Metal-dependent folding of a single zinc finger from transcription factor IIIA

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ABSTRACT A 30-amino acid peptide, which corresponds to the second "zinc finger" domain of transcription factor IIIA, has been synthesized and purified. This peptide folds in the presence of zinc: adding Zn2+ significantly changes the circular dichroism spectrum, and Zn2+ protects the peptide from tryptic digestion. The peptide also binds Co2+, and the absorption spectrum of the Co2+ complex suggests that a tetrahedral binding site is formed by two cysteines and two histidines. Experiments at higher temperatures (60–75°C) suggest that these folded metal-peptide complexes are quite thermostable. The peptide shows some sequence-specific effects in DNase and methylation protection experiments. However, it does not give a clear "footprint," and some effects are observed in the absence of added zinc.

Zinc binding domains appear to be important structural elements in many nucleic acid binding proteins (1–8). Such domains were first noted in Xenopus transcription factor IIIA (TFIIIA) (1, 2). This protein contains nine tandem repeats of a 30-amino acid motif, and each repeat has two invariant cysteines and two invariant histidines. When isolated, TFIIIA contains Zn2+ (8), with 7–11 ions in the ribonucleoprotein complex containing TFIIIA (1). Based on these observations, it was suggested that each repeat was a Zn2+ binding domain (a "zinc finger") and that phosphohistidine side chains from these domains could make specific contacts with nucleic acids (1, 2). Sequences containing similar patterns of cysteines and histidines have been found in a wide variety of proteins involved in nucleic acid binding or gene regulation (3–7).

There is substantial evidence that the nine domains in TFIIIA are independent units and are stabilized by Zn2+. Partial proteolysis of TFIIIA yields fragments that differ in molecular size by 3 kDa (1), a size difference consistent with the removal of successive 30-amino acid domains. The sequence of the TFIIIA gene shows that most of these domains are encoded by separate exons (9), suggesting that these units have structural significance (10). X-ray absorption spectra of the ribonucleoprotein complex show that the coordination sphere of the Zn2+ ions contains two cysteine and two histidine residues (11). Removal of Zn2+ from the protein destroys site-specific DNA binding activity (8). To simplify physical and structural characterization of these zinc domains, we have prepared a peptide corresponding to one zinc finger from TFIIIA. This peptide folds in the presence of Zn2+. The peptide also binds DNA with some sequence specificity, but binding shows no clear dependence on Zn2+.

MATERIALS AND METHODS

Preparation of DNA. Plasmid DNAs [ptrpLE' (16), pTFIIIA-2 (see below), and pXBS201 (19)] were purified from Escherichia coli MM294 cells by the rapid alkaline extraction procedure of Birnboim and Doly (12) and equilibrium centrifugation in CsCl/ethidium bromide gradients (13). The gene encoding TFIIIA-2 was synthesized in four fragments on an Applied Biosystems (Foster City, CA) model 380A DNA synthesizer. These oligonucleotides, which were 39–60 bases long, were purified on 15% polyacrylamide/urea gels.

Construction of the pTFIIIA-2 Plasmid. The TFIIIA-2 gene was constructed by phosphorylating two of the synthetic oligonucleotides, annealing with the other two oligonucleotides, and ligating. Duplex DNA of the correct size was purified on a 1% agarose gel. This DNA was phosphorylated and inserted into EcoRI-cleaved ptrpLE', and the ligation mixture was used to transfect CaCl2-treated MM294 cells (14). Transformants were selected on LB (Luria Broth) agar plates containing ampicillin at 100 µg/ml. Plasmid DNA was prepared from several colonies, and the presence of TFIIIA-2 gene was confirmed by restriction endonuclease analysis. Several plasmids containing an insert of the proper size were sequenced by the method of Maxam and Gilbert (15), and the plasmid containing the TFIIIA-2 gene was named pTFIIIA-2 (Fig. 1). Phosphorylation reactions were performed using T4 polynucleotide kinase, and ligation reactions were carried out at 14°C using T4 DNA ligase. Enzymes were purchased from New England Biolabs, Pharmacia P-L Biochemicals, and Boehringer Mannheim.

