Intrinsic Structural Disorder of the C-Terminal Activation Domain from the bZIP Transcription Factor Fos†

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Received October 11, 1999; Revised Manuscript Received December 14, 1999

ABSTRACT: The bZIP proto-oncoprotein c-Fos activates transcription of a wide variety of genes involved in cell growth. The C-terminal activation domain of c-Fos is functionally independent of the remainder of the protein. Fos-AD corresponds to the C-terminal activation domain of human c-Fos (residues 216−380). Fos-AD suppresses (squelches) transcription in vitro, as expected for a functional activation domain lacking a DNA-binding domain. Fos-AD is unstructured and highly mobile, as demonstrated by circular dichroism spectra indicative of unfolded proteins, a lack of ^{1}H chemical shift dispersion, and negative ^{1}H-^{15}N heteronuclear nuclear Overhauser effects. The hydrodynamic properties of Fos-AD are also consistent with an extended structure. We conclude that the C-terminal domain of human c-Fos is biologically active yet intrinsically disordered. Our results suggest that conformational disorder is an integral aspect of the diverse contributions to transcriptional regulation by c-Fos.

The enzymatic synthesis of protein-coding RNA is performed in eukaryotes by a large protein complex called RNA polymerase II (*1*). Although assembly of polymerase II and the general transcription factors at the promoter is sufficient for transcription at basal levels in vitro, transcription at elevated levels requires DNA-binding transcriptional activator proteins. Activators may enhance various steps in transcription, such as assembly of the polymerase II preinitiation complex at the promoter, clearance, and elongation (*1*). Moreover, activators may be generally required in vivo to overcome the repressive effects of chromatin and suppresser proteins and for competitive recruitment of general transcription factors (*1*).

Members of the Fos family of mitogen-inducible transcriptional activators dimerize with the Jun or ATF/CREB activators to form the transcriptional regulator AP-1[1] (*2*, *3*). AP-1 activates transcription of genes involved in a diverse range of biological processes, including cell proliferation, differentiation, neuronal development, and apoptosis (*2*, *3*). The Fos proteins have a strong oncogenic potential, especially when transduced by retroviruses (*4*, *5*), and have provided a widely used model system to understand the mechanism of transcriptional activation using molecular biology and genetic approaches (*6*−*17*).

Human c-Fos has a modular structure consisting of an N-terminal activation domain, a bZIP dimerization/DNA-binding domain, and a C-terminal domain (Figure 1). The C-terminal domain of c-Fos contains both acidic and proline-rich activation domains (*9*) and is functionally independent of the rest of the molecule. For example, the C-terminal domain activates transcription when the bZIP domain is replaced with the LexA DNA-binding domain (*15*) and when the N-terminal domain is deleted (*9*).

Several transcriptionally important protein−protein interactions involving c-Fos are mediated by the C-terminal domain. In particular, the C-terminal domain interacts with TBP, a central component of the general transcription factor TFIID, the acetyltransferase and coactivator CBP, and the regulatory transcription factor Smad3 (*12*, *13*, *17*). In addition, regions of the C-terminal domain that participate in activation have been mapped in several deletion and mutagenesis studies and regulatory effects of phosphorylation of the C-terminal domain have been described (*7*−*17*).

While the C-terminal domain of Fos has been characterized extensively using molecular biology techniques, the domain has yet to be defined in structural terms. We show here that the C-terminal activation domain of human c-Fos (residues 216−380; Figure 1), although functionally active, is essentially devoid of the stable helical or β-sheet structure typical of globular proteins. We conclude that c-Fos contains an intrinsically disordered yet biologically active activation domain.

EXPERIMENTAL PROCEDURES

Jun-bz was expressed in *Escherichia coli* strain BL21 (DE3) using the plasmid pJJ70 (gift of P. S. Kim) and purified from the soluble fraction with ion-exchange chromatography. Fos-bzAD and Fos-AD were expressed in *E.*

† Supported by the Cancer League of Colorado and the donors of the Petroleum Research Fund, administered by the American Chemical Society (Grant 32789-G4), and by NIH equipment grants RR11847 and RR11981.

