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High-Yield Expression in *E. coli* and Refolding of the bZIP Domain of Activating Transcription Factor 5

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Abstract

Activating Transcription Factor 5 (ATF5) recently has been demonstrated to play a critical role in promoting the survival of human glioblastoma cells. Interference with the function of ATF5 in an *in vivo* rat model caused glioma cell death in primary tumors but did not affect the status of normal cells surrounding the tumor, suggesting ATF5 may prove an ideal target for anti-cancer therapy. In order to examine ATF5 as a pharmaceutical target, the protein must be produced and purified to sufficient quantity to begin analyses. Here, a procedure for expressing and refolding the bZIP domain of ATF5 in sufficient yield and final concentration to permit assay development and structural characterization of this target using solution NMR is reported. Two-dimensional NMR and circular dichroism analyses indicate the protein exists in the partially α -helical, monomeric x-form conformation with only a small fraction of ATF5 participating in formation of higher-order structure, presumably coiled-coil homodimerization. Despite the persistence of monomers in solution even at high concentration, an electrophoretic mobility shift assay showed that ATF5 is able to bind to the cAMP response element (CRE) DNA motif. Polyacrylamide gel electrophoresis and mass spectrometry were used to confirm that ATF5 can participate in homodimer formation and that this dimerization is mediated by disulfide bond formation.

Keywords

ATF5; ATFx; bZIP; recombinant expression; protein refolding; disulfide bond

Activating Transcription Factor 5 (ATF5) recently has been recognized for its importance in neurological development and contribution to brain cancer. This protein functions to maintain the cell in a proliferative state and must be down regulated in order for differentiation to occur in neural progenitor cells [1–3]. While ATF5 is highly expressed in developing neurons, it is not expressed to any detectable level in healthy mature neural tissue [1,3]. Increased levels of ATF5 have, however, been observed in primary brain tumors, and expression is elevated to particularly high levels in human glioblastoma [4]. It also is overproduced in several human and rat glioma cell lines [4]. The absence of ATF5 expression in mature neurons and its prominence in brain tumors has made it an appealing target for anti-cancer therapy. Importantly, an *in vivo* rat model has demonstrated that interference with ATF5 function caused glioma cell death in primary tumors, while it did not affect the status of normal cells surrounding the tumor [4]. This data suggests ATF5 is a prime target for pharmaceutical intervention.

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In general, there are two main approaches used to identify and develop lead pharmaceutical candidates. The first relies on empirical testing of chemical compounds and requires a screening assay that is capable of quantifying the effect compounds have on a given pharmaceutical target. The second entails rational drug design, which is based upon high-resolution structural information about a pharmaceutical target. The first step that must be taken to begin the analysis using either approach is production of the pharmaceutical protein target. In many cases involving multi-domain proteins, it is acceptable or advantageous to simplify the analysis by focusing the characterization specifically on the domain(s) that confer activity. In the case of ATF5, the portion responsible for DNA binding is of interest for assay development and structural characterization. ATF5 belongs to the bZIP family of transcription factors, which are composed of an unstructured N-terminal activating domain followed by a C-terminal bZIP domain [5,6]. The bZIP domain consists of a basic sequence that directly binds the DNA. The DNA binding sequence is followed by a helical leucine zipper region, which facilitates dimerization, increasing the DNA binding affinity [7–13]. The large, unstructured N-terminal domain typically is involved in more complex aspects of transcriptional regulation [5,6,14, 15]. The bZIP domain from numerous transcription factors has been shown to be amenable to structural characterization, and there are many instances where study of a bZIP protein has been performed using only this C-terminal domain [7–13].

The goal of the research described here was to produce the bZIP domain of ATF5 in sufficient yield and quantity to make high-resolution structure analysis and assay development feasible. We have developed a process to generate and maintain a soluble and stable solution of protein at sufficiently high concentration to permit such analyses. This paper describes the procedure whereby the bZIP domain of ATF5 was successfully expressed, purified and concentrated sufficiently to perform two-dimensional NMR analysis. SDS-PAGE and mass spectrometry were used to confirm that we successfully produced and isolated the intact bZIP domain of ATF5. The CD data indicate that ATF5 contains alpha helical content, showing that the protein obtained using our method has the expected secondary structure. 2D ¹H-¹⁵N NMR data was acquired and the spectrum shows the protein is predominantly in the monomeric x-form state, indicating that structural analysis can be performed on ATF5 that has been produced using the method presented here [16]. The functionality of ATF5 prepared under these conditions was confirmed using an electromobility shift assay where the protein specifically bound to the cyclic AMP response element (CRE), which is a known DNA binding site of ATF5 [17].

