Modulation of Transcription Factor Ets-1 DNA Binding: DNA-Induced Unfolding of an α Helix

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Conformational changes, including local protein folding, play important roles in protein-DNA interactions. Here, studies of the transcription factor Ets-1 provided evidence that local protein unfolding also can accompany DNA binding. Circular dichroism and partial proteolysis showed that the secondary structure of the Ets-1 DNA-binding domain is unchanged in the presence of DNA. In contrast, DNA allosterically induced the unfolding of an α helix that lies within a flanking region involved in the negative regulation of DNA binding. These findings suggest a structural basis for the intramolecular inhibition of DNA binding and a mechanism for the cooperative partnerships that are common features of many eukaryotic transcription factors.

Sequence-specific recognition of DNA by transcription factors is mediated by independent protein modules. The autonomy of DNA-binding domains has recently been challenged by the finding that inhibitory regions, which lie outside functionally defined binding motifs, can alter binding strength or specificity. Deletion mutants of various transcription factors, including TATA-binding protein (TBP), p53, nuclear factor kB (NF-kB), and islet-1 (Isl-1), show enhanced binding activity relative to that of full-length proteins (1, 2). In the case of TBP and p53, conformational changes also have been linked to the presence of inhibitory sequences (1, 3). Understanding the mechanisms of intramolecular inhibition requires the study of DNA-binding modules within their native structural contexts rather than in isolation.

The ets family of transcriptional regulators illustrates this inhibition phenomenon. Members of this gene family are defined by sequence homology within an 85-amino acid region termed the ETS domain (4). This region is a minimal DNA-binding domain that forms a winged helix-turn-helix (wHTH) motif (5). In at least seven members of the ets family, the DNA-binding affinity of the ETS domain is negatively regulated by inhibitory sequences. Inhibition can be released through partial proteolysis or by the formation of complexes with protein partners (6–11). Models of intramolecular inhibition propose that inhibitory regions can either sterically or allosterically affect the function of the DNA-binding domain. Both mechanisms predict that conformational changes accompany DNA binding. To test for such changes, we performed structural studies on Ets-1, the founding member of the ets family.

Ets-1 contains inhibitory regions both NH2-terminal and COOH-terminal to the ETS domain (7–10, 12). Quantitative studies of deletion mutants indicate that inhibition requires both regions (13). To examine the structural basis for inhibition, we analyzed repressed (ΔN280) and activated (ΔN331) forms of Ets-1 (Fig. 1A). ΔN331 is the minimal fragment that retains both high solubility during bacterial expression and high-affinity DNA binding. ΔN280 shows characteristics of a structural domain and retains low-affinity binding because both NH2-terminal and COOH-terminal inhibitory regions are present (13, 14). Both polypeptides were expressed in bacteria and purified protein was used in all studies (15).

To measure the extent of inhibition, we determined the equilibrium dissociation constants (Kd) of ΔN331 (3.1 × 10−11 M) and ΔN280 (4.4 × 10−10 M) (Fig. 1B). The binding affinities of ΔN280 and ΔN331 for a sequence-specific 21-base pair binding site differed by a factor of 15 (16). This lower affinity is similar to that of full-length Ets-1 (13). Thus, ΔN280 retains the inhibitory function of native Ets-1.

The structural origins of DNA-binding inhibition were first investigated by circular dichroism (CD) spectroscopy (17). The CD spectra of ΔN331, the fragment that lacks inhibitory function (Fig. 1A), showed no change between 205 and 225 nm upon sequence-specific DNA binding (Fig. 2A). These results suggest that the wHTH motif of Ets-1 assumes the same secondary structure in the bound and free states. This picture is consistent with nuclear magnetic resonance (NMR)–based structures of the ETS domain proteins Fli-1 and Ets-1 that were determined in the presence and the absence of DNA, respectively (5). The change in the CD spectra between 250 and 295 nm (Fig. 2, A and B) corresponds to a protein-induced alteration of the DNA structure (18).

