}N$ HSQC spectra recorded throughout the 48 h incubation period with compound **1** revealed only a minor decrease in the intensity of PKC$\delta_{231-280}$ amide signals (Figure 4). These results confirm what was observed by UV/visible spectroscopy with the cobalt-refolded PKC$\delta_{231-280}$; the thioester compounds are unable to eject significant quantities of tetrahedrally coordinated metal from PKC$\delta_{231-280}$ over a 48 h time period.

Thioester compounds do not react with classical Cys_2His_2 ZBDs. To examine the reactivity of the thioesters with other ZBDs, we incubated compound **2** with the cobalt-refolded ZBD (Cys_2His_2) from the DNA-binding protein GAGA ($GAGA_{310-372}$) (Figure 2D). As described for MMTV NCp10 and FOG-$1_{950-995}$, we monitored changes in the UV/visible spectrum of cobalt-refolded $GAGA_{310-372}$ over a period of 3 h. In the case of the cobalt-refolded $GAGA_{310-372}$, we failed to observe any significant changes in the absorbance at λ_{max} due to ejection of the tetrahedrally coordinated cobalt (Table 1). Based on this result, we concluded that compound **2** was unable to eject coordinated cobalt from the Cys_2His_2 ZBD of $GAGA_{310-372}$ to any significant extent over the period studied.

Since compound **2** was unable to eject cobalt from $GAGA_{310-372}$, we next used NMR spectroscopy to look at interactions involving a second classical Cys_2His_2 zinc-binding protein, MBP-1, with compound **1**. The goal of these studies was to determine if other Cys_2His_2 ZBDs were also resistant to metal ejection by thioester compounds, as well as examine in more precise detail if there were any interactions between the thioester compounds and the proteins that did not involve metal ejection. MBP-$1_{2085-2142}$ contains two tandem Cys_2His_2 ZBDs separated by a seven amino acid linker (Figure 4E).[48] Zinc-refolded ^{15}N-labeled MBP-$1_{2085-2142}$ was incubated with compound **1** for 48 h at 25 °C. 2D $^1H-^{15}N$ HSQC experiments were used to observe changes in the $^1H-^{15}N$ correlations of the protein over time. Upon initial addition of compound **1** to zinc-refolded ^{15}N-labeled MBP-$1_{2085-2142}$, there was no change in amide signal intensity and chemical shift in the 2D $^1H-^{15}N$ HSQC spectrum (data not shown). This result indicates that there was no structural change in MBP-$1_{2085-2142}$ upon addition of compound **1**. In addition, the results also demonstrate that there was no covalent modification of the thiols of MBP-$1_{2085-2142}$ when incubated with compound **1**.

The MBP-$1_{2085-2142}$ amide signals in the 2D $^1H-^{15}N$ HSQC spectra did not change significantly over the 48 h incubation period (Figure 5). If compound **1** was interacting with MBP-$1_{2085-2142}$, differences in the intensity or chemical shift of the amide signals would be expected, similar to what we observed for MMTV NCp10. Thus, the lack of change in the amide signals of MBP-$1_{2085-2142}$ demonstrates that the protein did not undergo any conformational change during the incubation period with compound **1**. It is unlikely, then, that compound **1** was able to interact with MBP-$1_{2085-2142}$ to any degree, similar to what we observed in the UV/visible spectroscopy experiments with Cys_2His_2 ZBD of $GAGA_{310-372}$. Thus, we did not observe significant metal ejection from these ZBDs, suggesting that the classical Cys_2His_2 ZBDs are less susceptible to covalent modification by the thioester compounds than the Cys_2HisCys ZBDs.

GATA Cys_4 ZBDs interact with thioester compounds. In the previous experiments, we examined the interaction of thioester compounds **1** and **2** with structural ZBDs containing either two or three cysteine residues. We subsequently looked at the ability of compounds **1** and **2** to interact with structural ZBDs that bound metal with four cysteine residues (Cys_4). Compound **2** was incubated with cobalt-refolded $cGATA_{158-223}$,

(55) Hommel, U.; Zurini, M.; Luyten, M. *Nat. Struct. Biol.* **1994**, *1*, 383−87.

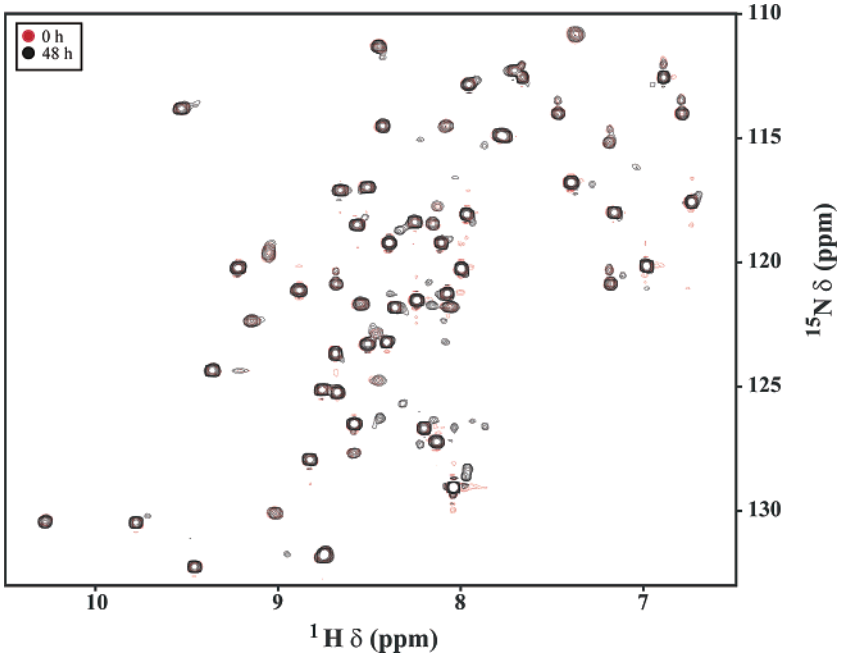

Figure 4. 2D ^{1}H−^{15}N HSQC spectrum of free PKC$\delta_{231-280}$ (red) superimposed on the 2D ^{1}H−^{15}N HSQC spectrum of PKC$\delta_{231-280}$ after 48 h of incubation with compound **1** (black).

