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# Role of the Zinc(II) Ions in the Structure of the Three-Finger DNA Binding Domain of the Sp1 Transcription Factor†

Jun Kuwahara‡ and Joseph E. Coleman*
*Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06510*
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ABSTRACT: The transcription factor Sp1 from Hela cells contains near the C-terminus of this protein of 778 amino acids three contiguous repeats of an amino acid sequence, -Cys-$X_4$-Cys-$X_{12}$-His-$X_3$-His-, typical of the $Cys_2His_2$-type zinc-finger DNA binding domain first found in transcription factor TFIIIA. A DNA sequence corresponding to the codons from residue 614 to residue 778 of Sp1 (encompassing the three zinc-finger motifs) has been cloned and overproduced in *Escherichia coli*. The fragment of Sp1 containing the C-terminal 165 residues plus 2 from the cloning vector, designated Sp1(167*), can be extracted with 5 M urea and then refolded in the presence of Zn(II) to a protein of specific conformation containing 3.0 ± 0.2 mol of tightly bound Zn(II)/mol of protein. Gel retardation assays using a labeled 14-bp DNA sequence containing a consensus Sp1 binding site show that the refolded Zn(II) protein specifically recognizes the "GC box" sequence in the presence of a large excess of calf thymus DNA. Treatment of Zn(II)Sp1(167*) with 10 mM EDTA results in removal of Zn(II) and the formation of an apoprotein which does not specifically recognize DNA. Cd(II) can be exchanged for Zn(II) in the refolded protein with full retention of specific DNA recognition. This is the first $Cys_2His_2$-type "finger" protein where this substitution has been accomplished. Titration of the Zn(II) protein with 6 mol of *p*-mercuribenzenesulfonate/mol of protein results in the complete release of the three Zn(II) ions. Release of Zn(II) is highly cooperative. Reaction of only two of the sulfhydryl zinc ligands with the organic mercurial releases 75% of the Zn(II), suggesting that the Zn(II)-induced folding of the three fingers is probably cooperative. Circular dichroism shows the $Zn(II)_3$ protein to contain ~20% α-helix, ~20% β-sheet, and ~60% random coil as the secondary structure of the zinc-finger domain. A large part of the secondary structure is lost when the metal ions are removed.

Transcription factor Sp1 is a protein of 105 kDa containing 778 amino acids which activates a reasonably large subset of mammalian genes containing "GC box" upstream promoter elements (Dynan & Tjian, 1985; Kadonaga et al., 1986, 1987; McKnight & Tjian, 1986; Courey et al., 1989). The C-terminal 168 amino acid residues of Sp1 contain three contiguous zinc-finger motifs, -Cys-$X_4$-Cys-$X_{12}$-His-$X_3$-His-, which are believed to bind Zn(II). The C-terminal domain has been shown to constitute the DNA binding domain, while the N-terminal 610 amino acid residues form the trans activating domain. Structural features of the latter include two distinct glutamine-rich regions (Courey et al., 1989). Deletion mutations and domain swapping experiments on Sp1 show that the two domains function independently (Courey & Tjian, 1988; Courey et al., 1989). In order to study the structure and metal ion binding properties of the DNA binding domain of Sp1, the C-terminal 165 amino acids were cloned into an overproduction vector under the control of a T7 RNA polymerase promoter. While expressed in high yield, the 165-residue construct is insoluble as expressed in *Escherichia coli* but can be resolubilized and refolded after extraction with 5 M urea. Attempts to solubilize the finger domain of Sp1 by fusing it to the highly soluble C-terminal domain of glutathione-*S*-transferase resulted in an insoluble fusion protein. Refolding of the 165 amino acid fragment in the presence of metal ions, however, results in a protein that contains three Zn(II) ions in a stable secondary structure which specifically recognizes the GC box of the Sp1 binding site. The properties of that protein are described in this paper.

