Metal and DNA Binding Properties of a Two-Domain Fragment of Neural Zinc Finger Factor 1, a CCHC-type Zinc Binding Protein†

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ABSTRACT: Neural zinc finger factor 1 (NZF-1) is a member of a family of neural-specific transcription factors that contain multiple copies of a relatively uncharacterized zinc binding motif. We have studied the metal binding and DNA binding properties of a fragment of NZF-1 containing two adjacent zinc binding domains. Partial proteolysis with endoproteinase Lys-C identified metal-stabilized fragments containing either one or both of the zinc binding domains. Both domains were required for specific DNA binding to the β-retinoic acid receptor element, producing a DNase I footprint covering predominantly one strand. The metal binding site was probed via cobalt(II) substitution. The visible absorption spectrum of the cobalt(II) complex is consistent with Cys-Cys-His-Cys coordination of the metal. The two domains appear to have similar affinities for metal and bind cobalt(II) and zinc(II) with dissociation constants of 4 ($\pm$ 2) $\times$ 10^{-7} M and 1.4 ($\pm$ 0.8) $\times$ 10^{-10} M, respectively. The domains fold upon the addition of zinc, as observed by ^{1}H NMR. However, an additional weak binding site causes line broadening in the presence of excess zinc, presumably due to aggregation.

An essential role of zinc in biology involves the stabilization of small structural domains, many of which function by binding nucleic acids (*1*). One of the most common of these DNA binding structural motifs is the Cys_2His_2-type zinc finger found in eukaryotic transcription factors and characterized by sequences of the form Cys-X_{2-4}-Cys-X_{12}-His-X_{3-5}-His. These fingers contain a single zinc ion coordinated by the cysteine and histidine residues to form a stable domain consisiting of two antiparallel β strands followed by a helix. These domains almost always occur in tandem repeats. Zinc binding domains found within the steroid hormone receptor family contain two zinc ions, each coordinated by four cysteine ligands to form two structurally distinct units (*2*−*4*). Unlike the Cys_2His_2 zinc fingers, in which the domains form independent, noninteracting structures, these two zinc binding domains are folded together into a single stable DNA binding unit. GAL4 represents a family of fungal transcription factors that also contain a single cysteine-rich DNA binding domain. These domains contain a metal cluster with two zinc ions surrounded by a total of only six cysteine ligands; two of the cysteines bridge two ions to satisfy tetrahedral zinc coordination (*5*−*7*).

A new structural class of zinc binding transcription factors has emerged, characterized by the presence of multiple Cys-X_4-Cys-X_4-His-X_7-His-X_5-Cys sequences (*8*). These domains have been found in transcription factors with neuronal expression patterns. Myelin transcription factor (MyT1),[1] the first identified member of this family (*8*), was cloned from a human fetal brain-derived phage expression library upon screening for proteins that bind to regulator sequences for the proteolipid protein (PLP) gene. A similar method was used to isolate the gene for neural zinc finger factor 1 (NZF-1) from a rat pituitary cell line in a search for proteins that bind retinoic acid response elements (*9*). Several other members of this NZF/MyT1 gene family have since been identified through homology cloning (*10*−*13*).

The six putative zinc binding domains present in MyT1 are arranged into clusters of two and four, separated by an apparent transactivation region. NZF-1 has similarly spaced clusters of two and three domains and a single domain residing near the amino terminus of the protein. Specific DNA binding has been observed for expressed protein fragments containing either of the cluster regions within MyT1 or NZF-1, whereas a single domain binds DNA only nonspecifically (*8*, *9*).

The amino acid sequences of the NZF/MyT1-type domains distinguish them from the Cys_2His_2 zinc fingers. Most notably, these sequences include *five* absolutely conserved potential zinc binding ligands: three cysteines and two histidines. The metal binding stoichiometry by such domains has not been investigated and many structures can be envisioned, assuming tetrahedral metal ion coordination. These domains also contain several additional invariant residues, particularly a set of glycine and proline residues.