Purification of TFIIIA-2. TFIIIA-2 was purified by growing E. coli MM294 cells containing the pTFIIIA-2 plasmid in M9 medium containing ampicillin at 100 µg/ml and tryptophan at 100 µg/ml. During late logarithmic phase, cells were centrifuged, resuspended in medium lacking tryptophan, and grown for 3–12 hr more at 37°C. Removal of tryptophan induces production of the fusion protein (Fig. 1), which can be seen as granules by phase-contrast microscopy (16). Cells were harvested and lysed, and the granules were isolated by centrifugation as described (16). The crude protein pellet from an 8-liter culture was treated with 100 mg of cyanogen bromide in 40 ml of 70% (vol/vol) formic acid for 24 hr at room temperature (17) and lyophilized. To identify TFIIIA-2, cyanogen bromide-treated samples from both pTFIIIA-2 and ptrpLE' preparations were electrophoresed on a native 15% polyacrylamide gel (30 mM sodium acetate, pH 4.5) and stained with Coomassie blue. An additional band was observed in the pTFIIIA-2 preparation. To purify TFIIIA-2, dried, cyanogen bromide-treated samples were resuspended in water and centrifuged to remove insoluble material. This crude mixture was loaded onto a Vydac C4 HPLC column in 0.1% trifluoroacetic acid and eluted with an acetonitrile gradient (0–30%). The absorbance was monitored at 214 nm, peaks were collected, and TFIIIA-2 was identified by native gel electrophoresis. The purified peptide was sequenced using an Applied Biosystems automated

Abbreviations: TFIIIA, transcription factor IIIA; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

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peptide sequencer, and its concentration was determined by amino acid analysis. Approximately 1 mg of purified peptide was obtained per liter of cell culture.

The number of free thiols in TFIIIA-2 was determined by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (18). Reactions were carried out in 100 mM Tris-HCl (pH 8.0) with DTNB at 0.2 mg/ml. When necessary, TFIIIA-2 was separated from excess dithiothreitol by chromatography on a Pharmacia Superose 12 column in 1 mM acetic acid/50 mM NaCl before adding DTNB and buffer.

**Tryptic Cleavage of TFIIIA.** Proteolysis of TFIIIA-2 was carried out at room temperature in 20 mM Tris-HCl (pH 7.0) with various ZnCl2 concentrations. Each sample contained 2.5 μg of peptide. After equilibrating the peptide with buffer and ZnCl2 for 30 min, 20 μg of trypsin was added, and proteolysis proceeded for 3 min. Reactions were stopped by adding 10 μg of phenylmethylsulfonyl fluoride, and the samples were electrophoresed on a 20% polyacrylamide gel containing 30 mM Tris-HCl (pH 7.0) at 12 V/cm for 3 hr. Reduced TFIIIA-2 was prepared by treating the peptide with 10 mM dithiothreitol at 90°C for 30 min and lyophilizing. In tryptic digestions with oxidized TFIIIA-2, an equivalent amount of dithiothreitol was added to the reaction mixtures at the time of trypsin addition.

**Circular Dichroism of TFIIIA-2.** Circular dichroism spectra were measured using an Aviv model 60DS spectropolarimeter. Spectra were recorded from 300 nm to 200 nm and averaged over five scans. Samples were in 10 mM Tris-HCl (pH 7.0, degassed with helium), and peptide concentrations varied from 20 μg/ml to 100 μg/ml. Temperature was controlled using a water-jacketed cuvette holder and an external water bath. Reduced TFIIIA-2 was prepared by treating the peptide with 250 mM dithiothreitol at 90°C for 30 min. The reduced peptide was purified by HPLC, lyophilized, and stored at −80°C.

**Absorption Spectra with Cobalt.** Absorption spectra were measured on a Bausch & Lomb Spectronic 2000 spectrophotometer and on a Perkin–Elmer Lambda 3A spectrophotometer. Temperature was controlled using a water-jacketed cuvette holder and an external water bath. Reduced TFIIIA-2 was prepared as described in the previous section. Lyophilized peptide was resuspended in 10 mM Tris-HCl (pH 7.0, degassed with helium), and CoCl2 was added as indicated in the figure legend. For experiments at 60°C, the buffer was titrated to compensate for the temperature dependence of Tris ionization.