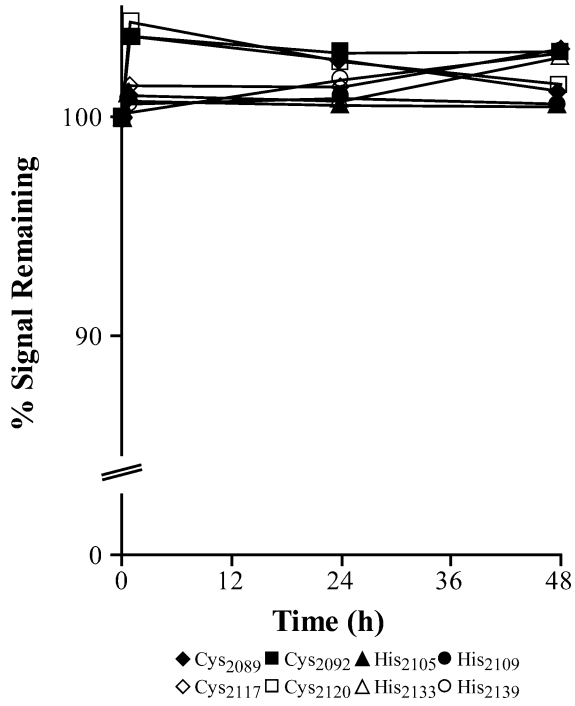

Figure 5. Changes in 2D ^{1}H−^{15}N HSQC signal intensity of MBP-1$_{2085-2142}$ zinc-coordinating residues after addition of compound **1**.

a fragment of the chicken GATA-1 transcription factor that contains one Cys$_4$ ZBD (Figure 2F).[42,56] As with the other ZBDs, we monitored the change in UV/visible absorbance over a 3 h period of time. The rate of absorbance loss observed for cGATA-1$_{158-223}$ was similar to that observed for FOG-1$_{950-995}$ and MMTV NCp10 (Table 1). This observed rate of absorbance loss for cGATA-1$_{158-223}$ was ∼2.5 times slower than that observed for HIV-1 NCp7.[30] Thus, the Cys$_4$ ZBD of cGATA-1$_{158-223}$ is able to interact with compound **2** to a similar degree as the various Cys$_2$HisCys domains studied.

To gain a more detailed understanding of the interaction between the thioester compounds and the Cys$_4$ ZBD of GATA-1, we again used NMR spectroscopy. Zinc-refolded hGATA-1$_{200-317}$ was incubated with compound **1** at 25 °C for 48 h. hGATA-1$_{200-317}$ is a human GATA-1 fragment that contains two Cys$_4$ ZBDs separated by a 29 amino acid linker (Figure 4G).[36] The 2D ^{1}H−^{15}N HSQC spectrum (Supporting Information Figure 2A) recorded immediately after addition of compound **1** did not display any significant changes in the amide signals of the ^{15}N-labeled hGATA-1$_{200-317}$. The lack of change demonstrates that the structure of hGATA-1$_{200-317}$ remained intact upon addition of compound **1**, indicating that compound **1** did not covalently modifiy the thiols of hGATA-1$_{200-317}$.

The 2D ^{1}H−^{15}N HSQC spectra recorded throughout the compound **1** incubation with hGATA-1$_{200-317}$ showed a loss of signal intensity similar to what was seen with MMTV NCp10 (Figure 6A). By 24 h, all eight zinc-coordinating cysteine residues had lost >60% of their original signal intensity. After 48 h, many signals had almost completely disappeared. The rates of signal intensity loss are very similar for both ZBDs of hGATA-1$_{200-317}$ (Table 2), indicating that compound **1** was capable of covalently modifying cysteine residues in both ZBDs. The rate of signal intensity loss calculated for the two ZBDs of hGATA-1$_{200-317}$ was ∼2.5 times greater than that observed for MMTV NCp10 (Table 2). Our results demonstrate that both Cys$_4$ ZBDs of GATA-1 are susceptible to metal ejection by the thioester compounds which results in loss of protein structure.

Binding of DNA to GATA prevents and is prevented by interaction with the thioester compounds. As GATA-1 is a transcription factor, it was important to determine if the DNA bound conformation of the GATA-1 protein would have an effect on the interaction with the thioester compound. We first determined that the unbound carboxyl-terminal ZBD of chicken

(56) Omichinski, J. G.; Trainor, C.; Evans, T.; Gronenborn, A. M.; Clore, G. M.; Felsenfeld, G. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1676−80.