We present here the characterization of the two-domain fragment of NZF-1. A fragment encoding residues 487−606 was isolated by PCR from a rat 3-week brain cDNA library. Metal-stabilized structured regions were identified

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[1] Abbreviations: NZF-1, neural zinc finger factor 1; MyT1, myelin transcription factor; PLP, proteolipid protein; PCR, polymerase chain reaction; β-RARE, β-retionic acid response element; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacryamide gel electrophoresis; NTA, nitrilotriacetic acid; DMS, dimethyl sulfate.

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by partial proteolysis and examined for binding to the β-retinoic acid response element (β-RARE). DNase I footprinting was used to further characterize the DNA binding site. The cobalt(II) and zinc(II) binding properties of the metal binding domains were investigated, and zinc-induced protein folding was observed by ^{1}H NMR.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of an NZF-1 Fragment. Primers 5′-GCACCGCATATGCATGTCAAAAAGCCATACTATGATCCC-3′ and 5′-TATTATGTCGACTATCATGGCCTGAGGACTCGGTCTGAGGCCTG-3′ were used to amplify 360 base pairs of NZF-1 from a rat 3-week brain cDNA library. The PCR product was then cloned into the T7 expression vector pG5 (*14*) to construct plasmid pNZF-1a. pNZF-1b was subcloned from pNZF-1a and corresponds to the removal of 22 amino acids from the C-terminus of the expressed protein. Plasmids were transformed into BL21 (DE3) bacteria. Protein expression and partial purification were carried out as previously described (*15*), followed by stepwise elution with KCl from an SP-Sepharose Fast Flow column (Pharmacia). Protein was then reduced at 55 °C with excess dithiothreitol (DTT) and further purified by HPLC on a diphenyl reversed-phase column (Vydac). Molecular masses were confirmed by electrospray mass spectrometry. Protein concentrations were measured by use of an extinction coefficient of 5390 M^{-1} at 275 nm, determined by quantitative amino acid analysis.

Partial Proteolysis with Endoproteinase Lys-C. Purified NZF-1a (12 μM) was treated with endoproteinase Lys-C (Boehringer Mannheim) at a substrate:protease ratio of 400:1 (w/w). The reaction was carried out in 25 mM Tris-HCl (pH 8.0), 100 mM KCl, 100 μM $ZnCl_2$, and 2 mM DTT at 37 °C for up to 3 h and terminated by the addition of 1 μM leupeptin. Following reduction with DTT, the proteolytic fragments were separated by HPLC on a diphenyl reversed-phase column. Three isolated fragments were identified by N-terminal sequence analysis and electrospray mass spectrometry.

Construction and Preparation of the β-RARE DNA Binding Site. Complementary deoxyoligonucleotides containing the β-retinoic acid response element (β-RARE; GTAGGGTTCACCGAAAGTTCACTC) (*9*) were synthesized, annealed, and cloned into a modified version of the pEMBL vector (*16*). A 51 base pair DNA fragment containing β-RARE was excised from the plasmid by digestion with *Eco*RI and *Hin*dIII and end-labeled with [α-^{32}P]dATP by using the Klenow fragment of DNA polymerase.

Electrophoretic Mobility Shift Analysis. Protein was combined with 1−5 ng of labeled DNA in 20 μL of 25 mM Tris-HCl (pH 8.0), 100 mM KCl, 50 μM $ZnCl_2$, 2 mM DTT, 10% (v/v) glycerol, 2 mg/mL BSA, and 40 μg/mL poly(dI·dC). Reaction mixtures were incubated at room temperature for 25 min, followed by electrophoresis on Tris−glycine 8% polyacrylamide gels. The gels were dried and autoradiographed.