**DNase I and Dimethyl Sulfate Protection Experiments.** DNase I digestions were done at room temperature in 20 mM Tris-HCl, pH 7.0/20 mM NaCl/10 mM MgCl2/1 mM ZnCl2 with various concentrations of TFIIIA-2. After equilibrating for 30 min, 100 ng of 32P-labeled pXBS201 DNA was added, and, after another 30 min, 3 ng of DNase I was added. The reaction was stopped after 1 min by adding 1 ml of 0.3 M ammonium acetate, 5 μg of tRNA, 70% (vol/vol) ethanol. DNA was ethanol precipitated twice, resuspended in loading buffer (15), and electrophoresed on an 8% polyacrylamide/urea sequencing gel.

Dimethyl sulfate protection reactions were carried out at room temperature in the same buffer as the DNase I reactions, except that MgCl2 was not included. After equilibrating in buffer for 30 min and then with 100 μg of 32P-labeled pXBS201 DNA for another 30 min, 1 μl of 2% (vol/vol) dimethyl sulfate was added and allowed to react for 2 min. The stop mixture (15) was added, and the DNA was treated to cleave at methylated guanines (15) and electrophoresed.

Reduced and oxidized samples of TFIIIA-2 were prepared as for the partial proteolysis experiments. 32P-labeled pXBS201 (label on the coding strand of the 5S rRNA gene) was prepared as described by Smith et al. (19).

**RESULTS**

**Production and Purification of TFIIIA-2 Peptide.** We produced a single zinc finger by synthesizing its gene, expressing it in *E. coli* as part of a fusion protein, and releasing the peptide by cyanogen bromide cleavage. This peptide, named TFIIIA-2, corresponds to the second of the nine repeats in TFIIIA. It contains residues 42–71 from the intact protein and has the sequence:

Pro-Pro-Cys-Lys-Glu-Glu-Gly-Cys-Glu-Lys-Gly-Phe-Thr-Ser-Leu-His-Leu-Thr-Arg-His-Ser-Leu-Thr-Gly-Glu-Lys,

where underscores indicate conserved residues. This second domain contains all the conserved residues found when the nine repeats are aligned, including the two cysteines and two histidines thought to be involved in coordinating the zinc ion, two highly conserved aromatic residues, and one highly conserved leucine.

We constructed a plasmid that would encode the TFIIIA-2 peptide fused to the carboxyl terminus of the trp LE' protein. The LE' protein is produced at very high levels in *E. coli* and has been used in a similar way to make other peptides (16, 20). The plasmid pTFIIIA-2 is sketched in Fig. 1. The codon for methionine was placed at the protein-peptide junction so that the peptide could be released with cyanogen bromide. All other codons were chosen on the basis of their preferential usage in *E. coli* (21). This plasmid was used to produce the desired peptide, which was cleaved from the fusion protein and purified.

The peptide, as initially isolated, contained an internal disulfide bond. Treatment with DTNB indicated there were no free thiols in the purified peptide (18). A reduced form was generated by treating the peptide with 250 mM dithiothreitol at 90°C for 30 min. A Pharmacia Superose 12 column was then used to separate the peptide from dithiothreitol. Collecting the peptide peak and immediately adding DTNB revealed that there were 2 μmol of free thiol per mol of peptide. The elution times of the oxidized and reduced peptide were identical on the Superose 12 sizing column, and they also had identical mobilities on native 20% polyacrylamide gels. These results suggest that the oxidized peptide is a monomer with the two cysteines forming an internal disulfide bond. To test other protocols for reduction of TFIIIA-2, we treated it with dithiothreitol at several concentrations and temperatures and chromatographed the peptide on a C4 reverse-phase HPLC column. Eluting with acetonitrile resolved the reduced and oxidized peptides (Fig. 2). We found that incubating at 90°C for 30 min with a 5-fold molar excess of dithiothreitol gave 80–90% reduction of the disulfide.
Stability to Proteolytic Digestion. Since a stable, folded structure generally makes a polypeptide less accessible to proteolytic digestion, trypsin was used to monitor folding of TFIIIA-2. These experiments (Fig. 3) compared trypsin cleavage of the reduced and oxidized peptide at various ZnCl₂ concentrations. ZnCl₂ clearly inhibits digestion of the reduced peptide (lanes 6–9). Control experiments revealed that at least 20-fold higher ZnCl₂ concentrations were needed to inhibit proteolysis of the oxidized peptide (lanes 2–5).