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[1] Abbreviations used: AP-1, activating protein 1; bZIP, basic region leucine zipper; CBP, CREB-binding protein; CREB, cyclic AMP response element binding protein; CD, circular dichroism; DQF COSY, double-quantum-filtered correlation spectroscopy; HSQC, heteronuclear single-quantum coherence spectroscopy; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE spectroscopy; ppm, parts per million; TBP, TATA-binding protein.

10.1021/bi9923555 CCC: $19.00 © 2000 American Chemical Society
Published on Web 02/15/2000

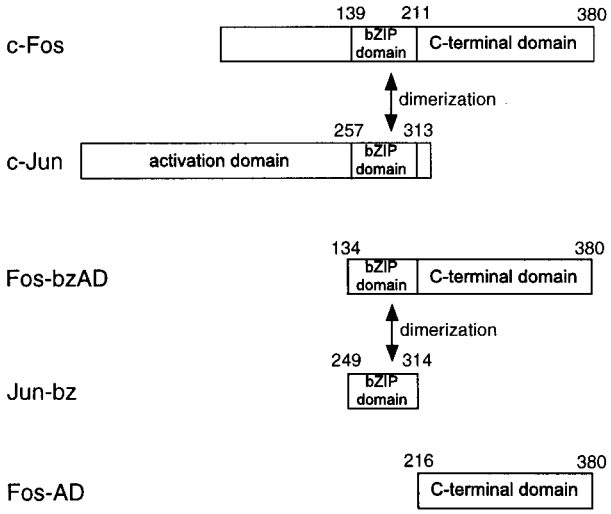

FIGURE 1: Schematic representation of the deletion mutants of human c-Fos and c-Jun. The leucine zipper of the bZIP domain of c-Fos moderates dimerization with c-Jun (*32*) and the basic region of the bZIP domain binds DNA (*5*). The N- and C-terminal domains activate transcription (*9*). Fos-AD spans the human c-Fos C-terminal activation domain (residues 216−380) and Fos-bzAD spans the human c-Fos bZIP and C-terminal domains (residues 134−380). Jun-bz corresponds to the bZIP domain of human c-Jun (residues 249−314).

coli strain BL21 (DE3) as N-terminal His-tag fusion proteins. The pET15b expression plasmids for Fos-bzAD (called pHFosbzAD) and Fos-AD (called pHFosAD) were constructed using PCR products derived from pFF70 (which encodes the bZIP domain of human c-Fos with codon usage optimized for *E. coli*.; gift of P. S. Kim) and pGEX2TKN-hcfos (encoding human c-Fos; gift of J. A. Goodrich). DNA sequences were confirmed with dRhodamine sequencing. The Fos-bzAD and Fos-AD fusion proteins were purified using His-bind resin (Novagen), and the His-tag sequence cleaved with thrombin. Final purification was by reversed-phase C_4 high-performance liquid chromatography. The identity of Fos-AD was confirmed with electrospray mass spectrometry. ^{15}N-labeled Fos-AD was prepared in the same way, except cells were grown in M9T medium containing $(^{15}NH_4)_2SO_4$ as the sole nitrogen source (*18*). Fos-AD and Fos-bzAD have three additional N-terminal amino acids (Gly-Ser-His) derived from the His-tag sequence.