Materials and methods

Cloning and construction of the expression plasmid

The cDNA of ATF5 was obtained through ATCC (**MGC-842**). The portion encoding the bZIP domain was amplified using PCR for insertion to a plasmid using the following primers: 5' GCGCGCCCATGGGCCCTGCCACCACCCGA3' (forward primer with NcoI restriction site), 5'GCGCGCCATATGCCTGCCACCACCCGAGGG3' (forward primer with NdeI restriction site), 5'CGCGCGGGATCCTCAGCTACGGGTCCTCTG3' (reverse primer with BamHI restriction site). The amplified fragments were digested with the corresponding endonucleases, gel purified and ligated into the pET-42b vector (Novagen) by overnight incubation with T4 Ligase at 16°C. The NcoI site was used to insert the ATF5 gene following a Glutathione-S-Transferase (GST) tag, whereas insertion at the NdeI site generated a construct from which untagged ATF5 could be expressed. The ligation product was transformed into competent Novablue *E. coli* (Novagen) using the standard heat shock protocol. Transformed colonies were grown overnight at 37°C on LB agar plates containing 30 µg/ml kanamycin. Individual colonies were selected and used to inoculate 5 ml M9ZB containing 0.4% glucose, 1 mM MgSO₄ and 30 µg/ml kanamycin. Cultures were incubated with shaking overnight at

37°C. Plasmid DNA was isolated from the overnight cultures using a Qiagen miniprep kit. Purified DNA was verified to be correct by bidirectional sequencing (Northwoods DNA).

Protein expression

BL21(DE3) *E. coli* (Novagen) were transformed with the pET-42b-ATF5 bZIP plasmid using the standard heat shock protocol. Transformed colonies were selected and grown overnight at 37 °C on LB agar plates containing 30 µg/mL kanamycin. Colonies were selected and used to inoculate 5 mL LB containing 0.4% glucose, 1 mM MgSO₄, and 30 µg/mL kanamycin. Cultures were grown with shaking overnight at 37°C. Two starter cultures were used to inoculate one 500 ml culture containing minimal media consisting of deionized water supplemented with 0.1% ammonium chloride, 1% glucose, 10 mM MgCl, 40 µg/mL thiamine HCl, 30 µg/mL kanamycin plus additional salts (100 mM KH₂PO₄, 57 mM K₂HPO₄, 63 mM Na₂HPO₄, 14 mM K₂SO₄) and trace minerals (200 µM CaCl₂·H₂O, 100 µM FeSO₄·H₂O, 50 µM MnCl₂·6H₂O, 15 µM CoCl₂·6H₂O, 10 µM ZnSO₄·7H₂O, 9 µM CuCl₂·2H₂O, 1.5 µM H₃BO₄, 1.2 µM (NH₄)MoO₇O₂₄·4H₂O, 65 µM Na₂EDTA). Isotopic labeling was accomplished via substitution with ¹⁵N-labeled ammonium chloride (Spectra Isotopes). Cultures were grown to an OD₅₅₀ of 0.6 to 0.8 before inducing protein expression with a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were incubated with shaking at 37°C for 4 additional hours post induction, harvested by centrifugation at 3000 × g and stored at -80° C.

Purification of the GST-tagged ATF5 construct

Cell pellets were thawed on ice and resuspended in cold phosphate buffered saline (PBS), pH 7.3 (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) containing 5 mM dithiothreitol (DTT). Each pellet derived from 1 L culture was taken up in 25 mL solution and lysed via passage through a french pressure cell. Lysates were centrifuged for 1 hour at 21,000 × g and 4°C. The supernatant was decanted and passed through a 0.2 µm filter. The filtrate was loaded onto a 5 mL GSTrap column (GE Healthcare) at a flow rate of 1 mL/min. The column was rinsed with 10 column volumes (CV) of loading buffer or PBS, pH 7.3 containing 5 mM DTT at 3 mL/min. Elution was performed with 3 CV of elution buffer or 50 mM Tris HCl, pH 8.0 containing 10 mM reduced glutathione (GSH) and 5 mM DTT at 3 mL/min. Chromatographic separation was carried out at 4–8°C. Fractions were collected and stored cold prior to analysis by SDS-PAGE.

Eluted fractions containing ATF5 were concentrated by ultrafiltration using a 3500 MWCO Millipore ultrafilter with a Biomax membrane and centrifuged at 4000 × g and 4°C. Subsequently, Factor Xa (Novagen) was added to each sample at a concentration of 10 units/mL and incubated overnight (approximately 16 hours) at room temperature to cleave the GST tag. Cleavage progress was monitored by SDS-PAGE. The GST tag was removed by incubating the cleaved protein with glutathione-agarose beads (BD Biosciences) for 30 minutes at room temperature. The sample was centrifuged at 16,000 × g for 10 minutes to pellet the agarose beads and recover the ATF5 protein.