DNase I Footprinting of NZF-1b. The 51 base pair DNA fragment containing the β-RARE site was labeled with ^{32}P exclusively at one end. Binding reactions were prepared essentially as described above but in 40 μL of binding buffer: 37.5 mM Tris-HCl (pH 8.0), 100 mM KCl, 75 μM $ZnCl_2$, 2 mM DTT, 20 μg/mL poly(dI·dC), 50 μg/mL BSA, 5 mM $MgCl_2$, and 1 mM $CaCl_2$. One microliter of 1 unit/μL DNase I (FPLC pure, Pharmacia) was added and digestion was terminated after 1 minute with 150 μL of stop solution (700 mM NH_4OAc and 70 μg/mL tRNA in ethanol). Reactions were precipitated and resuspended in 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF. Radioactivity levels were counted on a Beckman LS 6000SE scintillation counter, and equal counts of each sample were electrophoresed on denaturing 10% polyacrylamide gels. The gels were dried and visualized by autoradiography with intensifying screens or with the use of a phosphoimager.

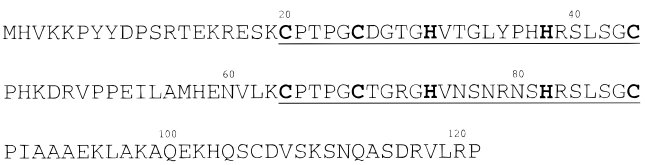

FIGURE 1: Sequence of the expressed NZF-1a protein fragment. Zinc finger domains are underlined, and conserved cysteine and histidine residues are shown in boldface type.

Titration of NZF-1b with Cobalt(II) and Zinc(II). Metal titrations were performed in an anaerobic atmosphere containing 5−6% hydrogen in nitrogen to prevent cysteine oxidation. Reactions were carried out in 100 mM HEPES (pH 6.9) and 50 mM NaCl at 25 °C. The d−d transitions of the metal/peptide complexes were optically monitored over the range of 470−820 nm by using a Perkin-Elmer Lambda 9 spectrophotometer. Approximately 80 nmol of NZF-1b was titrated to saturation of the d−d transitions with $CoCl_2$. Cobalt(II) was then added to 1000-fold excess, and $ZnCl_2$ was titrated in while the decrease in absorbance of the cobalt(II)−protein complex was monitored. Changes in the absorption spectra were used to evaluate the dissociation constants for the cobalt(II) and zinc(II) complexes by nonlinear least-squares analysis.

Zinc(II) Titration of NZF-1b by ^{1}H NMR. NMR samples containing 470 μM NZF-1b were prepared in ^2H_2O in the absence of zinc and the pH was adjusted to approximately 7.0 with deuterated Tris. Zinc(II) was titrated into samples of NZF-1b until saturation. Protein aggregation caused by excess zinc(II) was reversed by the addition of nitrilotriacetic acid (NTA), a metal chelator, to 1.2 mM. ^{1}H NMR spectra were recorded at 20 °C with a Varian UNITY Plus 500 MHz NMR spectrometer.

RESULTS

The transcription factor NZF-1 is highly expressed in the rat brain and pituitary (*9*). We have cloned a cDNA encoding a 121 amino acid portion of NZF-1 from a rat 3-week forebrain cDNA library (Figure 1). The expressed protein fragment, called NZF-1a, encompasses the two zinc binding domain cluster and binds specifically to the β-RARE DNA binding site as shown by gel shift analysis (data not shown).

NZF-1a contains extensive N- and C-terminal sequences flanking the two highly conserved zinc binding domains. To identify a more well-defined structural domain that is stabilized upon binding zinc, NZF-1a was subjected to partial proteolytic cleavage with endoproteinase Lys-C in the presence and absence of zinc (Figure 2A). Digests were