Transcription assays were performed in *Saccharomyces cerevisiae* whole-cell extract from strain BJ926 (*19*, *20*) with a template [pS(GCN4)2CG$^-$ (*21*)] containing a G-less transcript under control of two AP-1 (Gcn4p) sites (gift of R. D. Kornberg). Reaction mixtures (30 μL) contained 5 nM (300 ng) pS(GCN4)2CG$^-$, yeast whole-cell extract (250 μg protein), 10 μCi α-^{32}P UTP, 0.25 units RNAse Inhibitor (5′-3′, Inc.), 50 mM HEPES-KOH, pH 7.6, 100 mM potassium glutamate, 10 mM magnesium acetate, 2.5 mM DTT, 5 mM EDTA, 1% PEG 3350, 70 μM ATP, 70 μM CTP, 4 μM UTP, 30 mM creatine phosphate, creatine kinase (1.4 units/mL), and 10% glycerol. The whole-cell extract and DNA template were supplemented with equimolar (0−40 nM) Jun-bz and Fos-bzAD, Jun-bz (0−50 nM) or Fos-AD (0−50 nM) and incubated at 21 °C for 15 min prior to transcription initiation by the addition of nucleotides. Reactions were run an additional 15 min at 21 °C, treated with ribonuclease T1 (50 units in 120 μL 10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM EDTA) for 15 min at room temperature, followed by addition of 8 μL 10% SDS, 17.5 μL proteinase K (2.5 mg/mL), and a ^{32}P end-labeled 550 base pair DNA recovery standard and incubation at 37 °C for 20 min. A 2.5 μL sample of glycogen (6 mg/mL) was added, and the transcript and recovery standard precipitated with ethanol. Transcripts were resolved on 8% polyacrylamide/6 M urea (6 M) gels, visualized with a Molecular Dynamics PhosphorImager, and quantified with NIH Image (http://rsb.info.nih.gov/nih-image/). Reactions were performed three times with reproducible results.

CD spectroscopy was performed with a Jasco J-720 spectrometer. Samples were prepared in 10 mM sodium phosphate, 0−1 M NaCl, 1 mM DTT, pH 7.0, and contained 10 μM Fos-AD. Protein concentrations were determined by absorbance in 6 M GuHCl, 10 mM sodium phosphate, pH 6.5 at 25 °C using an extinction coefficient at 280 nm of 9530 M^{-1} cm^{-1} (*22*).

NMR spectroscopy was performed with a Varian Unity Inova spectrometer operating at 500.1 MHz for ^{1}H. Samples were prepared in 10 mM sodium phosphate, 150 mM NaCl, 1 mM d_{10}-DTT, pH 6.5. Homonuclear ^{1}H experiments were performed on 0.35 mM Fos-AD. Heteronuclear ^{1}H-^{15}N experiments were performed on 90 μM or 0.93 mM ^{15}N-labeled Fos-AD. Data were collected at 25 °C using States-TPPI phase discrimination in the indirectly detected dimension. The water resonance was suppressed using gradient techniques rather than solvent presaturation to minimize cross saturation of the amide NH protons. ^{1}H chemical shifts were referenced with internal DSS at zero ppm (*23*). Magic-angle-gradient DQF-COSY spectra (*24*) consisted of 256 complex increments defined by 16 transients. 3-9-19 WATERGATE (*25*) NOESY spectra (*26*) consisted of 256 increments defined by 96 transients and were collected using a 200 ms mixing time. Sensitivity-enhanced gradient HSQC spectra (*27*) consisted of 256 complex increments defined by 24 (0.93 mM Fos-AD) or 96 (90 μM Fos-AD) transients. Steady-state ^{1}H-^{15}N NOE values were measured at 25 °C from 2D ^{1}H-^{15}N NOE correlation spectra (*28*). Spectra were recorded with and without a 3 s ^{1}H saturation period, using relaxation delays of 5 and 8 s, respectively, and consisted of 128 complex increments defined by 24 transients.

Sedimentation equilibrium was performed at 25 °C with a Beckman XL-I analytical ultracentrifuge. Samples were dialyzed against the reference buffer (10 mM sodium phosphate, 150 mM NaCl, 1 mM DTT, pH 7.0). Initial loading concentrations of 26, 52, and 87 μM were used. Data were collected at 30 and 40 krpm using 12 mm path length six-sector centerpieces and an An-60Ti rotor. Data were analyzed with ORIGIN (Beckman Instruments). Discrimination between different models (i.e., ideal vs nonideal single species) was based on the distribution of residuals. Partial molar volumes and solvent densities were calculated as described elsewhere (*29*).