Protein extraction and refolding

Cell pellets were thawed, resuspended in PBS, pH 7.3 containing 5 mM DTT and lysed as described above. The insoluble pellet was resuspended by vortexing it in 4 mL of 50 mM sodium phosphate, pH 6.5 containing 100 mM NaCl, 6 M guanidine HCl and 5 mM DTT. The solution was incubated at room temperature for 3 hours to permit denaturation to occur before separating the soluble and insoluble material by centrifugation for 1 hour at 21,000 × g and 4° C. The solubilized protein was refolded by dilution into 40 mL of 50 mM sodium phosphate, pH 6.5 containing 100 mM NaCl and 5 mM DTT [18]. Insoluble components were pelleted by centrifugation for 1 hour at 21,000 × g and 4°C. The supernatant containing refolded ATF5

was dialyzed using 1000 MWCO dialysis tubing with a cellulose membrane (Spectra) overnight at 4°C into 2 L of phosphate buffer (50 mM sodium phosphate, pH 6.5, 100 mM NaCl, 5mM DTT) to remove the guanidinium. The ratio of protein to dialysis solution was 0.04 L protein: 2 L dialysis buffer, which results in a 50-fold dilution of guanidinium to a final concentration of 12 mM. Any precipitation generated during dialysis was removed by centrifugation for 1 hour at $21,000 \times g$ and 4°C. The supernatant containing soluble protein was collected and concentrated using a 15 mL 3500 MWCO Millipore ultrafilter with a Biomax membrane and exchanged into the same buffer solution (50 mM sodium phosphate, pH 6.5, 100 mM NaCl) without DTT. The solution was concentrated to approximately 1 mL and then exchanged into 20-fold excess buffer to further remove guanidinium, reducing the final concentration to less than 1 mM. Sample purity was assessed by SDS-PAGE analysis.

Denaturing Gel Electrophoresis

SDS-PAGE was performed according to the Laemmli procedure [19]. GST-tagged ATF5 was separated on a discontinuous system consisting of a 5% (v/v) stacking gel and a 12% (v/v) resolving gel, whereas untagged ATF5 was resolved using a 15% (v/v) gel. Samples were diluted with 2× Laemmli buffer and heated at 90°C for 10 minutes before being loaded onto the gel. To examine the presence of disulfide-linked species a non-reducing loading buffer was used, which did not contain any reducing agent. An unstained molecular weight marker was used for reference (Bio-Rad Precision Plus standards). Coomassie staining was performed to visualize protein content.

Densitometry was performed on Coomassie stained SDS-PAGE gels using a Typhoon Trio Variabel Mode Imager (Amersham Biosciences) and the amount of protein in each band was determined using ImageQuant TL software (Amersham Biosciences).

Protein Concentration Determination

The molar concentration of the purified ATF5 solution was determined using the Beer-Lambert Law ($A=\epsilon lc$). A theoretical molar absorptivity value (ϵ) corresponding to 280 nm was calculated to be $4470 \text{ M}^{-1}\text{cm}^{-1}$ using the ExPASy Proteomics Server [20]. A_{280} absorption readings were measured in a 1 cm, quartz cuvette using a Cary 100 UV-Visible spectrophotometer.

Liquid Chromatography and Mass Spectrometry

Non-reduced samples of ATF5 were prepared at a concentration of 0.2 mM in 100 mM ammonium bicarbonate buffer, pH 6.0. In order to prepare reduced samples, 5 mM DTT was added to the non-reduced stock and the solution was heated at 37°C for 20 minutes prior to injection on the column. Microbore HPLC/MS experiments were performed to determine the mass of the ATF5 product and investigate the existence of intermolecular disulfide bonds. The HPLC step was used to desalt and separate proteins chromatographically (Waters Acquity). Solvents A and B contained 99% H_2O , 1% CH_3CN and 99% CH_3CN , 1% H_2O , respectively, and both contained 0.08% formic acid. Separations were performed on a 1 mm ID $\times$ 5 cm long C4 reverse phase column (Vydac C4, 300Å pore size, 3.5 μM particles packed by MicroTech Scientific, Vista, CA) at 110 $\mu\text{L}/\text{min}$. The gradient was held at 1% B for 3 min, then ramped to 23% B by 4 min, 45% by 14 min, and 90% B by 15 min. Partial resolution of the ATF5 monomer from dimer in the non-reduced sample was observed near 28 % B with this gradient.