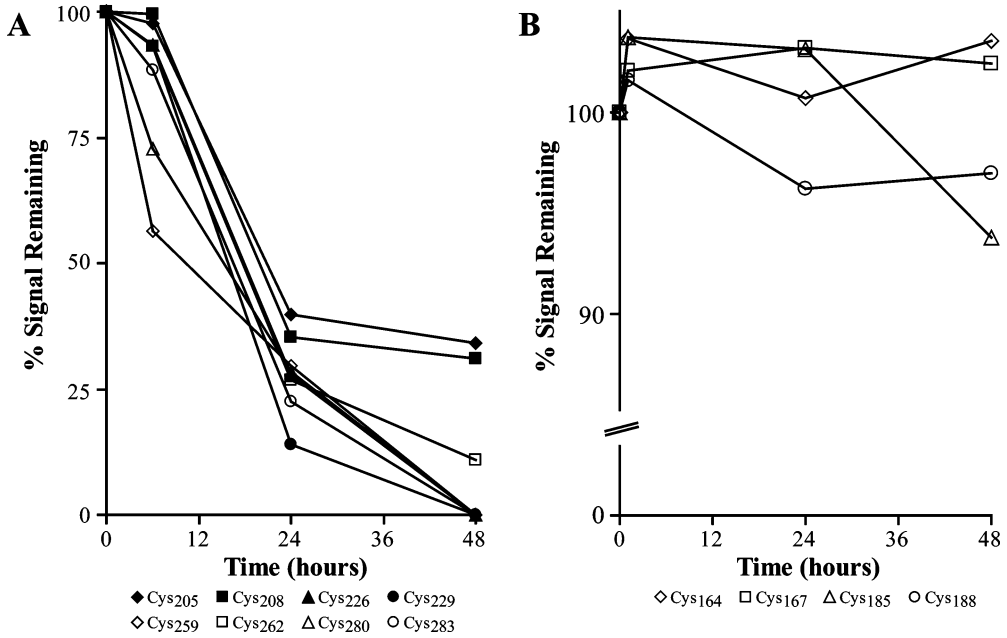

Figure 6. Changes in 2D $^{1}H-^{15}N$ HSQC signal intensity of zinc-coordinating residues of hGATA-$1_{200-317}$ (A) and AGATAA DNA-bound cGATA-$1_{158-223}$ (B) after addition of compound **1**.

GATA-1 (cGATA-$1_{158-223}$) was able to interact with compound **1** in a manner identical to that observed by NMR spectroscopy with the two ZBDs of hGATA-$1_{200-317}$ (data not shown). Previous studies have shown that the domain comprised by cGATA-$1_{158-223}$ is the minimum domain needed for specific DNA binding to target double-stranded AGATAA DNA sequences[56] and the NMR solution structure of the cGATA$_{1158-223}$/AGATAA DNA complex has been determined.[42] The zinc-refolded ^{15}N-labeled cGATA-$1_{158-223}$/AGATAA DNA complex was incubated with compound **1** for 48 h at 25 °C. The 2D $^{1}H-^{15}N$ HSQC spectrum recorded immediately after addition of compound **1** to the zinc-refolded ^{15}N-labeled cGATA-$1_{158-223}$/AGATAA DNA complex did not undergo any changes in the amide signals of the DNA-bound cGATA-$1_{158-223}$ (data not shown). This again indicated that the structure of the complex was unchanged upon addition of compound **1** and that cGATA-$1_{158-223}$ and compound **1** did not covalently modify the protein thiols. The 2D $^{1}H-^{15}N$ HSQC spectra did not change during the entire 48 h incubation with compound **1** (Figure 6B). The signal intensities of the zinc-coordinating residues remained the same for the entire incubation period. By 48 h, all signals of the DNA-bound cGATA-$1_{158-223}$ retained at least 94% of their original intensity, which is dramatically different from what was observed when compound **1** was incubated with either the free cGATA-$1_{158-223}$ or the free hGATA-$1_{200-317}$ (Figure 6). Thus, over the 48 h incubation period, compound **1** is unable to eject significant quantities of zinc from cGATA-$1_{158-223}$ when it is bound to its target AGATAA DNA sequence.

We used gel mobility shift assays to determine if preincubation of cGATA-$1_{158-223}$ with compound **1** would have an effect on the ability of cGATA-$1_{158-223}$ to bind DNA. Compounds **1** and **2** were incubated with zinc-refolded cGATA-$1_{158-223}$ for 30 s, 4 h, and 24 h at 25 °C. After the incubation period, 5′-^{32}P-labeled DNA was added to the zinc-refolded cGATA-$1_{158-223}$. The native gel ran after the 30 s incubation with compound **1** revealed that the amount of DNA bound to cGATA-$1_{158-223}$ was not significantly changed (Figure 7A). After 4 h of incubation with compound **1**, there was a small decrease in the amount of DNA bound (<5%), and after 24 h, we observed a significant decrease in the amount of DNA bound to cGATA-$1_{158-223}$ (~20%) (Figure 6B). In contrast, after a 30 s preincubation with compound **2**, there was already a significant decrease in the amount of bound DNA (~10%) (Figure 7A). After 4 h, only ~50% of the DNA remained bound to cGATA-$1_{158-223}$. However, longer incubations with compound **2** (24 h) did not seem to cause much decrease in bound DNA (Figure 7B). These results demonstrate that after thioester-induced metal ejection, the cGATA-$1_{158-223}$ protein loses its ability to bind specifically to its target DNA sequence. This indicates that the thioester compounds likely disrupt the structure of the ZBD, preventing sequence specific DNA binding. In this assay, compound **2** seems to have a more pronounced effect than compound **1**, a result that has also been observed previously with HIV-1 NCp7.[28,30]