The Stokes' radius of Fos-AD was determined by gel filtration (*30*) on Sephacryl S-100 equilibrated in 10 mM sodium phosphate, 150 mM NaCl, 1 mM DTT, pH 7. The column was calibrated with bovine ribonuclease A, bovine chymotrypsinogen A, hen ovalbumin, bovine serum albumin, and Blue Dextran 2000.

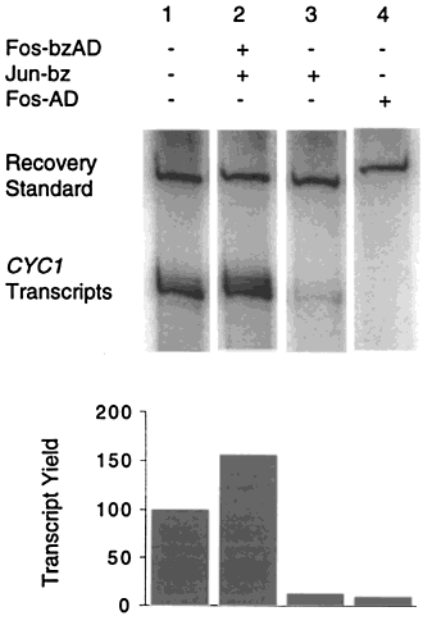

FIGURE 2: Transcriptional activity at an AP-1 promoter in yeast whole-cell extract. The extract contains endogenous AP-1 activity (i.e., Gcn4p) responsible for the intrinsic transcription of a 350-nucleotide gene product under control of the AP-1 site (lane 1). The presence of several bands indicates multiple start sites. Fos-bzAD paired with Jun-bz activates transcription (lane 2). Jun-bz alone suppresses transcription (lane 3). Fos-AD suppresses (squelches) transcription (lane 4). Results are shown for 40 nM Jun-bz/Fos-bzAD (lane 2), 50 nM Jun-bz (lane 3), or 50 nM Fos-AD (lane 4). The bar chart illustrates the average of three independent experiments.

RESULTS AND DISCUSSION

Transcriptional Activity of Fos-AD. Since transcriptional activators must bind to regulatory DNA elements to function efficiently (*31*), it is generally not possible to assay directly transcriptional activation by an activation domain lacking a DNA-binding domain. However, a functional activation domain lacking a DNA-binding domain would be expected to suppress (squelch) transcription by sequestering target transcription factors and making them unavailable to participate in transcription (*31*).

A yeast-based transcription assay for c-Fos activity at an AP-1 promoter was used, since human c-Fos activates transcription in yeast (*6*). To show that the yeast extract was responsive to transcriptional activation by the human c-Fos C-terminal domain, we used an N-terminal deletion mutant of human c-Fos corresponding to the bZIP DNA-binding domain and C-terminal activation domain (called Fos-bzAD; Figure 1). Fos-bzAD activates transcription from an AP-1 regulated promoter when paired with Jun-bz (a peptide corresponding to the human c-Jun bZIP domain; Figure 1), in accord with previous studies (*9*). Jun-bz lacks an activation domain and actually suppresses transcription in the absence of Fos-bzAD (Figure 2). Suppression by Jun-bz presumably occurs because the c-Jun bZIP domain can form a stable homodimer (*32*) that competes with endogenous AP-1 activity for DNA-binding sites. The suppression by Jun-bz indicates that transcriptional activation by Fos-bzAD/Jun-bz is not due to Jun-bz alone. Thus, the c-Fos C-terminal domain can activate transcription in the assay when bound to DNA.