## Materials and Methods

*Cloning of the DNA Binding Subdomain of Sp1.* The plasmid Sp1-1 coding for the C-terminal 696 amino acids of transcription factor Sp1 was kindly supplied by James Kadonaga and Robert Tjian of the University of California, Berkeley. A number of different fragments from the C-terminal region of Sp1 containing the three zinc-finger sequences were cloned into pAR3039 plasmid under the control of a T7 promoter and the products overexpressed in the host *E. coli* BL21(DE3), which carries a chromosomal copy of the T7 gene 1 under the control of the lac promoter and is subject to induction by IPTG (Studier & Moffatt, 1986). The constructs are listed below as Sp1($x + y$), where $x$ refers to the number of codons from the Sp1 gene while $y$ indicates the number of extra codons from the cloning vector. The following constructs resulted in overexpressed products: Sp1(167 + 12) containing residues 612–778 of Sp1 and obtained from a *Bam*H1/*Hin*dIII fragment of the Sp1 gene with an additional 12 residues from T7 gene 10 contributed from the cloning vector at the N-

† This work was supported by NIH Grants DK09070 and GM21919.
* Corresponding author.
‡ Present address: Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan.

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terminal end. The resultant plasmid is designated pJK4. Plasmid pJK4 was further modified by digesting the plasmid with *Nhe*I and *Bam*HI, which remove a small DNA fragment 5′ to the Sp1 sequences. The ends of the linearized plasmid were then digested with mung bean nuclease to create blunt ends, and the plasmid was recircularized. This combined procedure removes 10 of the 12 codons transcribed at the 5′-end of the Sp1 construct. The resulting plasmid, pJK5, codes for Sp1(165 + 2), a protein that contains N-terminal Met-Ala-followed by 165 residues from Sp1 (residues 614–778 of Sp1).

Both pJK4 and pJK5 overproduce the indicated subdomains of Sp1 when IPTG is added to the culture, but all protein products are found in the pellet after cell lysis and have to be extracted with 5 M urea. Following the extraction with urea, the products can be maintained in solutions of 5–10 μM in buffer and salt after removal of the urea (see below). A refolded and metal-reconstituted Sp1(165 + 2) overproduced from pJK5, termed Sp1(167*), was the protein utilized for the experiments on the refolded DNA binding domain of Sp1 described in this paper.

*Cloned Fusion Proteins Containing the Three Zinc Fingers of Sp1.* In an attempt to solve the solubility problem, we constructed several fusions of the gene for a soluble protein, glutathione-*S*-transferase, with the DNA fragments coding for the three zinc fingers of Sp1. All were overproduced, but the Sp1 sequence has the property of rendering insoluble all fusion proteins that have been constructed thus far. These fusion proteins are briefly described below. A *Bam*HI/*Eco*RI fragment was isolated from pJK5 and ligated into pGEX1N, which carries a glutathione-*S*-transferase gene (coding a 26-kDa polypeptide) followed by a 10-codon polylinker (Smith & Johnson, 1988). Thus, residues 612–778 of Sp1 were fused to the C-terminus of glutathione-*S*-transferase. The fusion protein is overproduced but is insoluble. Two other fusion constructs between Sp1 sequences and the C-terminal end of glutathione-*S*-transferase were overproduced but were also insoluble. One of these had 34 codons removed from the 3′-end of the Sp1 sequences, i.e., the region downstream from the codons for the three zinc fingers. The second had 92 residues removed from the 3′-end, which removes the condons for 15 amino acids of the third finger. Unfortunately, neither of these modifications increased the solubility of the fusion protein.

*Purification of Sp1(167*).* IPTG was added to a culture of BL21(DE3)/pJK5 when an OD of 1 at 600 nm was reached. The cells were harvested 4 h later at which time Sp1(167*) represents ~10% of the total *E. coli* protein. Cells (10 g wet wt) were harvested from 2.4 L of LB ampicillin (100 μg/mL) broth and resuspended in 20 mL of 10 mM Tris-HCl/1 mM β-mercaptoethanol/1 mM EDTA/50 mM NaCl/pH 7.5 and lysed with lysozyme and deoxycholate (0.04%) followed by DNaseI (50 μg/mL) treatment. Following centrifugation, the pellet was resuspended in 5 M urea/10 mM Tris-HCl/50 mM NaCl/1 mM β-mercaptoethanol/pH 7.5 and loaded onto a Trisacryl SP column preequilibrated with the same buffer at 25 °C. The column was eluted with a 50 mM to 1 M gradient of NaCl, 25 °C. The fractions emerging at ~0.4 M NaCl contained Sp1(167*) that was approximately 90% pure. Several repeats of the Trisacryl column can be used to produce ultrapure protein. The procedure yields ~2 mg of Sp1(167*)/g wet wt of cells. Gels of the cells induced for 4 h, of the supernatant and pellet, and of the purified protein are shown in Figure 1.