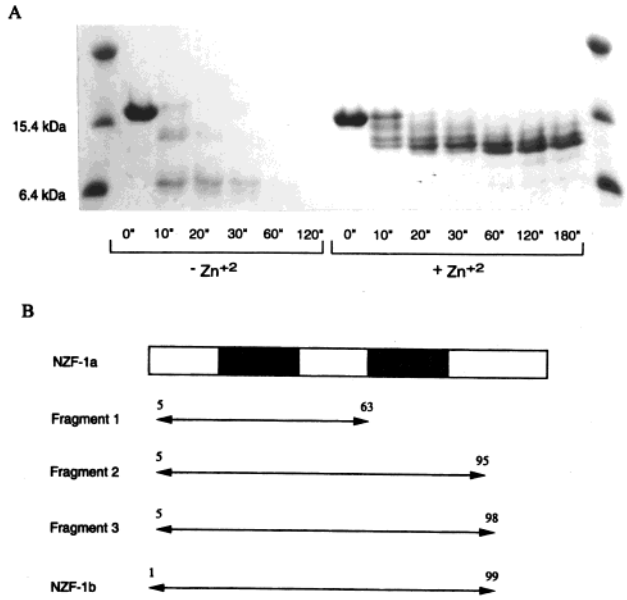

FIGURE 2: Proteolysis of NZF-1a. (A) NZF-1a was digested with endoproteinase Lys-C at a w:w ratio of 400:1 in the presence and absence of Zn^{2+}. (B) Identities of the isolated proteolytic fragments, determined by amino acid analysis and electrospray mass spectrometry.

analyzed by SDS−PAGE. Proteolytic cleavage of NZF-1a in the absence of zinc initially produced multiple bands. By 60 min the protein was completely digested. In the presence of zinc, multiple bands were observed at early time points. By 1 h, the major products were two fragments of approximately 10 kDa each. These were stable for up to 3 h of digestion.

The proteolytic digest at the 3 h time point was subjected to reversed-phase HPLC. Three products were isolated and further characterized by mass spectrometry and amino-terminal sequence analysis (Figure 2B). Fragment 1 contained the first zinc binding domain and the entire 19-residue linker, while fragments 2 and 3 retained both domains. All three fragments shared a common N-terminus, consisting of 15 residues adjacent to the first metal binding cysteine. Despite the presence of four lysines in the N-terminal flanking sequence of NZF-1a, cleavage by endoproteinase Lys-C was observed exclusively after the first lysine residue. Glutamate residues within this sequence were also inaccessible to endoproteinase Glu-C (data not shown).

Within the linker, only one of two potential cleavage sites was cut by endoproteinase Lys-C. Fragment 1 was formed by cleavage at the lysine adjacent to the first metal-binding cysteine of the second domain. As noted above, a lysine at the equivalent position of the first domain was protected from cleavage. A corresponding cleavage product containing the second domain was not isolated. Fragments 2 and 3 represent the major endoproteinase Lys-C cleavage products. The C-termini of these fragments were significantly shortened by proteolysis, with the removal of 26 and 23 amino acids, respectively. Gel shift analysis with the β-RARE DNA binding site showed that both fragments are functional DNA binding domains. Fragment 1 did not bind DNA strongly, on the basis of gel shift analysis (Figure 3). Further characterization of the protein was performed with the construct NZF-1b, which corresponds to residues 1−99 of NZF-1a and is five residues longer than fragment 3.

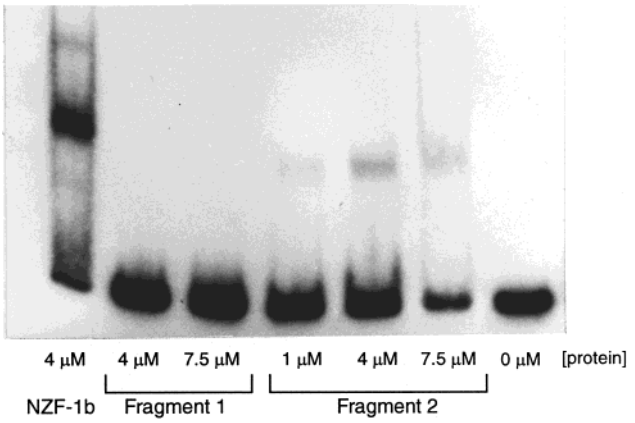

FIGURE 3: Binding of the proteolytic fragments 1 and 2 to the β-RARE-DNA binding site. ^{32}P-labeled β-RARE-containing DNA fragments were incubated with the isolated proteolytic fragments at the indicated concentrations and analyzed on a Tris−glycine 8% polyacrylamide gel. Results similar to those for fragment 2 were obtained for fragment 3 (not shown). NZF-1b served as a positive control.