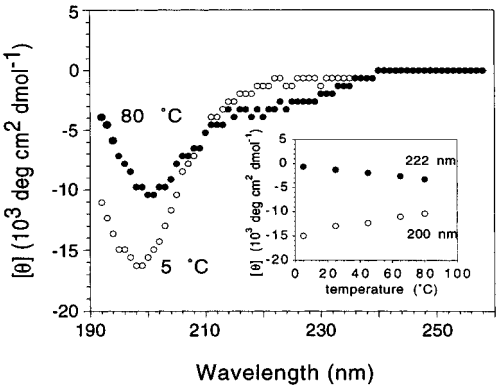

FIGURE 3: CD spectra of Fos-AD (10 mM sodium phosphate, 1 mM DTT, pH 7.0). The CD spectra at 5 °C (○) and 80 °C (●) are characteristic of an unfolded protein (*33*). Changes in the CD spectrum with temperature are linear (insert), indicating that any structure formation is not cooperative.

In contrast to Fos-bzAD/Jun-bz, Fos-AD suppresses transcription of an AP-1-regulated gene (Figure 2), as expected for a functional activation domain lacking a DNA-binding domain (*31*). We conclude that Fos-AD is functional for interactions with the target transcription factors of c-Fos in a biological context.

Structural and Dynamic Properties of Fos-AD. CD provides information on protein secondary structure. The CD spectrum of Fos-AD at neutral pH lacks the spectral features in the far-UV region that are characteristic of significant helical or β-sheet secondary structure (Figure 3). Instead, the spectrum contains a negative band at 198 nm and a shoulder centered at 220 nm (above 5 °C), which are indicative of a disordered conformation (*33*). Changes with temperature in the CD spectrum of Fos-AD are linear up to at least 80 °C (Figure 3), which is also characteristic of an unfolded protein. In addition, the increase in negative ellipticity at 222 nm with temperature is often observed in peptides with extended or disordered conformations (*33*).

The CD spectrum of Fos-AD is independent of pH in the range 2−8 (except at pH 4, where Fos-AD is insoluble) and ionic strength in the range 0−1 M NaCl (data not shown). Thus, in contrast to the behavior of some peptide fragments of activation domains, helical or β-sheet structure is not induced in Fos-AD by changes in pH or ionic strength (*34*). The CD data, therefore, indicate a lack of stable, cooperatively folded helical or β-sheet structure in Fos-AD.

The ^{1}H chemical-shift provides information on secondary and tertiary structure formation in proteins (*35*). ^{1}H chemical shifts of Fos-AD were obtained from DQF-COSY and ^{1}H-^{15}N HSQC spectra. The NH chemical shifts fall between 7.6 and 8.5 ppm (Figure 4). The H$^\alpha$ chemical shifts fall between 3.8 and 4.7 ppm, and the aliphatic chemical shifts fall between 0.6 and 3 ppm in DQF COSY spectra (data not shown). The ^{1}H chemical shifts of Fos-AD are within the chemical shift ranges expected for unfolded proteins (*35*). The chemical shift analysis is limited by ^{1}H chemical shift degeneracy and cannot rule out formation of marginally stable secondary structure that exists in fast exchange on the chemical-shift time scale with a random-coil conformation. Nonetheless, the chemical shift data suggest strongly that Fos-AD lacks stable secondary and tertiary structure typical of globular proteins.

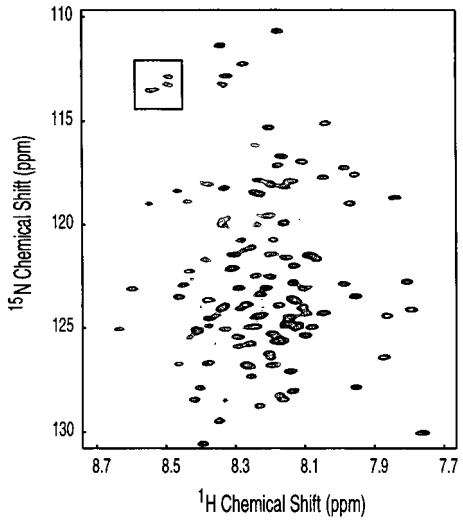

FIGURE 4: ^{1}H-^{15}N HSQC spectrum of Fos-AD (0.93 mM Fos-AD, 10 mM sodium phosphate, 150 mM NaCl, 1 mM DTT, pH 6.5, 25 °C). The water resonance was suppressed using gradients (*27*). The lack of NH chemical shift dispersion suggests that Fos-AD lacks stable secondary structure. Boxed cross-peaks are plotted at a lower contour level.