Circular Dichroism Spectra. Circular dichroism spectroscopy was also used to monitor peptide folding. As shown in Fig. 4A, the circular dichroism spectrum changes markedly when zinc is added to reduced TFIIIA-2. In 50 μM ZnCl₂ (a slight molar excess), there are the following two major changes: (i) a significant increase in negative molar ellipticity near 220 nm and (ii) a dramatic reduction in the negative molar ellipticity near 200 nm. Increasing the concentration of ZnCl₂ (up to 5 mM) did not give further changes in the spectrum. Very similar spectra were obtained with CoCl₂ (data not shown). As a test for aggregation, spectra were recorded at a series of peptide concentrations (6–30 μM) in the presence of ZnCl₂. No changes in molar ellipticity were observed as the peptide was diluted. To analyze the thermal stability of the folded structure, we recorded the circular dichroism spectra over a range of temperatures. Remarkably, increasing the temperature to 75°C only gave small changes in the spectrum (Fig. 4A). The original spectrum was recovered when the sample was cooled.

The spectrum of the oxidized peptide (Fig. 4B) changes very little in 50 μM ZnCl₂. Larger changes are seen as more ZnCl₂ is added. However, even at the highest concentration tested (5 mM) the spectral changes with the oxidized peptide were less pronounced than with the reduced peptide, and the final spectrum with the oxidized peptide was significantly different than that obtained with the reduced peptide.

Absorption Spectrum of the Co²⁺–Peptide Complex. Cobalt was used to probe the metal binding site in TFIIIA-2. Since cobalt complexes have distinctive optical absorption spectra, cobalt substitution has been used in many studies of metalloproteins (22). Fig. 5 shows the spectrum obtained when a stoichiometric amount of CoCl₂ was added to reduced TFIIIA-2. Adding a molar excess of Co²⁺ (6.7-fold) did not change the spectrum. Several absorption bands were observed: There is a maximum near 635 nm with an extinction coefficient (ε) of 400 M⁻¹ cm⁻¹ and a shoulder near 575 nm. These absorption bands are responsible for the blue color of the sample. The spectrum also has an absorption maximum near 310 nm with a shoulder at 340 nm. Spectra recorded at 60°C were essentially identical to those at room temperature (data not shown). In control experiments, cobalt was added to the oxidized peptide. No spectral changes were observed, even with a 70-fold excess of CoCl₂.

Competition experiments show that zinc readily displaces cobalt from the complex. As shown in Fig. 5, adding Zn²⁺ eliminates the absorption at 635 nm and at 575 nm and reduces absorption at 310 nm and at 340 nm. Zinc must bind
FIG. 5. Optical absorption spectra of TFIIIA-2 with Co\(^{2+}\). Solid line shows the spectrum of 147 \(\mu\)M reduced TFIIIA-2 with 150 \(\mu\)M CoCl\(_2\). Additional CoCl\(_2\) was added (to 1 mM) with no changes in the spectrum. Dashed line shows the spectrum after 150 \(\mu\)M ZnCl\(_2\) was added to the sample containing 147 \(\mu\)M reduced peptide and 1 mM CoCl\(_2\). The spectra have been corrected by subtracting the spectra of reduced peptide and of CoCl\(_2\).

significantly more tightly than cobalt, since this solution contained a stoichiometric amount of ZnCl\(_2\) and a 6.7-fold excess of CoCl\(_2\).

DNA Binding. Does a single zinc finger show sequence-specific binding? Intact TFIIIA binds to the internal control region of the SS rRNA gene (23–26), and we used DNase- and methylation-protection experiments to monitor peptide binding to this region. DNase protection experiments with high concentrations of TFIIIA-2 show sequence-dependent effects (Fig. 6). It is interesting that methylation protection experiments also show effects in the same regions (data not shown). However, effects are seen with the oxidized peptide and in the absence of ZnCl\(_2\). No conditions gave a clearly defined “footprint,” and these DNA binding experiments are difficult to interpret.