*Synthesis of a Double-Stranded Oligonucleotide Containing the Consensus GC Box Recognized by Sp1.* A representative

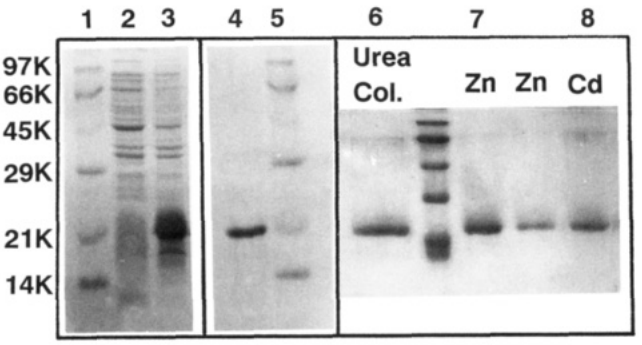


FIGURE 1: SDS–acrylamide gels of Sp1(167*) in various stages of purification. Columns are (1) standard molecular weight markers as indicated, (2) uninduced cells containing plasmid pJK5, (3) the same cells induced with IPTG for 4 h, (4) homogeneous $Zn_3$Sp1(167*) after two passages through the Trisacryl–urea column and refolding in the presence of 50 μM Zn(II), (5) repeat of molecular weight markers, (6) pooled fractions directly from the Trisacryl–urea column, (7) refolded $Zn_3$Sp1(167*), and (8) sample 7 after exchange with 50 μM Cd(II). The MW standards are repeated on the short gel between columns 6 and 7.

DNA sequence recognized by Sp1 is 5′-CCGCCC-3′. Hence, two separate oligonucleotides of 14 bases to form the double-stranded DNA

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5'-GATCGCCCCGCCCC-3'

3'-CGGGGCGGGGCATG-5'
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were synthesized on an Applied Biosystems 380A DNA synthesizer, purified to homogeneity by gel electrophoresis, and 5′-end labeled with [γ-$^{32}$P]ATP for use in the gel retention assays.

*Gel Retention Assay.* Gel retention assays to detect binding of Sp1 to DNA were performed on 6% acrylamide gels as described by Fried and Crothers (1981).

*Zinc and cadmium analyses* were performed by atomic absorption spectroscopy with an Instrumentation Laboratory (Lexington, MA) IL157 spectrometer.

## RESULTS

*Zinc Content of the Refolded DNA Binding Domain of Sp1.* ZnSp1(167*) was reconstituted by dialysis of the protein in 5 M urea against 10 mM Tris-HCl, 0.2 M NaCl, 1 mM β-mercaptoethanol, 50 μM $ZnSO_4$, 5% glycerol, pH 7.5, 4 °C. After two changes of dialyzate, the protein was dialyzed for four changes of 24 h each against the same buffer rendered metal free. Metal analyses of several preparations of the reconstituted protein after dialysis against metal-free buffer are shown in Table I. The reconstituted Zn(II) protein can be completely exchanged with Cd(II) by dialysis against metal-free buffer containing 50 μM Cd(II), followed by dialysis, two changes, against metal-free buffer. After this dialysis the protein contained 2.89 ±0.03 cadmium ions (Table I). The reconstituted proteins with this metal content were used for all the experiments described below. The Cd(II) protein has strong absorption bands centered near 250 nm as expected for $-S^-$ to Cd(II) charge-transfer bands (Figure 2). The difference spectrum, $Cd_3$Sp1(167*) minus $Zn_3$Sp1(167*) shows a maximum at ~246 nm with $\epsilon_{246}$ = ~5 × $10^4$ $M^{-1}$ $cm^{-1}$.