Compound 1 is modified upon incubation with select ZBDs. The 1D difference water-sLED spectrum was used to monitor changes in compound **1** when mixed with an equimolar concentration of ^{15}N-labeled ZBDs.[44] The difference water-sLED experiment is run so that the difference spectrum shows resonances only from the more quickly diffusing part of the sample, i.e., the thioester compound. When the thioester compound is able to covalently modify the protein, it will diffuse more slowly with the protein, and the resonances for the thioester will no longer be observed. The 1D difference water-sLED spectrum that resulted from the spectra collected immediately after addition of compound **1** to MBP-$1_{2085-2142}$, MMTV NCp10, hGATA-$1_{200-317}$, cGATA-$1_{158-223}$/AGATAA DNA complex, or PKC$\delta_{231-280}$ was identical to the spectrum of the free thioester compound (data not shown). Therefore, compound **1** did not change upon addition to any of the proteins. Over the 48 h incubation of compound **1** with MBP-$1_{2085-2142}$, there was no change in ^{1}H chemical shifts or signal intensities attributed to compound **1** in the 1D difference water-sLED spectra at any time (data not shown). Thus, compound **1** did not undergo any

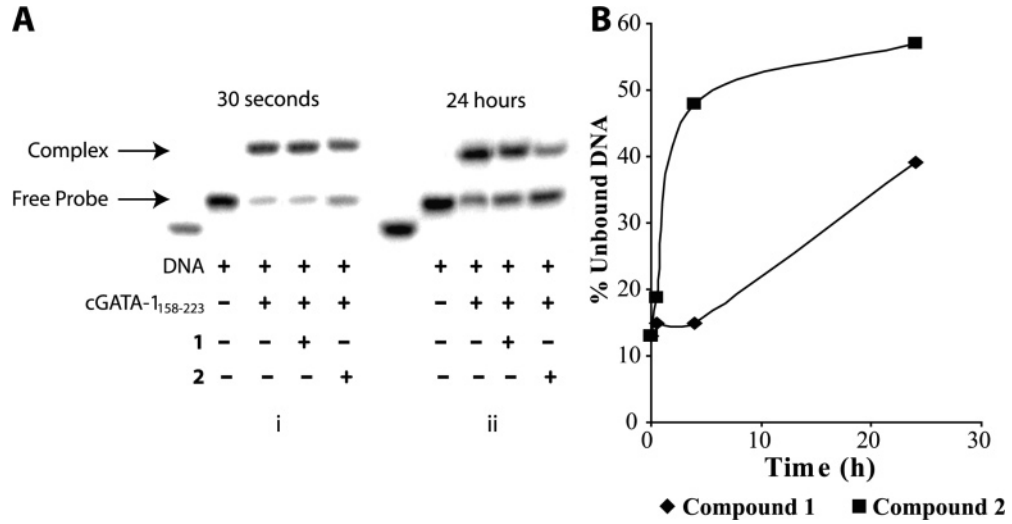

Figure 7. Gel mobility shift assay of binding of cGATA-$1_{158-223}$ to 5′-^{32}P-labeled AGATAA DNA upon incubation with thioester compounds. (A) cGATA-$1_{158-223}$ was incubated with compound **1** or **2** for 30 s (i) or 24 h (ii). AGATAA DNA was then added and incubated for 30 min before being run on an 8% native polyacrylamide gel. In each gel, the first lane is a control of labeled DNA prior to annealing. (B) Plot of change in free DNA over thioester incubation time. The density of each band was measured and used to calculate the percent free DNA as described in the Experimental Procedures.

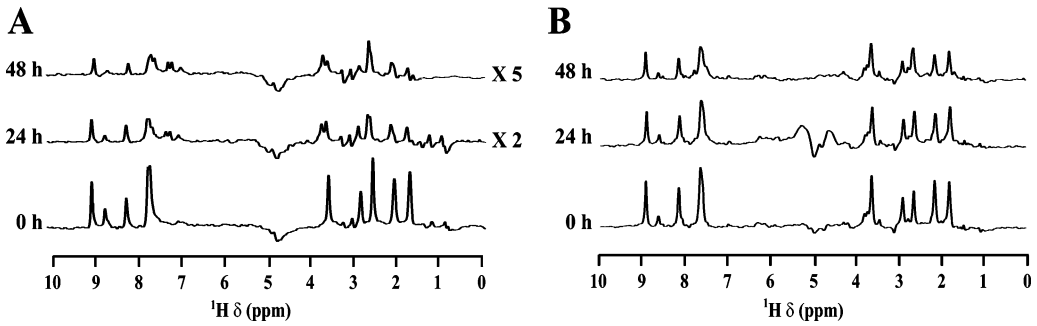

Figure 8. 1D difference water-sLED spectra of compound **1** incubated with hGATA-$1_{200-317}$ (A) or AGATAA DNA-bound cGATA-$1_{158-223}$ (B). In both A and B, spectra are shown after 0 h, 24 h, and 48 h.

alterations that would affect its diffusion during the incubation with MBP-$1_{2085-2142}$. These results further support our earlier conclusions that compound **1** fails to interact with MBP-$1_{2085-2142}$.

During the 48 h incubation of MMTV NCp10 with compound **1**, the 1D difference water-sLED spectra show that over time the signals of compound **1** lost some intensity (data not shown). After 48 h, the signals of compound **1** were almost indistinguishable from subtraction artifacts. Changes in the 1D difference water-sLED spectra could be due to either alterations in the diffusion of the thioester compound in solution or degradation of compound **1**. Since compound **1** is completely stable in NMR buffer over the 48 h incubation period,[30] the loss of signal intensity observed in the 1D difference water-sLED spectra is caused by an interaction of compound **1** with MMTV NCp10 that leads to covalent modification of the protein. This would then result in changes in the diffusion of the thioester compound. Based on these results, we conclude that compound **1** interacts in some fashion with the $Cys_2HisCys$ ZBD of MMTV NCp10.