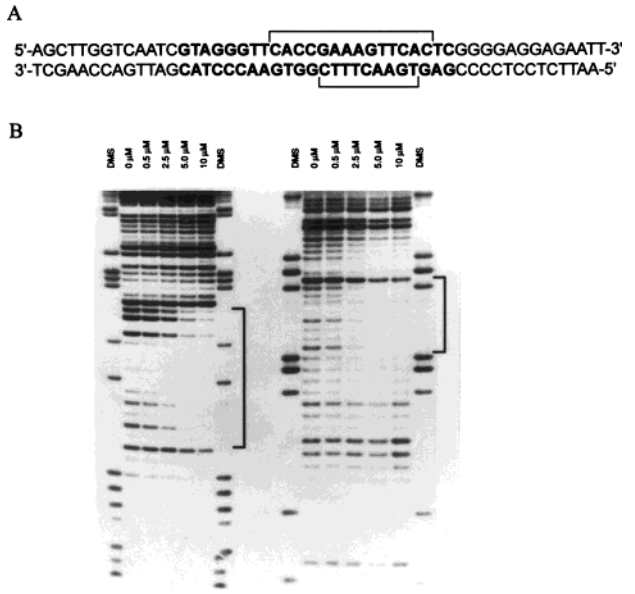

FIGURE 4: DNase I footprinting analysis of the NZF-1b/β-RARE complex. The 51 base pair DNA fragment containing β-RARE (panel A; in boldface type) was ^{32}P-labeled on one strand. Incubation of the DNA with NZF-1b at the indicated concentrations was followed by digestion with DNase I and electrophoresis on a denaturing 10% polyacrylamide gel (shown in panel B). Brackets indicate the observed footprints on the sense (left) and antisense (right) strands. DMS lanes show the guanine positions derived by reaction with dimethyl sulfate (DMS).

An expressed protein fragment of NZF-1 containing the two domain cluster has been shown to bind the β-RARE DNA binding site specifically (*9*). We performed DNase I footprinting experiments in order to further define the DNA binding site (Figure 4). A strong footprint extending over 14 bases was observed on the sense strand. A somewhat weaker footprint over 9 bases on the antisense strand overlapped this region. Binding isotherms were fit to a dissociation constant of 1.6 μM for this protein−DNA complex.

Because the zinc-bound forms of these peptides have no readily accessible spectroscopic signals in the visible region, cobalt(II) was substituted into the metal binding domains to probe the ligand environment (Figure 5). The visible absorp-