Analysis of ΔN280, the fragment that contains both inhibitory regions (Fig. 1A), provided evidence for the unfolding of the NH2-terminal inhibitory region upon DNA binding. Addition of DNA to ΔN280 caused a decrease in protein molar ellipticity between 205 and 225 nm (Fig. 2B), which suggests a loss of α-helical secondary structure. This difference cannot be attributed to a change in DNA that is induced by ΔN280 but not by ΔN331, because comparison of the DNA spectra (between 250 and 295 nm) in the presence of ΔN280 or ΔN331 showed identical ellipticity values (Fig. 2E) (19). In addition, ΔN280 and ΔN331 make identical contacts to the DNA (20). Because there was no difference in ellipticity between bound and free ΔN331 (Fig. 2A), the simplest explanation for the decrease in ΔN280 ellipticity (between 205 and 225 nm) is the loss of α-helical structure in the NH2-terminal flanking region.

We used partial proteolysis combined with NH2-terminal sequencing to further define the conformational changes within the Ets-1 polypeptides and to map the region that undergoes a structural transition.

Fig. 1. Equilibrium binding assays demonstrate a ∼15-fold difference in affinity (Kd) between ΔN331 and ΔN280 (16). (A) Schematic representation of full-length Ets-1, ΔN280, and ΔN331. Both fragments retain the native COOH-terminus and the 85-amino acid ETS domain (shaded region). The NH2-terminal and COOH-terminal inhibitory regions are represented by the striped lines. (B) Binding isotherms for ΔN280 (●) and ΔN331 (○). The fraction of DNA bound at each protein concentration was calculated from mobility shift assays. Data points are the average (±SD) of three independent titrations.
Chymotrypsin and trypsin were used in these studies because both Ets-1 polypeptides contain potential cleavage sites scattered throughout their length (Fig. 3A). \( \Delta N280 \) and \( \Delta N331 \) were exposed to a fixed concentration of protease in the presence and absence of cognate DNA (21). The \( \Delta N280 \) domain in \( \Delta N331 \) was resistant to proteolytic cleavage under these conditions (Fig. 3B). Consistent with the CD analyses, the protease studies detected no structural rearrangement of the ETS domain or the COOH-terminal sequences upon DNA binding. In contrast, \( \Delta N280 \) showed enhanced proteolytic sensitivity in the presence of specific DNA. Both trypsin and chymotrypsin proteolysis yielded two cleavage products, a major and a minor species (Fig. 3C). Quantification of the trypsin fragments showed that only 8% of free \( \Delta N280 \) was cleaved during the 10-min incubation interval, compared with 84% of bound \( \Delta N280 \) (21). These data demonstrate that DNA binding stabilizes an altered conformation of \( \Delta N280 \) that exhibited enhanced susceptibility to proteolytic attack.

NH\textsubscript{2}-terminal sequencing of the cleavage products (Fig. 3A) showed that trypsin cleaved after residues Arg\textsuperscript{309} and Arg\textsuperscript{311} whereas chymotrypsin cleaved after residues Phe\textsuperscript{304} and Tyr\textsuperscript{307} (22). These cleavage sites all lie within the NH\textsubscript{2}-terminal inhibitory region. Thus, the conformational change was localized to the NH\textsubscript{2}-terminal inhibitory sequences rather than the DNA-binding domain or the COOH-terminal region. These findings were consistent with the CD analyses that suggested that the sequences present in \( \Delta N280 \) but not those in \( \Delta N331 \), undergo a loss of \( \alpha \)-helical structure upon DNA binding.