In the incubation of compound **1** with hGATA-$1_{200-317}$, the 1D difference water-sLED spectra showed a progressive loss of intensity in the signals of compound **1**. This loss in signal intensity was observed for all ^{1}H signals of compound **1**, and by 48 h the signals had lost over 90% of their original signal intensity (Figure 8A). In contrast, during the incubation of compound **1** with the cGATA-$1_{158-223}$/AGATAA DNA complex, there was a smaller change in the intensity of the signals from the thioester compound (Figure 8B). Thus, these data support the fact that compound **1** modifies the unbound GATA-1 proteins at a faster rate than the DNA-bound GATA-1 protein.

After the 48 h incubation of compound **1** with PKC$\delta_{231-280}$, there were only minor changes in the 1D difference water-sLED spectra (data not shown). The signals of compound **1** lost only a small percentage of their original signal intensity. The primary change was the appearance of new signals (data not shown). The new signals were relatively weak and were only observed at the end of the incubation period, and may reflect a very weak interaction with PKC$\delta_{231-280}$. Overall, the signals of compound **1** remain intact, indicating that PKC$\delta_{231-280}$ does not interact significantly with the thioester compound.

Calculated electrostatic screening and protein packing values correlate with ZBD reactivity. Since the ZBDs of GATA-1, FOG-$1_{950-995}$, and MMTV NCp10 were found to be reactive with the thioester compounds, it was important to try to ascertain why these specific domains were reactive while other domains were not. The electrostatic (V_s) and steric (ρ_s) screening of the zinc-binding cores within these domains were calculated from experimentally determined structures. These metrics have been proposed to serve as predictors of the redox reactivity of a ZBD,[11] since exposed zinc-coordinated cysteine thiolates of a zinc-binding core would be more susceptible to

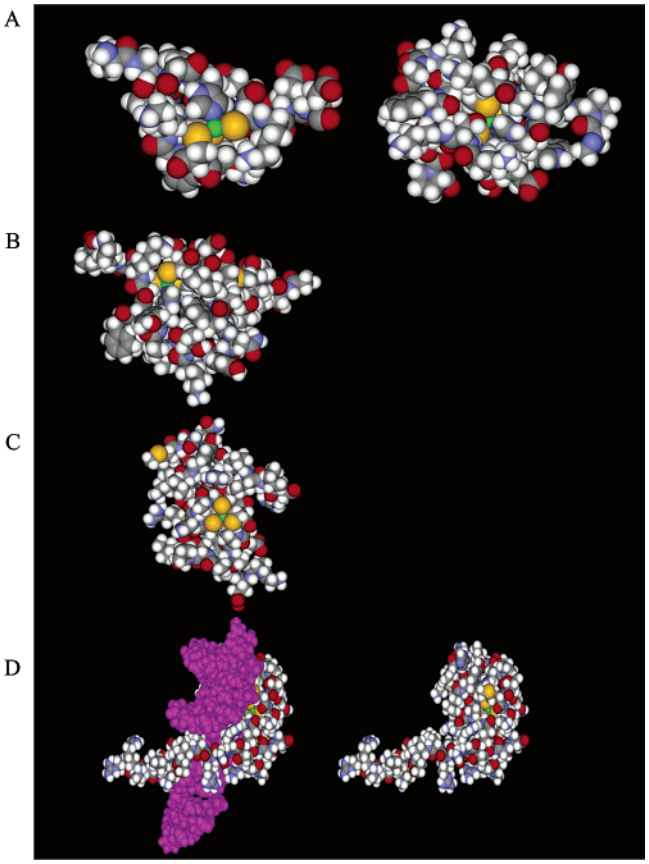

Figure 9. Comparison of the zinc-binding core environments of MMTV NCp10 ZD1 (A left, PDB: 1DSQ) and ZD2 (A right, PDB: 1DSV), FOG-1 (B, PDB: 1FU9), free GATA-1 (C, PDB: 1GNF), and DNA-bound GATA-1 (D, PDB: 3GAT). The zinc-coordinating cysteines are shown in gold, and the zinc in green. In D, the DNA is colored magenta in the left image and has been removed in the image at the right.

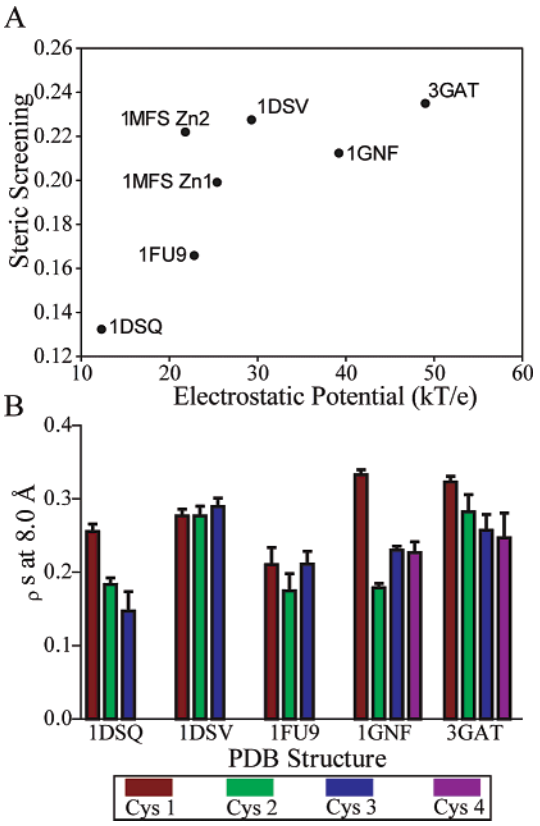

Figure 10. Comparison of calculated ZBD screening. (A) Plot of steric screening versus electrostatic screening for MMTV NCp10 ZD1 (PDB: 1DSQ) and ZD2 (PDB: 1DSV), FOG-1 (PDB: 1FU9), HIV-1 NCp7 (PDB: 1MFS), free GATA-1 (PDB: 1GNF), and DNA-bound GATA-1 (PDB: 3GAT). (B) Plot of steric screening at each zinc-coordinating cysteine at 8.0 Å. Error bars represent the variation in the calculated screening over the ensemble of structures for each ZBD.

reaction than cores sterically or electrostatically stabilized by the surrounding protein.