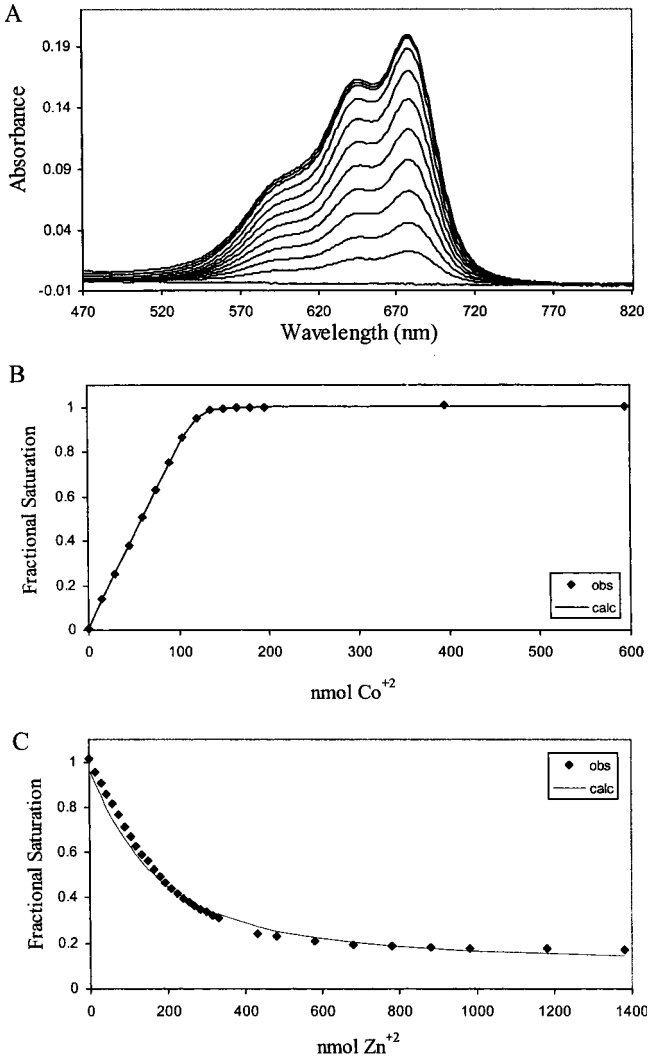

FIGURE 5: Cobalt(II) and zinc(II) binding by a two-domain fragment of NZF-1. Aliquots of $CoCl_2$ were added to a solution of the reduced protein in 100 mM HEPES (pH 6.9) and 50 mM NaCl. $ZnCl_2$ was subsequently back-titrated into the protein-cobalt(II) complex, in the presence of excess cobalt(II). (A) Representative titration of NZF-1b with cobalt(II), showing the d−d absorption region. The fractional saturation of the NZF-1b/cobalt(II) complex as a function of cobalt(II) and zinc(II) concentrations is shown in panels B and C, respectively. The curves were fit to the data by nonlinear least-squares methods.

tion spectrum of the NZF-1b−cobalt(II) complex is consistent only with tetrahedral cobalt(II) coordination, based on band positions and intensities. This spectrum is quite similar to that obtained for a peptide corresponding to a single NZF-1 domain (*17*). The three transitions observed correspond to $^4A_2$ to $^4T_1$ (P) ligand field transitions (*18*, *19*). Studies with zinc finger peptide variants and with model cobalt(II) complexes have shown that these transitions are shifted to lower energies upon increasing the number of cysteine ligands (*18*, *20*). The absorption envelope of the NZF-1b/cobalt(II) complex, extending from 550 to 725 nm, is red-shifted compared to that of the Cys_2His_2 zinc fingers (*21*) and is similar to that of the retroviral nucleocapsid CCHC box proteins (*22*, *23*). These observations support a CysCysHisCys ligand configuration for the NZF1-type zinc binding domains, indicating that one of the conserved histidines is involved in metal ion binding whereas the other plays some other as-yet-undetermined role.

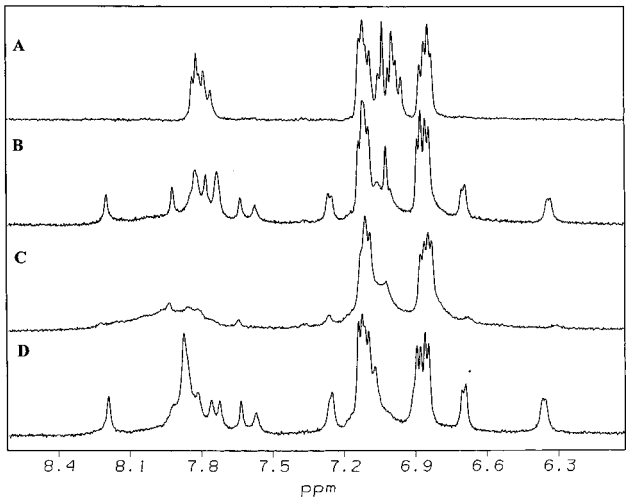

FIGURE 6: ^1H NMR titration of NZF-1b with zinc. NZF-1b appears to be largely unfolded in the absence of zinc (A). Upon addition of zinc to the apoprotein, substantial increases in spectral dispersion were observed (B). The addition of more than 2 equiv of zinc(II) per protein caused substantial broadening of the spectrum (C), which was reversed by the subsequent addition of NTA (D). The causes of the differences between spectra B and D have not been identified.