Similar experiments were used to examine structural changes induced by protein binding to a DNA duplex that lacks an Ets-1 binding site (23). Nonspecific DNA added at the same concentration as specific DNA caused only a modest increase in the protease sensitivity of \( \Delta N280 \) (Fig. 3C). Under these conditions, and assuming an affinity (K\textsubscript{D}) in the micromolar range (24), only \(-35\%\) of the protein would be bound to DNA. However, nonspecific DNA added at concentrations sufficient to ensure \( 99\% \) protein occupancy converted all of the \( \Delta N280 \) to the proteolytic-sensitive form (25). CD analyses performed in the presence of nonspecific DNA provided similar results; \( \Delta N280 \) exhibited a decrease in ellipticity that was proportional to the fraction of protein bound to DNA (Fig. 2D). These results indicated that both specific and nonspecific DNA can stabilize an altered conformation of the NH\textsubscript{2}-terminal inhibitory region of \( \Delta N280 \). Nonspecific DNA binding had no effect on the structure of \( \Delta N331 \) in either assay (Figs. 2C and 3B).

The inhibitory region of \( \Delta N280 \) that becomes hypersensitive to proteolytic cleavage in the presence of DNA spans residues 304 to 311. NMR analysis of \( \Delta N280 \) indicates that this region (residues 303 to 310) forms an \( \alpha \) helix in the free protein (26). In the presence of DNA, almost all potential trypsin and chymotrypsin sites in this region became accessible to cleavage (Fig. 3A), which suggests that this helix is unfolded in the bound state. Our CD analyses are also consistent with the unfolding of this \( \alpha \) helix. The mean residue ellipticity of \( \Delta N280 \) decreased by 20% in the presence of DNA; this corresponds to the unfolding of \(-11\) helical amino acids (of the total 62 NMR-assigned helical residues), in good agreement with the size (eight amino acids) of the metastable \( \alpha \) helix.

The similar DNA-binding affinities of \( \Delta N280 \) and full-length Ets-1 imply that the structural transition observed in \( \Delta N280 \) also occurs in full-length Ets-1. Indeed, partial proteolysis of full-length Ets-1 in the presence of DNA shows increased trypsin susceptibility only between residues 309 and 312 (13). In our model of full-length
Ets-1 (Fig. 4), the NH2-terminal inhibitory region makes intramolecular contacts with the COOH-terminal inhibitory region and the ETS domain in the absence of DNA (27). In support of this proposal, NMR studies and modeling of the secondary structure elements of ΔN280 suggest that the two helices within the NH2-terminal inhibitory region interact directly with the first helix in the ETS domain and the helix in the COOH-terminal inhibitory region, possibly forming a four-helix bundle (26). DNA binding allosterically alters these structural elements. Specifically, the α helix at residues 303 to 310 within the inhibitory region unfolds. Refolding restores the repressed state, thereby causing the dissociation of the DNA-protein complex. Stabilization of the repressed conformation by a partner protein may serve to couple binding to adjacent transcription factors. The localized folding could create a hinge that facilitates interactions with the partner. Alternatively, the inhibitory sequences could be the contact surface for the interaction with the partner protein. Thus, inhibition could be relieved by partner-protein interactions within a regulatory pathway.

Our findings demonstrate that Ets-1 contains an inhibitory sequence that becomes disordered upon DNA binding. In contrast to previous studies of transcription factors (28), both specific and nonspecific DNA can act as effectors of Ets-1 conformational changes. Consequently, sampling of the decondensed conformation can precede specific site recognition. Our results also are distinct from structural and biophysical analyses of specific DNA-protein interactions that implicate local folding as a key to site-specific DNA recognition (29, 30). Although many DNA-binding proteins fold upon contact with DNA, our findings provide evidence that the inhibitory region of Ets-1 unfolds upon DNA binding (31). This unfolding represents a distinct mechanism for regulating transcription factor activity. Autoinhibitory domains also have been described in kinases, phosphatases, and a calmodulin-dependent Ca2+ pump (32). Allosterically induced conformational change has been implicated in the derepression of these proteins. Our study of Ets-1 demonstrates that conformational equilibria also can govern the repression and derepression of transcription factor-DNA interactions.