The reactive ZD1 of MMTV NCp10 is clearly much more open and solvent-exposed than ZD2 (Figure 9A). Indeed, both V_s and ρ_s are substantially lower for ZD1 than for ZD2 (Figure 10A). V_s is over 2 times greater and ρ_s is 1.5 times greater for ZD2 than for ZD1. Analysis of the steric screening around each of the cysteines in the two zinc-binding cores of MMTV NCp10 found Cys_{44} in ZD1 to be the least screened (Figure 10B). In addition, all of the cysteine residues in ZD2 are equally screened and have higher steric screening than any cysteine in ZD1 (Figure 10B). Thus, for MMTV NCp10, the less screened ZD1 is also the more reactive ZBD toward the thioester compounds.

The Cys_3His domain of FOG-1 is exposed on two sides of the protein, providing two areas for interaction (Figure 9B). This domain of FOG-1 has a similar degree of electrostatic screening to ZD2 of HIV-1 NCp7, though it is less sterically screened (Figure 10A). V_s and ρ_s are also midway between the two domains of MMTV NCp10 (Figure 9A). The cysteines of the zinc-binding core of FOG-1 are all in a similar steric environment, though the second of the three cysteines is slightly less screened (Figure 9B). Thus, the screening of the Cys_3His zinc-binding core of FOG-1 suggests that this ZBD should be reactive toward electrophiles, consistent with our experimental evidence.

The structure of the amino-terminal Cys_4 ZBD of GATA-1 illustrates that the zinc-coordinating residues are fairly well exposed and accessible in the free protein (Figure 9C). This is contrasted with the almost complete blockage of the zinc-binding core when the protein is bound to DNA (compare Figure 9, panels C and D). Further, V_s and ρ_s are both higher when the protein is in the DNA-bound conformation (Figure 10A). Interestingly, only the second cysteine is significantly more shielded in the DNA-bound form than in the free form (Figure 10B). Overall, the difference in screening of GATA-1 in the free and DNA-bound forms correlates well with the observed reactivity of GATA-1 with the thioester compounds: the thioester compounds can only interact with GATA-1 when it is not bound to DNA. In addition, the degree of calculated steric and electrostatic screening correlates with the reactivity of the ZBD; the zinc-binding cores of reactive domains are less sterically and electrostatically screened.

Discussion

In an attempt to develop new drug therapies to resistant strains of the HIV-1 virus, a number of different classes of small molecules have been designed to specifically target the HIV-1 NCp7 protein.[14−25] In most cases, these compounds are electrophilic and function by covalently modifying one or more cysteine thiolates in the HIV-1 NCp7 zinc-binding domains. The covalent modification of the cysteine thiolates by the thioester compounds ultimately leads to zinc ejection and loss of protein function, since the zinc ions play critical roles in maintaining the structure of the ZBDs.[19] Though it is unlikely

that the thioester compound would interact with the zinc itself as the coordination of zinc to the ZBD is quite tight (K_D ranging from 10^{-10}−10^{-12} M[57−59]), the thiolate that is released after thioester reaction could interact with the dissociated zinc ion, tending to prevent reassociation into the ZBD. Given the reactive nature of these electrophilic compounds, it is essential to experimentally measure their reactivity with ZBDs from cellular proteins in both their free and bound states. The thioester compounds are also extremely valuable probes for assessing the reactivity of structural ZBDs that have various zinc-coordination motifs.

Despite their importance in the cell, there have been surprisingly few studies addressing the reactivity of ZBDs from cellular proteins.[20,22] Theoretical studies suggest that the various classes of ZBDs have widely varying reactivity with electrophilic compounds, and it has been predicted that the reactivity toward ZBDs for a given group of electrophiles is highly dependent upon both first and second shell interactions with the zinc ion.[6,11,34,60] Unfortunately, previous experimental studies have never thoroughly examined the reactivity of these compounds with a diverse group of ZBDs from cellular proteins.[20,22] While the reactivity of a particular group of electrophilic compounds has been examined with one or two zinc-binding proteins specifically bound to nucleic acids,[20] no attempt has been made to compare the reactivity with the proteins in their unbound states. This lack of rigorous experimental data has limited the refinement of the initial theoretical predictions. In this study, we have investigated the reactivity of two electrophilic thioester compounds with ZBDs from six different proteins. The ZBDs originate from five cellular proteins and one retroviral protein, providing a diverse set of structural motifs.