The cobalt(II) and zinc(II) binding affinities of NZF-1b were examined by visible spectroscopy. Titration of cobalt(II) into the apoprotein produced the absorption spectra shown in Figure 5A. The metal binding domains showed a greater affinity for zinc(II) than for cobalt(II), as observed by a decrease in the absorbance of the NZF-1b−cobalt(II) complex upon titration with zinc(II). Nonlinear least-squares analysis of the fractional saturation data yielded dissociation constants of 4 ($\pm$ 2) $\times$ 10^{-7} M for cobalt(II) complex and 1.4 ($\pm$ 0.8) $\times$ 10^{-10} M for zinc(II) complex.

Folding of NZF-1b was followed by ^1H NMR spectroscopy (Figure 6). The spectrum of the apoprotein showed two groups of resonances in the aromatic region corresponding to histidine and tyrosine protons. Titration of zinc(II) into the apoprotein led to dispersion of these resonances, indicative of structure formation. Surprisingly, the addition of zinc in small excess relative to the metal binding sites caused a significant broadening of the spectrum, apparently due to protein aggregation. A similar but not identical spectrum with sharp lines was recovered by the addition of a stoichiometric amount of a weak metal chelator.

DISCUSSION

NZF-1 is a member of a class of neural-specific transcription factors containing multiple copies of a novel zinc binding motif. These domains have five potential zinc binding ligands, three cysteines and two histidines. The visible absorption spectrum of the cobalt(II)-bound form of these domains indicates a CysCysHisCys coordination scheme but does not establish which of the two conserved histidines is bound to the metal. Other investigations have suggested that the first rather than the second histidine is involved in metal ion binding, but this point must be more clearly established through additional studies (*17*).

The NZF-1b domains appear to be relatively unstructured in the absence of metal, based on lack of dispersion in the

^{1}H NMR spectrum. Folding of the protein upon the addition of zinc(II) was accompanied by dispersion of the aromatic resonances. Because the NZF-1b construct contains eight histidine residues, peaks corresponding to the two histidines involved in metal ligation could not be unambiguously identified. Additionally, the ^{1}H NMR spectrum broadened as zinc(II) was added beyond 2 equiv per protein. This appears to be the result of protein aggregation caused by zinc binding to additional sites on the protein fragments. A similar secondary zinc binding site was observed for the Nanos metal binding domain (*24*). The aggregation of NZF-1b was abolished by the addition of a stoichiometric amount of a metal chelator, NTA, without removing a substantial amount of the zinc(II) from the main binding sites.

Upon titration of NZF-1b with cobalt(II) to saturation of the metal sites, the shape of the visible absorption spectrum did not change. The dispersed aromatic resonances within the ^{1}H NMR spectrum also grew in evenly as zinc was added. Thus, the two metal binding sites in NZF-1b could not be distinguished and appear to have roughly equivalent affinities for both cobalt(II) and zinc(II). The metal ion affinities are comparable to those of other zinc binding domains (*20*, *22*, *23*).

It has been demonstrated for members of this family that two zinc binding domains are sufficient for specific DNA binding. Furthermore, a minimum of two domains is required, as shown with NZF-1 (*9*) and by the proteolysis and gel shift experiments reported here. The two domain cluster of NZF-1 produced a DNase I footprint that extended over approximately 1.5 helical turns of DNA, primarily protecting the sugar−phosphate backbone of the sense strand. This contact region encompasses the GAAAGTT element within β-RARE previously identified as important by gel-shift analysis with mutant binding sites (*9*).

Thus, a fragment of NZF-1 that represents a relatively well-defined structural domain and is capable of sequence-specific interactions with DNA has been identified. Metal binding studies revealed that this fragment binds two cobalt(II) ions in tetrahedral sites that appear to have CysCysHis-Cys metal coordination and that zinc(II) and cobalt(II) binding occurs with affinities comparable to those observed for other classes of zinc binding domains that have been more well-characterized structurally. Finally, metal-induced protein folding was detected by NMR. The use of excess of zinc(II) that does not, in general, affect NMR spectra of other zinc binding domains was found to broaden peaks in the NZF-1b NMR spectrum. This appears to be due to metal binding to secondary sites, leading to peptide aggregation. The significance of this observation in light of the conservation of an additional histidine residue that does not appear to be involved in primary interactions with metal ions within this family remains to be determined.