*Binding of Apo-Sp1(167*), Zn(II)Sp1(167*), and Cd(II)Sp1(167*) to the -CCGCCC- Sequence Contained in a 14-mer ds DNA As Assayed by Gel Retardation.* The refolded Zn(II)Sp1(167*) efficiently retains the $^{32}$P-labeled 14-mer ds DNA, incorporating a GC box even in the presence of a 120-fold molar excess of calf thymus DNA (Figure 3). The

Table I: Metal Content of the C-Terminal Zinc-Finger Domain of Sp1

| protein sample | treatment | metal content (mol/mol of protein) Zn | Cd |
|---|---|---|---|
| Sp1(167*) | refolded in the presence of 50 μM Zn(II), followed by dialysis against metal-free buffer: | | |
| prepn 1 | 10 mM metal-free phosphate,[a] pH 7.5, 4 °C | 2.98 ± 0.10 | |
| prepn 2 | metal-free TNG buffer,[b] pH 7.5, 4 °C | 3.02 ± 0.11 | |
| prepn 3 | metal-free TNG buffer, pH 7.5, 4 °C | 2.70 ± 0.10 | |
| prepn 4 | 10 mM metal-free phosphate, pH 7.5, 4 °C | 3.28 ± 0.10 | |
| prepn 5 | metal-free TNG buffer plus 5 mM EDTA, pH 7.5, 4 °C | 1.33 ± 0.10 | |
| Sp1(167*) | $Zn(II)_3$Sp1(167*) exchanged against 50 μM Cd(II) followed by dialysis against metal-free TNG buffer, pH 7.5, 4 °C | <0.1 | 2.89 ± 0.03 |
| apo-Sp1(167*) | | 0.03 | |

[a] Phosphate buffer = 10 mM phosphate/0.2 M NaCl/1 mM β-mercaptoethanol/5% glycerol, pH 7.5. [b] TNG buffer = 10 mM Tris-HCl/0.2 M NaCl/1 mM β-mercaptoethanol/5% glycerol, pH 7.5.

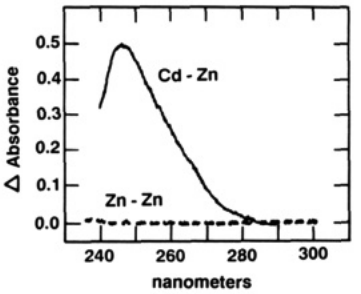


FIGURE 2: Ultraviolet difference spectrum: (—) $Cd_3$Sp1(167*) minus $Zn_3$Sp1(167*); (--) $Zn_3$Sp1(167*) minus $Zn_3$/Sp1(167*). The buffer was 10 mM phosphate/0.2 M NaCl/5% glycerol, pH 7.5.

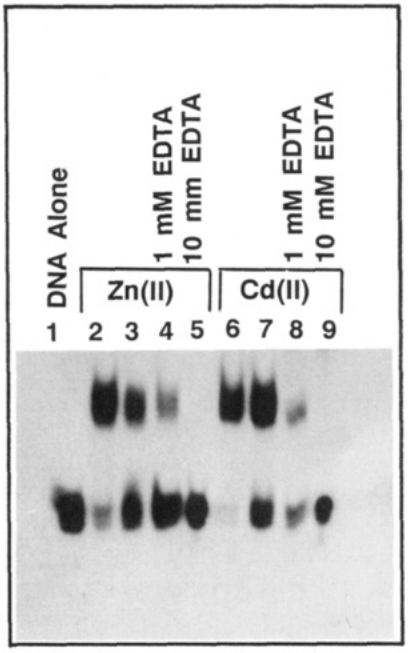


FIGURE 3: Gel retardation of specific double-stranded DNA by $Zn(II)_3$Sp1(167*) and $Cd(II)_3$Sp1(167*). Columns: (1) $^{32}$P-labeled 14-mer DNA; (2) plus $Zn(II)_3$Sp1(167*) + 5-fold excess calf thymus DNA; (3) plus $Zn(II)_3$Sp1(167*) + 120-fold excess calf thymus DNA; (4) same as (3) + 1 mM EDTA; (5) same as (3) + 10 mM EDTA; (6) $^{32}$P-labeled 14-mer plus $Cd(II)_3$Sp1(167*) + 5-fold excess of calf thymus DNA; (7) plus $Cd(II)_3$Sp1(167*) + 120-fold excess calf thymus DNA; (8) same as (6) + 1 mM EDTA; (9) same at (6) + 10 mM EDTA. The reactions consisted of $^{32}$P-labeled 14-mer ds DNA (100 nM) containing a GC box and Sp1(167*) (1.0 μM) in 10 mM Tris–borate/50 mM NaCl/1 mM β-mercaptoethanol/2.5% glycerol, pH 8.3. The reactions were started by the addition of protein, incubated for 20 min at 25 °C, and then loaded on to a 6% polyacrylamide gel and electrophoresed at 150 V.