DISCUSSION

Our results show that a single zinc finger—a peptide containing just 30 amino acids—can fold in the presence of zinc. Our first evidence came from proteolytic digestion experiments. Adding ZnCl\(_2\) protected the reduced peptide from tryptic digestion. The oxidized peptide (which contains an internal disulfide bond) also was protected, but at least 20-fold higher ZnCl\(_2\) concentrations were needed (Fig. 3). We presume that the peptide folds in the presence of zinc and that the folded conformation is less susceptible to proteolytic cleavage. Zinc also stabilizes the gene 32 protein and protects it from tryptic digestion (27).

Circular dichroism spectra show that TFIIIA-2 folds in the presence of zinc. Without zinc, the spectrum of the reduced peptide resembles that of a random coil; adding stoichiometric amounts of zinc gives a very different spectrum (Fig. 4A). We believe this is a more ordered structure, but the observed changes do not allow a simple assignment of secondary structure. (We presume that the spectral changes reflect changes in the peptide structure, since zinc and cobalt gave similar circular dichroism spectra.) Stoichiometric amounts of zinc have relatively little effect on the circular dichroism spectrum of the oxidized peptide (Fig. 4B). At 20- to 100-fold higher zinc concentrations, there are significant changes in the spectrum of oxidized TFIIIA-2. These results, along with the trypsin digestions, suggest that the oxidized peptide becomes partially structured at high ZnCl\(_2\) concentrations.

To determine the coordination state of the bound metal, we studied a cobalt–peptide complex. Cobalt can frequently be substituted for zinc, and the optical absorption spectrum is very sensitive to the nature of the coordination site. The spectrum observed for the complex between reduced TFIIIA-2 and a stoichiometric amount of Co\(^{2+}\) (Fig. 5) clearly shows that the metal binding site is tetrahedral. The intensities of the d-d transitions (e.g., \(\varepsilon = 400 \text{M}^{-1}\text{cm}^{-1}\) at 635 nm) rule out higher coordination numbers (22). The spectrum is entirely consistent with the structure proposed for the zinc sites in TFIIIA (1, 2, 11). The charge-transfer bands near 310 nm and 340 nm indicate that cysteinate ligands are involved.

This spectrum is quite similar to the spectrum for the Co\(^{2+}\)-substituted gene 32 protein (27) and to the “blue hybrid” of liver alcohol dehydrogenase (28). Competition experiments show that Zn\(^{2+}\) binds to the same site with higher affinity since it readily displaces Co\(^{2+}\) from the complex (Fig. 5).

In contrast to the reduced peptide, no spectral changes were observed when CoCl\(_2\) was added to the oxidized peptide (even with a 70-fold excess of CoCl\(_2\)). This clearly demonstrates that the metal binding site in the reduced peptide is quite different from any metal binding sites in the oxidized peptide. Obviously, since the oxidized peptide has an internal disulfide bond, the cysteines are not available to act as metal ligands. The complex observed with the oxidized peptide at high ZnCl\(_2\) concentrations (in the tryptic digestions and circular dichroism experiments) must result from binding to a low-affinity site.

The folded structure, observed in the presence of metals, appears to be quite thermostable. Circular dichroism spectra of the zinc–peptide complex were recorded from 4°C to 75°C. There is remarkably little change in the spectrum over this temperature range, and the small changes observed are reversible. The cobalt–peptide complex also is thermostable. Absorption spectra recorded at 60°C are essentially identical with spectra recorded at room temperature.

Our DNA binding experiments are difficult to interpret. The effects seen in DNA protection experiments are subtle (Fig. 6). There never is a clear footprint and changes are noticed both inside and outside of the control region recognized by intact TFIIIA. Some effects are observed even in the absence of added zinc. However, the results are intriguing because the effects are reproducible and because there is a clear relationship between the DNase protection experiments and the methylation protection experiments (data not shown). It is not surprising that this isolated zinc finger shows very little specificity in DNA binding. Intact TFIIIA, which binds tightly to the internal control region of the SS rRNA gene, contains nine zinc fingers. Tight binding and site-specific recognition typically require a set of cooperative contacts. For example, repressors and restriction enzymes...
bind as dimers, making extensive contacts along an entire face of the protein (29, 30). 

As discussed above, many observations have suggested that zinc fingers are independent structural units. Our results directly demonstrate that an isolated zinc finger domain can fold in the presence of metal ions. TFIIIA-2 and related peptides should facilitate physical and structural studies of the zinc finger motif and its role in nucleic acid recognition.

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