Two thioester compounds were tested with three classes (Cys_2His_2, Cys_3His, and Cys_4) of ZBDs, including the classical zinc finger proteins. Classical zinc fingers chelate zinc with 2 molar equiv of cysteine and 2 mol of histidine to form a very compact $\beta\beta\alpha$ fold.[61] This is an extremely important and abundant class of proteins in mammalian systems, so it is important that a prospective therapeutic agent show little or no reactivity toward this class of ZBDs. We examined two zinc finger Cys_2His_2 ZBDs ($GAGA_{310-372}$ and $MBP\text{-}1_{2085-2142}$) and demonstrated that both were essentially unreactive toward the thioester compounds. These results are consistent with what has previously been published with other thioester and disulfide compounds.[20,22] The only difference is that in our studies we have looked at the reactivity of the cellular zinc fingers in their unbound states, whereas other studies examined their reactivity in their DNA-bound forms. These results are also consistent with theoretical and other experimental studies that suggest that Cys_2His_2 ZBDs are the least reactive type of zinc-binding cores, and therefore ZBDs that contain these cores are inherently resistant to redox reactions relative to ZBDs containing Cys_3His and Cys_4 motifs.[11,34,62] Thus, the experimental and theoretical data indicate that free or DNA-bound classical zinc finger proteins do not appear to interact readily with most electrophilic compounds.

We also tested the reactivity of three ZBDs that coordinate zinc with three cysteine residues and one histidine residue (Cys_3His). This class of ZBDs is structurally very diverse, and the three ZBDs that we selected were chosen to experimentally explore this structural diversity. Theoretical studies reveal that Cys_3His zinc-binding cores are more likely to be reactive toward electrophilic compounds than the Cys_2His_2 zinc-binding cores due to the extra negative charge from the additional cysteine thiolates.[11] The variation in reactivity of a given zinc-binding core has been suggested to depend on the second shell interactions with other residues around the zinc, as these residues can provide stabilization to the zinc-binding core.[6,11,60] As predicted, we did observe a wide range of reactivities between our thioester compounds and the three different ZBDs containing Cys_3His cores, indicating the importance of the protein environment. We determined that $FOG\text{-}1_{950-995}$ and ZD1 of MMTV NCp10 reacted with the thioesters, and their reactivities were similar to what we previously observed with the same thioesters for the Cys_3His ZD2 of HIV-1 NCp7. On the other hand, the interleaved Cys_3His ZBDs of $PKC\delta_{231-280}$ and MMTV NCp10 ZD2 possess low reactivity toward the thioester, analogous to what we observed for ZD1 of HIV-1 NCp7 (Table 2). The variation in reactivity suggests that not only are the second shell interactions around the zinc-binding core important but also there may be an element of amino acid sequence specificity that directs the interaction with the compounds. We are currently exploring this potential sequence specificity, focusing on amino acid residues that vary between both the reactive and nonreactive Cys_3His ZBDs of HIV-1 NCp7 and MMTV NCp10.

Our results are in good agreement with previous theoretical predictions of the potential reactivity of the Cys_3His ZBDs of HIV-1 NCp7 and $PKC\delta_{231-280}$,[11] which showed that the interleaved domains of PKCδ were more electrostatically and sterically screened than ZD2 of HIV-1 NCp7. These predictions were not previously made for the Cys_3His domains of MMTV NCp10 and FOG-1, since structures were not available at the time. Here, we have calculated the screening for these Cys_3His zinc-binding cores, to predict the reactivity of the corresponding ZBD. A ZBD is predicted to be more stable chemically as its zinc-binding core is more electrostatically and sterically screened by the surrounding protein. We found the amino-terminal zinc-binding core of MMTV NCp10 to be 2 times less electrostatically screened and 1.5 times less sterically screened than ZD2 (Figure 9A). The shielding of the carboxyl-terminal zinc-binding core is provided by a series of hydrophobic and electrostatic interactions between the ZBD and a β-hairpin that is located immediately adjacent to it. Calculations performed with the Cys_3His domain of FOG-1 also show its zinc-binding core to be only moderately screened, similar to the carboxyl-terminal zinc-binding core of HIV-1 NCp7 (Figure 10A). Our experimental results with these Cys_3His ZBDs correlate with the trends in ρ_s and V_s screening of the zinc-binding cores. Interestingly, ZD1 of MMTV NCp10 is less screened than ZD2 of HIV-1 NCp7, yet we found the latter to be slightly more reactive than the former (Table 2). This result suggests that there may be specific protein sequence determinants for interaction with the thioester compounds. Notably, the computed steric and elec-

(57) Ghering, A. B.; Shokes, J. E.; Scott, R. A.; Omichinski, J. G.; Godwin, H. A. *J. Am. Chem. Soc.* **2004**, *43*, 8346−55.
(58) Krizek, B. A.; Merkle, D. L.; Berg, J. M. *Inorg. Chem.* **1993**, *32*, 937−40.
(59) Magyar, J. S.; Godwin, H. A. *Anal. Biochem.* **2003**, *320*, 39−54.
(60) Dudev, T.; Lin, Y.-L.; Dudev, M.; Lim, C. *J. Am. Chem. Soc.* **2003**, *125*, 3168−80.
(61) Lee, M. S.; Gippert, G. P.; Soman, K. V.; Case, D. A.; Wright, P. E. *Science* **1989**, *245*, 635−7.
(62) Wilker, J. J.; Lippard, S. J. *J. Am. Chem. Soc.* **1995**, *117*, 8682−3.

trostatic screening metrics only account for zinc-binding core/protein interactions in stabilizing a zinc-binding domain and do not address possible protein/ligand interactions that could alter stability. Moreover, ρ_s and V_s measure only the radial distribution of zinc-binding core/protein interactions and therefore are insensitive to angular variation in the protein screening of a zinc-binding core, such as a cleft within the ZBD structure that could permit specific zinc-binding core/thioester docking. These metrics are also based upon a small sample of static protein structures that do not fully account for protein dynamics. Rather than being absolute predictors of reactivity, ρ_s and V_s are more likely to be useful as qualitative metrics for quickly assessing relative trends in ZBD reactivity, based on nonspecific oxidation mechanisms, in keeping with their original intended use. Nonetheless, the experimental results strongly support theoretical predictions that zinc-binding core/protein interactions can significantly modulate the stability and reactivity of the zinc-coordination complex, in this case Cys_3His, within ZBDs.