formation of the specific Sp1(167*)–DNA complex is moderately affected by 1 mM EDTA, but complex formation is abolished by the addition of 10 mM EDTA (Figure 3). Al-

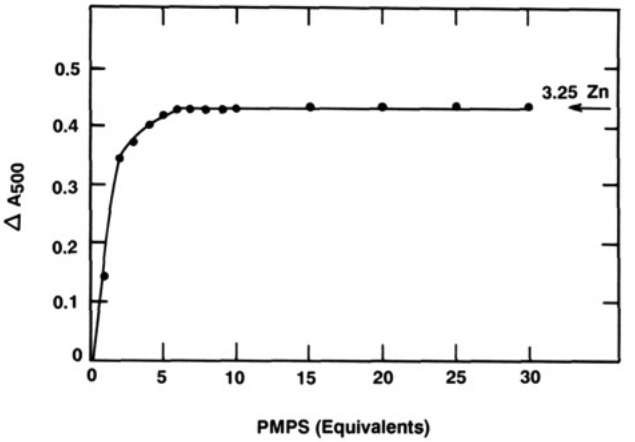


FIGURE 4: Titration of Zn(II)Sp1(167*) with PMPS. Refolded Zn(II)Sp1(167*) (2 μM) in 0.8 mL of TNG buffer was treated with successive 0.8-μL aliquots of PMPS (2 mM) to give the indicated molar ratios of PMPS/protein. The reaction solution contained 0.1 mM 4-(2-pyridylazo)resorcinol in both the protein and buffer blank cuvettes. The aliquots of PMPS were added to both the sample and blank cuvettes.

most identical behavior is shown by the Cd(II) derivative of Sp1(167*), but the Cd(II) derivative appears more susceptible to dissociation by EDTA (Figure 3).

*Zinc Release from Sp1(167*) during Titration with p-Mercuribenzenesulfonate (PMPS).* Zinc release accompanying reaction of PMPS with Zn(II)-binding sulfhydryl groups can be detected by including the Zn(II) complexing dye 4-(2-pyridylazo)resorcinol (PAR) in the reaction mixture. $Zn(II)PAR_2$ has a new absorption maximum at 500 nm (Hunt et al., 1984). The change in absorbance at 500 nm during the titration of Zn(II)Sp1(167*) with PMPS in the presence of PAR is shown in Figure 4. The increase in $A_{500}$ ceases after the addition of 6 equiv of PMPS/molecule of Sp1(167*). The total increase in $A_{500}$, 0.43 OD unit, corresponds to the release of 3.25 mol of Zn(II)/mol of Sp1(167*). Release of Zn(II) as a function of [PMPS] is not linear, rather ~75% of the Zn(II) is released upon reaction of only two molecules of PMPS with the protein, suggesting that there may be a cooperative release of metal ion from the zinc fingers after the structure of one of them is disturbed (see Discussion).

The cooperativity of metal ion release shown by the PMPS titration has an apparent counterpart when the specific DNA binding of the samples of protein during the progress of the PMPS titration is examined. Reaction of 1 equiv of PMPS with Sp1(167*) decreases but does not abolish DNA retention compared to the control. However, reaction of the protein with 2 equiv of PMPS completely abolishes complex formation. After the addition of 7 equiv of PMPS, which releases all the Zn(II), the addition of 0.1 mM DTT does not restore DNA