Homonuclear ^{1}H NOEs reflect secondary structure, even in transiently formed structures (*36*). Unambiguous NOEs are not observed in the region of the NOESY spectrum where $d_{NN}(i, i+1)$ NOEs are expected to be observed (data not shown), indicating that Fos-AD does not populate significantly the α-region of ϕ/ψ space or form helical secondary structure (*36*). Strong NOE connectivities are observed in the region of the spectrum where $d_{\alpha N}$ and $d_{\alpha N}(i, i+1)$ NOE connectivities are expected (data not shown). The presence of potential $d_{\alpha N}(i, i+1)$ NOE connectivities is consistent with Fos-AD adopting an extended conformation in the β-region of ϕ/ψ space (*36*).

The heteronuclear ^{1}H-^{15}N NOE provides information on mainchain flexibility. In particular, ^{1}H-^{15}N NOEs are positive in folded, globular proteins and reduced or negative in unfolded and disordered proteins (*37*−*41*). The ^{1}H-^{15}N HSQC spectrum of Fos-AD is expected to contain 147 amide ^{1}H-^{15}N correlations (168 residues minus Gly1 and 20 prolines). The HSQC spectrum of Fos-AD at pH 6.5 contains 95 completely resolved cross-peaks and numerous partially resolved or overlapped resonances (Figure 4). The ^{1}H-^{15}N NOE was measured for each completely resolved cross-peak. In all cases, the ^{1}H-^{15}N NOE was negative (Figure 5), ranging from −0.86 to −3.4. In addition, the bulk NOE of the partially resolved or degenerate cross-peaks was negative in all cases, ranging from −1.3 to −2.1. This result indicates that the Fos-AD mainchain is flexible, with motion on a timescale characteristic of unfolded proteins.

^{1}H-^{15}N HSQC spectra of Fos-AD were essentially identical at concentrations of 0.93 mM (where the ^{1}H-^{15}N NOE measurements were performed) and at 90 μM (where Fos-AD is monomeric; Figure 6), ruling out concentration-dependent effects on the NMR spectra of Fos-AD.

A global property of Fos-AD is the Stokes' radius, which reflects compactness. An apparent Stokes' radius of 35 Å for Fos-AD was obtained with gel filtration (data not shown). Given that Fos-AD has a dynamic structure, as indicated by the ^{1}H-^{15}N NOE measurements, the observed Stokes' radius of Fos-AD should be considered an average value of the ensemble of the structures accessible to Fos-AD. Sedimentation equilibrium indicates that Fos-AD is monomeric (Figure 6), and the expected Stokes' radius for a globular protein with this molecular weight is 17 Å (*30*). Fos-AD, therefore, has a Stokes' radius that is twice the size expected for a compact globular fold, consistent with the properties expected of an unfolded protein.

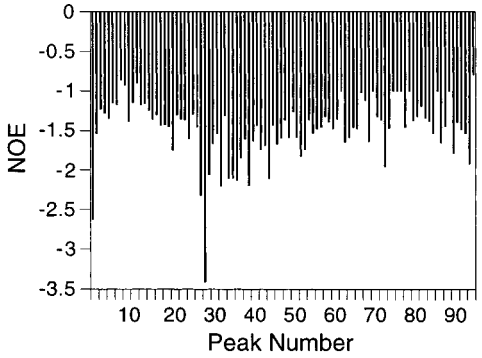

FIGURE 5: ^{1}H-^{15}N NOE intensities of the well-resolved ^{1}H-^{15}N cross-peaks of Fos-AD. Peaks are numbered in order of increasing NH chemical shift.