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REFERENCES

1. Berg, J. M., and Shi, Y. (1996) *Science 271*, 1081−1085.
2. Schwabe, J. W. R., Neuhaus, D., and Rhodes, D. (1990) *Nature 348*, 458−461.
3. Hard, T., Kellenbach, E., Boelens, R., Maler, B. A., Dahlman, K., Freedman, L. P., Carlstedt-Duke, J., Yamamoto, K. R., Gustafsson, J.-A., and Kaptein, R. (1990) *Science 249*, 157−160.
4. Luisi, B. F., Xu, W. X., Otwinowski, Z., Freedman, L. P., Yamamoto, K. R., and Sigler, P. B. (1991) *Nature 352*, 497−505.
5. Kraulis, P. J., Raine, A. R. C., Gadhavi, P. L., and Laue, E. D. (1992) *Nature 356*, 448−450.
6. Baleja, J. D., Marmorstein, R., Harrison, S. C., and Wagner, G. (1992) *Nature 356*, 450−453.
7. Marmorstein, R., Carey, M., Ptashne, M., and Harrison, S. C. (1992) *Nature 356*, 408−414.
8. Kim, J. G., and Hudson, L. D. (1992) *Mol. Cell. Biol. 12*, 5632−5639.
9. Jiang, Y., Yu, V. C., Buchholz, F., O'Connell, S., Rhodes, S. J., Candeloro, C., Xia, Y.-R., Lusis, A. J., and Rosenfeld, M. G. (1996) *J. Biol. Chem. 271*, 10723−10730.
10. Yee, K. S. Y., and Yu, V. C. (1998) *J. Biol. Chem. 273*, 5366−5374.
11. Bellefroid, E. J., Bourguignon, C., Hollemann, T., Ma, Q., Anderson, D. J., Kintner, C., and Pieler, T. (1996) *Cell 87*, 1191−1202.
12. Kim, J. G., Armstrong, R. C., Agoston, D. v., Robinsky, A., Wiese, C., Nagle, J., and Hudson, L. D. (1997) *J. Neurosci. Res. 50*, 272−290.
13. Weiner, J. A., and Chun, J. (1997) *J. Comp. Neurol. 381*, 130−142.
14. Alexander, P., Fahnestock, S., Lee, T., Orban, J., and Bryan, P. (1992) *Biochemistry 31*, 3597.
15. Desjarlais, J. R., and Berg, J. M. (1992) *Proteins*: *Struct., Funct. Genet. 12*, 101−104.
16. Dente, L., Cesareni, G., and Cortese, R. (1983) *Nucleic Acids Res. 19*, 1645−1654.
17. Blasie, C., and Berg, J. M. (2000) *Inorg. Chem.*, in press.
18. Corwin, D. T. J., Gruff, E. S., and Koch, S. A. (1988) *Inorg. Chim. Acta 155*, 5−6.
19. Bertini, I., and Luchinat, C. (1984) *Adv. Inorg. Biochem. 6*, 71−111.
20. Krizek, B. A., Merkle, D. L., and Berg, J. M. (1993) *Inorg. Chem. 32*, 937−940.
21. Krizek, B. A., Amann, B. T., Kilfoil, V. J., Merkle, D. L., and Berg, J. M. (1991) *J. Am. Chem. Soc. 113*, 4518−4523.
22. Green, L. M., and Berg, J. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4047−4051.
23. Bavoso, A., Ostuni, A., Battistuzzi, B., Menabue, L., Saladini, M., and Sola, M. (1998) *Biochem. Biophys. Res. Commun. 242*, 385−389.
24. Curtis, D., Treiber, D. K., Tao, F., Zamore, P. D., Williamson, J. R., and Hehmann, R. (1997) *EMBO J. 16*, 834−843.

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