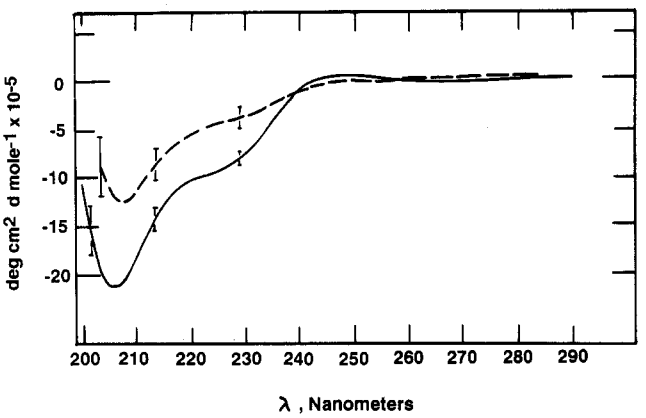


FIGURE 5: Circular dichroism of refolded $Zn(II)_3Sp1(167^*)$ (3 $\mu$M) (—) and of the same protein after removal of the metal ions with EDTA (5 mM) (--). The buffer was metal-free 10 mM Tris-HCl/0.2 M NaCl/5% glycerol, pH 7.5.

binding. However, the presence of 0.1 M DTT prior to the addition of PMPS completely preserves the DNA binding ability of the protein as assayed by gel retention, supporting the conclusion that PMPS is abolishing the gel retardation by reacting with the sulfhydryl groups of Sp1(167*).

*Metal-Dependent Conformation of Sp1(167*) As Determined by Circular Dichroism.* $Zn(II)_3Sp1(167^*)$ is a folded protein with a molar ellipticity, $\theta$, of $-20.5 \times 10^5$ deg cm$^2$ dmol$^{-1}$ at 208 nm and $-10 \times 10^5$ deg cm$^2$ dmol$^{-1}$ at 222 nm (Figure 5). The best fit for the ellipticity of $Zn(II)_3Sp1(167^*)$ by the method of Greenfield and Fasman (1969) is with ~20% $\alpha$-helix, ~20% $\beta$-sheet, and ~60% "random coil". Like a number of native proteins, the circular dichroism spectrum of $Zn(II)_3Sp1(167^*)$ is not perfectly fit by any combination of the spectra for the three conformations of poly(L-lysine). When the Zn(II) is removed by EDTA, a large part of the organized secondary structure disappears; e.g., the negative molar ellipticity at 222 nm decreases to $-5.0 \times 10^5$ deg cm$^2$ dmol$^{-1}$ upon Zn(II) removal (Figure 5). The metal-free polypeptide becomes hyperchromic in the far-ultraviolet as indicated by the significantly increased noise in the circular dichroism spectrum. Hyperchromia is expected if significant $\alpha$-helix unfolds.

## Discussion

The transcription factor Sp1, isolated from Hela cells, has been classed as one of the zinc-finger transcription factors (Kadonga et al., 1987). The DNA sequence of the gene for Sp1 shows it to contain 778 residues. Near the C-terminus there is a 81-residue sequence (residues 621–701) which contains three approximate repeats of a primary structural motif similar to the zinc-finger motif first described in TFIIIA from *Xenopus* oocytes (Miller et al., 1985). There is a conserved spacing in each repeat for the sequences Cys-$X_4$-Cys and His-$X_3$-His, with the pairs of Cys and His residues postulated to coordinate one Zn(II) in a tetrahedral complex (Miller et al., 1985; Kadonaga et al., 1986, 1987). Three-dimensional structures determined by 2D NMR techniques of Zn(II) complexes formed with synthetic peptides whose sequences match the putative $Cys_2His_2$ zinc fingers found in the yeast transcription factor ADR1 and the *Xenopus* transcription factor Xfin show the C-terminal His-containing segment to form a short three-turn $\alpha$-helix, while the N-terminal Cys-containing segment forms a turn and a short antiparallel $\beta$-sheet (Parraga et al., 1988; Lee et al., 1989). The 12 or so residues between the Cys-$X_4$-Cys and the His-$X_3$-His ligand sets are presumed to form the major part of the DNA binding surface. At present it is assumed that a structure similar to that of the synthetic peptide model is taken up by these fingers when inserted into the environment of a large folded native protein. Little structural data on a folded "native" DNA binding domain of any of the $Cys_2His_2$ zinc-finger proteins are available, with the exception of extended X-ray absorption edge spectroscopy on TFIIIA, which confirms a zinc absorption edge compatible with tetrahedral zinc complexes (Diakun et al., 1986).