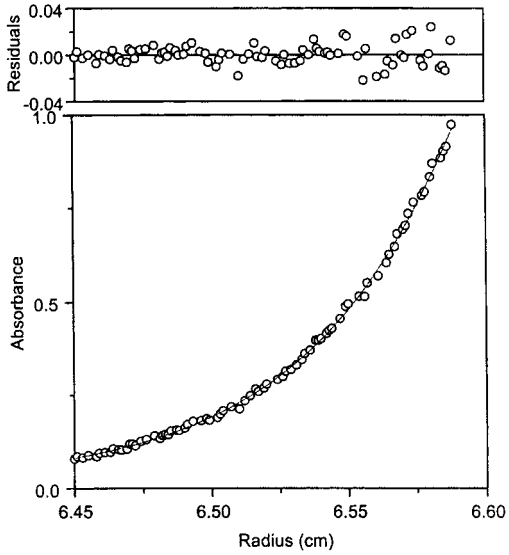

FIGURE 6: Sedimentation equilibrium data for Fos-AD (10 mM sodium phosphate, 150 mM NaCl, 1 mM DTT, pH 7, 25 °C). The data fit to single-species, nonideal model, as indicated by the random distribution of residuals, with a mass expected of a monomer (expected 17.61 kDa; observed 17.59 ± 0.23 kDa). The data shown span a concentration range of approximately 9−90 μM.

The spectroscopic and hydrodynamic data collectively indicate that Fos-AD lacks stable helical and β-sheet structure, has an extended conformation, and is monomeric. The data cannot rule out the formation of β-turn structure (*42*) or hydrophobic clusters (*43*). However, the negative amide ^{1}H-^{15}N NOEs indicate that the mainchain of Fos-AD is highly flexible and exists in a motional regime expected of an unfolded protein. We conclude that human c-Fos contains a biologically active yet intrinsically disordered domain.

Implications for Fos Function. Biological activity and molecular recognition often result from well-defined structures that place functional groups in key positions to interact with and discriminate between other molecules. Globular proteins, for example, typically lose biological activity when

unfolded. However, a combination of biophysical probes collectively indicate that the biologically active acidic and proline-rich C-terminal activation domain of human c-Fos is structurally disordered, with properties reminiscent of an unfolded protein. The entire activation domain was used, and so the disorder is not due to deletion of sequence required for structure. Structural disorder has also been observed for the full-length acidic activation domains from VP16, NF-κB, and p53 (*44*−*46*) as well as peptide fragments of other acidic activation domains at neutral pH (*34*, *47*). Our findings, along with results for the p53 proline-rich activation domain (*46*), also indicate that proline-rich activation domains can exhibit structural disorder.

Several mechanistic advantages to using disordered protein domains have been proposed, including increased binding specificity at the expense of thermodynamic stability, regulation by proteolysis, and the ability to recognize a range of different proteins (*48*−*50*). The C-terminal activation domain of c-Fos interacts directly with the transcription factors TBP, CBP, and Smad3 (*12*, *13*, *17*), and activates transcription in a diverse range of cellular processes (*2*, *3*). In addition, the c-Fos C-terminal domain contains a number of phosphorylation sites that moderate Fos activity (*7*, *8*, *14*). The structural requirements for Fos phosphorylation and for interactions of Fos with other transcription factors may well be different, as proposed for the intrinsically disordered KID activation domain of CREB (*51*, *52*). Our results raise the intriguing possibility that the conformational freedom of the structurally disordered C-terminal activation domain contributes to the functional diversity of c-Fos by permitting formation of a greater variety of protein complexes, both with other transcription factors and with regulatory kinases, than could be achieved with a single, predefined structure.

ACKNOWLEDGMENT

We thank C. D. Rithner, L. A. Stargell, and R. W. Woody for helpful discussions and J. A. Goodrich, P. S. Kim, and R. D. Kornberg for plasmids.

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BI9923555