Note added in proof: The Bam HI–DNA complex has been recently solved by crystallography (33). This structure reveals that an ordered α helix at the COOH-terminus of the free protein unfolds upon DNA binding.

REFERENCES AND NOTES

14. Thermal denaturation of ΔN280 monitored by CD shows a cooperative melting transition with an apparent midpoint of unfolding ~20°C higher than that observed for ΔN331 (J. M. Petersen and B. J. Graves, unpublished results).
15. The ΔN331 expression vector was constructed as follows: a fragment encoding ets-1 residues 331 to 415 was generated by the polymerase chain reaction (PCR) and cloned into the pAE44 expression vector [D. S. Deering, thesis, Massachusetts Institute of Technology (1993), expanded by the 5' PCR primer provided a methionine codon. The resulting clone was modified such that sequences encoding ets-1 residues 364 to 415 were replaced with a fragment encoding ets-1 residues 364 to 440. The ΔN280 expression vector was pET3A (H. Rosenberg et al., Gene 56, 125 (1993)). A DNA fragment encoding ets-1 residues 286 to 440 was ligated to a synthetic adapter encoding an NH2-terminal methionine (coding sequence for a site-specific recognition site) and ets-1 residues 285 to 285. Both proteins were expressed under the control of a T7 promoter in E. coli BL21(DE3) cells. The recombinant expression vector was induced by 0.5 μg/ml isopropylthiogalactopyranoside for 1 hour at 37°C. Cells were harvested by centrifugation and lysed by sonication in buffer containing 50 mM Tris (pH 7.9), 1 mM EDTA, 1 M KCl, 1 mM ATP, 1 mM DTT, and 0.1 mM PMSF, and applied to a DEAE-cellulose column (Fermento Chemicals). Both proteins flowed through DEAE-cellulose and were loaded directly onto an Sephacel column (Pharmacia) equilibrated in buffer containing 20 mM sodium citrate (pH 5.3), 1 mM EDTA, 1 M KCl, and 0.2 mM PMSF. Purified ΔN331 or ΔN280 was incubated with a radiolabeled top synthetic DNA duplex (top strand, 5’-A530GCAAItCACGAAATGTGTTG-3’) for 30 min at 4°C. Extinction coefficients were determined for the ΔN280 (37,850 M−1 cm−1) and ΔN331 (29,610 M−1 cm−1) as described [X. S. C. and X. F., in press (1990)]. Protein concentrations were measured spectrophotometrically at 280 nm. The total protein concentration was corrected for the fraction of active protein (>90%) by both for ΔN331 and ΔN280 as determined by DNA titration experiments. Free DNA and DNA-protein complexes were quantitated by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). DNA binding protein concentration is the total DNA concentration, [P] = [protein] + [free protein]. In all binding reactions mixtures of the DNA concentration was sufficiently low (2 x 10−10 M) to ensure that free protein was approximately equal to total protein.
16. All spectra were recorded in 50 mM KPO4 (pH 7.9), 65 mM KCl, and 0.1 mM EDTA at 4°C on an AVV 62DS spectrophotometer. All spectra were recorded in a double-layered mixing cuvette (Hellma, path length 0.874 cm) as follows: First, protein only was added to a single channel and the spectrum was recorded. Then, DNA (18, 23) was added to the second channel at a slow rate and a spectrum of the unmixed protein and DNA was recorded. Finally, the cuvette was inverted to mix protein and DNA samples and a final spectrum was recorded. To derive protein-only spectra in the presence of DNA (Fig. 2), we substracted the free DNA spectra (derived from the unmixed protein and DNA spectra) from the mixed protein-DNA spectra. To obtain DNA-only spectra (Fig. 2), we substracted the free DNA spectra from the mixed spectra. After each CD spectrum was recorded, samples were left in the mixing cuvette and checked for aggregation as detected by ultraviolet light scattering. No DNA concentration was determined before and after addition of DNA by Bradford assay (Bio-Rad) to check for precipitation; neither aggregation nor precipitation was observed. Data collection was performed in 100 mM sodium phosphate, pH 7.9, in steps with averaging times of 10 s from 250 to 190 nm and 3 s from 350 to 250 nm. Concentrations of specific DNA ensured 95% protein occupancy (Fig. 2).
2. A and B). On the basis of a Kd for nonspecific DNA binding in the 10^-4 to 10^-6 M range (24), the protein occupancy was estimated to be 36% for ΔN331 and 27% for ΔN280 (Fig. 2. C and D).