By cloning and overproducing a reasonably large polypeptide domain, 165 residues, encompassing the 81-residue three zinc-finger DNA binding domain of Sp1, we had hoped to be able to isolate a native folded zinc-containing finger domain of Sp1. Unfortunately, the constructs containing the finger domain of Sp1 overexpressed in *E. coli* are insoluble, even the fusion proteins. The Sp1 finger domain seems generally to have this property, since attempts to attach the Sp1 DNA binding domain to other proteins as "tethers" to GC boxes inserted in specific DNA sequences have also resulted in insoluble fusion proteins (Mark Biggin, personal communication).

After extracting the insoluble overproduced DNA binding domain with 5 M urea, we did find it possible, however, to refold in the presence of Zn(II) the 165 + 2 amino acid residue peptide to a stable reproducible conformation (Figure 5). This conformation retains three Zn(II) ions per molecule (Table I). Maintenance of this conformation requires the presence of the three Zn(II) ions (Figure 5). Most importantly, the conformation of the refolded Zn(II) protein is one which specifically recognizes the GC box DNA sequence characterizing Sp1 binding sites, and it does so even in the presence of excess nonspecific DNA (Figure 3). This ability of the refolded three-finger domain to recognize the DNA binding sequence to which it binds in vivo is in marked contrast to the refolded "single-finger" synthetic peptides which have not demonstrated specific DNA binding (Frankel et al., 1987; Parraga et al., 1988). These findings give us confidence that the refolded triple zinc-finger structure approximates the native fold of the finger domain in the intact Sp1. As has been found for a number of the $Cys_2Cys_2$-type zinc-finger proteins like the glucocorticoid receptor and GAL4 (Freedman et al., 1988; Pan & Coleman, 1989), Cd(II) is as efficient as Zn(II) in maintaining the specific DNA recognition surface of the Sp1 binding domain (Figure 3). This is the first $Cys_2His_2$-type finger where this substitution has been accomplished and the Cd(II) protein shown to be effective in specific DNA recognition. The exchange of Cd(II) for Zn(II) in the already refolded zinc protein proved to be more effective than refolding the apoprotein initially in the presence of 50 $\mu$M Cd(II). This may relate to alternate liganding patterns taken up by Cd(II) including cross-linking of $-S^-$ groups which may take a very long time for rearrangement, even if they are less stable than the native arrangement.

The yeast transcription factor SW15 from *Saccharomyces cerevesiae* also has three zinc fingers of the $Cys_2His_2$ type (Stillman et al., 1988). A peptide fragment of 89 amino acid residues containing these finger motifs has been overproduced by Nagai et al. (1988) as a fusion protein with a myosin light chain fragment. This fusion protein required extraction with 8 M urea from inclusion bodies in *E. coli*. After removal of the 89-residue zinc-finger peptide by cleavage with cyanogen bromide, the SW15 peptide has been reported to refold in the presence, but not in the absence, of Zn(II) to a conformation that correctly footprints a DNA carrying the base sequence recognized by SW15 (Nagai et al., 1988). Metal analyses or

other characterizations of the refolded SW15 fragment have not been reported.

The ability of the Sp1 DNA binding domain to recognize its specific DNA sequence is completely dependent on the presence of the metal ion. This is not surprising, since most of the secondary structure appears to require stabilization by the metal ions (Figure 5). If, as the model peptides suggest, each Zn-folded finger has ~12 residues in the $\alpha$-helical conformation, then the folded Sp1(167*) should have at least 22% $\alpha$-helix, in fair agreement with the 20% $\alpha$-helix predicted by the circular dichroism (Figure 5). The apparent cooperativity observed in the displacement of Zn(II) from the Sp1 DNA binding domain by PMPS (Figure 4) suggests that the whole protein may unfold and lose most of its Zn(II) as soon as one site has been displaced. Cooperative rather than independent folding of the multiple zinc fingers in the $Cys_2His_2$-type proteins where the number of fingers varies from 2 (ADR1) to 37 (Xfin) may be an important structural feature in the consideration of how these proteins bind and influence DNA topology.

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