The Cys_4 ZBDs were the third class of ZBDs for which the stability was probed with the thioester compounds. Experimental model systems[34,62] and theoretical predictions suggest that this class of ZBDs is the most reactive toward electrophiles by virtue of the four cysteine thiolates present in the ZBD. For these studies, we used the ZBDs from the GATA-1 protein as a test case. The GATA-1 protein contains two Cys_4 ZBDs separated by a 29-amino acid linker (Figure 2G). The two ZBDs are structurally very similar but serve distinct biological functions in hematopoietic development and appear to be structurally independent domains within the protein. GATA-1 was chosen to determine whether the two ZBDs display different relative reactivities toward the thioesters, as observed with the two ZBDs of HIV-1 NCp7 and MMTV NCp10. Based on the initial metal ejection experiments with protein fragments containing either one or both ZBDs from GATA-1, it was clear that both Cys_4 ZBDs in the GATA-1 protein are very susceptible to zinc ejection when incubated in the presence of the thioester compounds. Mass spectrometry studies showed that all four cysteine residues in $cGATA\text{-}1_{158-223}$ are modified by the thioester compounds and there does not seem to be a preference for a specific cysteine residue (Supporting Information Figure 3). This result demonstrates that covalent modification of GATA-1 by thioester compounds is nonspecific, in contrast to the apparent specificity observed in the reactions with the Cys_2-HisCys ZBDs of HIV-1 NCp7 and MMTV NCp10. Since the Cys_4 ZBD is inherently more reactive than the $Cys_2HisCys$ ZBD, this greater reactivity may result in the loss of specificity for the thioester modification.

Initially, it was believed that these results were in disagreement with earlier experimental studies and theoretical predictions that suggested that the carboxyl-terminal ZBD of GATA-1 would have low reactivity with electrophilic compounds. However, early experimental studies with disulfide compounds were done in the presence of DNA, and the theoretical predictions were made with the structure of the carboxyl-terminal ZBD of GATA-1 in the DNA-bound state. Therefore, we examined the reactivity of the carboxyl-terminal ZBD of GATA-1 in both the free and the DNA-bound state. Our results clearly demonstrated that the free carboxyl-terminal ZBD was susceptible to attack by the thioesters, whereas the DNA-bound form was not. In fact, the two ZBDs of free GATA-1 are the most reactive among the ZBDs that we studied. In addition, we determined that the disulfide compounds are also capable of ejecting metal from the free GATA-1 ZBDs (L.M.J., P.L., and J.G.O. unpublished data).

We further calculated the electrostatic and steric screening of the GATA-1 zinc-binding cores using the unbound structure of GATA-1. The GATA-1 zinc-binding cores are significantly less screened, in terms of both ρ_s and V_s, when no DNA is present compared with GATA-1 bound to DNA (Figure 9). The binding of the carboxyl-terminal ZBD of GATA-1 to DNA induces a significant structural change in regions adjacent to the ZBD.[42] In particular, there is a significant increase in the electrostatic screening around the zinc ion provided by several basic amino acids that make salt bridges to help stabilize the coordination of the zinc ion. Studies on zinc−thiolate analogues have demonstrated that hydrogen bonds forming between an amide nitrogen and a thiolate can reduce the reactivity of the thiolate by 1 or 2 orders of magnitude.[7] Thus, the trends in ρ_s and V_s correlate with the experimental reactivity toward the thioester compounds. In addition, the results with the GATA-1 ZBD are very similar to what was previously observed with HIV-1 NCp7, where binding to RNA significantly reduced the rate of zinc ejection from ZD2 of HIV-1 NCp7.[30] In summary, the GATA-1 Cys_4 ZBDs were highly reactive, but specific binding to their target nucleic acid significantly increased the stability of the ZBDs.

In conclusion, these results provide important information regarding the reactivity of various classes of ZBDs with thioester compounds specifically designed to eject zinc from HIV-1 NCp7. The results demonstrate that the thioester compounds have considerable in vitro reactivity with Cys_4 GATA ZBDs and some Cys_3His ZBDs found in both cellular and other retroviral proteins. In the case of GATA-1, reactivity with the thioester compounds appears to be inhibited by binding to its specific nucleic acid target, as observed for HIV-1 NCp7. The thioester compounds serve as excellent probes for determining the stability and reactivity of cysteine compounds within the zinc-binding core of ZBDs. Our experimental results greatly improve our understanding of the reactivity of thiolates present in various classes of ZBDs. In addition, the results provide valuable information that can be incorporated in the future design of therapeutic agents with improved reactivity and specificity for the structural zinc-binding domains.

Acknowledgment. This work was supported by Canadian Institutes of Health Grants MOP-135604 (J.G.O.), MOP-64341 (P.L.), and National Institutes of Health Grant RO1 GM60298-01 (to J.G.O and P.L.). P.L. holds a Canada Research Chair in Structural Biology of RNA. L.M.M.J. was supported by a Presidential Graduate Fellowship from the University of Georgia. This project was funded in part by the NIH Intramural AIDS Targeted Antiretroviral Program (IATAP).

Supporting Information Available: Complete refs 17 and 51, and supplementary figures referenced in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

JA063329E