18. DNA-only spectra of mixed DNA-protein samples containing specific DNA and either ΔN331 or ΔN280 show a difference in molar ellipticity between 250 and 295 nm as compared to the spectra of free DNA, which suggests a protein-induced change in the DNA. This difference was not observed in ΔN280 or ΔN331 samples mixed with nonspecific DNA (Fig. 2. C and D) (J. M. Petersen and B. J. Graves, unpublished results).

19. Specific DNA-only spectra in the presence of ΔN31 and ΔN280 were recorded between 250 and 295 nm because ΔN331 and ΔN280 make no important contributions to their CD spectra in this region (Fig. 2. A through D). In contrast, specific DNA shows a strong CD signal in this region (Fig. 2E).

20. The NH2-terminal deletion mutant ΔN336 and full-length Ets-1 show identical DNA contacts in protection and interference assays (8). DNA-binding studies of ΔN331 and ΔN280, including deoxycytidine or deoxyadenosine footprinting and binding site size selection, also indicate identical contact regions (Q. Xu and J. M. Petersen, unpublished results).

21. A time course of protease digestion was performed with fixed concentrations of trypsin (115 ng) or chymotrypsin (215 ng) in a buffer containing 50 mM KPO4, pH 7.9, 65 mM KCl, and 0.1 mM EDTA. Proteases were inactivated with 50 mM PMSF, 2% SDS, and 100 mM DTT. Polypeptides were fractionated on Tricine-SDS polyacrylamide gels (H. Schagger and G. von Jagow, Anal. Biochem. 166, 368 (1987)) and visualized by Coomassie blue staining. Stained bands were quantified by video capture and computer-based densitometric analysis with NIH image software (version 1.49). The concentrations of specific DNA (16) and protein that were sufficient to obtain 99% protein occupancy were 10 µM for reactions containing ΔN280 and 15 µM for reactions containing ΔN331. Nonspecific DNA (23) was used at equimolar concentrations relative to specific DNA. On the basis of a Kd for nonspecific DNA binding in the 10^-4 to 10^-6 M range (24), the protein occupancy was calculated to be 46% for ΔN331 and 35% for ΔN280.

22. Fragments were electroblotted from Tricine-SDS polyacrylamide gels to polyvinylidene fluoride membranes and sequenced by Edman degradation with an Applied Biosystems automated sequencer.

23. A 21-bp synthetic DNA duplex [5'-AG-GGCTGATTGGCCATATTG-3'] was used as the specific control DNA. This duplex lacks the core 5'-GGAG-3' motif required for Ets-1 sequence-specific DNA binding.


25. J. M. Petersen and B. J. Graves, data not shown.


27. The interaction between the NH2-terminal and COOH-terminal inhibitory regions is supported by the finding that the pattern of protease digestion within the NH2-terminal inhibitory region is altered in the absence of the COOH-terminal inhibitory region (13).


31. Because the wtTH motif of Ets-1 does not change secondary structure upon DNA binding (5), we predict that the change in heat capacity (ΔCp) that accompanies Ets-1 DNA binding will be affected by the unfolding of the a-helix (residues 303 to 310). We propose that ΔCp will be smaller in magnitude than the values observed for protein-DNA interactions that display a substantial induced fit or rigid body association (30).


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