Electrospray ionization (ESI) spectra were acquired on a Q-ToF-2 (Micromass Ltd, Manchester UK) hybrid mass spectrometer operated in MS mode and acquiring data with the time of flight analyzer. The instrument was operated with analyzer settings optimized for maximum sensitivity while infusing a 50% organic/water solution of lysozyme at the flow rate of the chromatography. The cone and collision cell voltages (35 and 20V, respectively) were a

compromise to maximize sensitivity and keep the M-H₂O/NH₃ ion below 20% of the primary ion in each charge state cluster. Argon was admitted to the collision cell using 16 psi on the supply regulator or 5.3×10^{-5} mBar on a penning gauge near the collision cell. Spectra were acquired at an 11,364 Hz pusher frequency, covering the mass range 800 to 3000 u and accumulating data for 5 seconds per cycle. Time to mass calibration was made with CsI cluster ions acquired under the same conditions. The resulting suite of charge states in the ESI spectrum were subject to charge state deconvolution to present a “zero” charge mass spectrum using the Transform or MaxEnt1 routine in MassLynx software.

NMR Spectroscopy

Two-dimensional ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) spectra were recorded at 25°C using a Bruker AVANCE 800 MHz spectrometer equipped with a triple-resonance CRYO-probe with pulse field gradients. Samples were prepared at a concentration of 0.2 mM in 50 mM sodium phosphate, pH 6.5, containing 100 mM NaCl, 5 mM DTT and 5% D₂O. Water suppression was accomplished using flip-back pulses. Data were acquired in 128 scans with 2048 points in ¹H and 128* increments in ¹⁵N. ¹H chemical shifts were referenced with respect to an external DSS standard in D₂O [21]. Indirect referencing relative to ¹H was determined for ¹⁵N, assuming a ratio of ¹⁵N/¹H = 0.101329118 [21]. Data were processed using NMRPipe and Sparky software [22,23].

Circular Dichroism Spectroscopy

Data were collected on a Jasco J-720 spectropolarimeter equipped with a Peltier temperature control unit. Samples consisted of 20 μM protein in 50 mM sodium phosphate buffer, pH 6.5, and 100 mM NaCl. Spectra were acquired at 10°C using a quartz cuvette with a 1 mm path length. Scans were collected in the range of 190 to 260 nm, a data pitch of 1 nm, a band width of 5 nm, a scanning speed of 50 nm/min and a response time of 8 seconds. Sample spectra were corrected by subtraction of the buffer spectrum, and molar ellipticity was calculated using a molecular weight of 9.33 kDa.

Electromobility Shift Assay

The DNA binding activity of the bZIP domain of ATF5 was determined using a 20 base-pair, double-stranded oligonucleotide probe containing the cyclic AMP response element (CRE) binding motif (5'-TAAAAATGACGTCATGGTAA-3'). The forward primer was modified at its 5' end with a 6FAM fluorescent tag for detection (IDT). The forward and reverse strands were preannealed to generate double-stranded DNA by heating at 90°C for 5 minutes before cooling to room temperature over the next 11 minutes. A 2 μL volume of a 1 mM stock of purified ATF5 was combined with 2 μL of a 1 μM stock CRE DNA containing the fluorescent tag in 12 μL of binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA 1 mM DTT, 5 mM MgCl₂, 10% (v/v) glycerol) and incubated at room temperature for 15 minutes. As a control, 100-fold excess of preannealed unlabeled CRE DNA was added to ATF5 as a competitive inhibitor in the above reaction. After the addition of loading buffer, 10 μL of each sample was loaded onto a 20% nondenaturing polyacrylamide gel. The gel was run at 100 V for one hour and the 6FAM-labeled DNA was detected using a Typhoon Trio Variable Mode Imager (Amersham Biosciences). Excitation of the fluorophore was done at a wavelength of 488 nm and emission was detected at 520 nm.

Results

Expression and purification of GST-tagged ATF5

The initial attempt to obtain the bZIP domain of ATF5 involved production of it in a fusion construct with a N-terminal GST-tag, as previously reported [17]. SDS-PAGE analysis of pre-

and post-induction samples demonstrated successful expression of this protein, as detected by the presence of a band at 38 kDa, which is the expected molecular weight for this construct (Figure 1A). Although the majority of recombinant protein was insoluble following cell lysis (Figure 1B, lane 9), we isolated the soluble fraction (lane 1) by centrifugation, which contained approximately 9 mg GST-ATF5. Affinity chromatography then was performed on the soluble fraction using a GSTrap column. Isolation of the fusion construct from other soluble proteins was achieved using this method; however, extensive secondary proteolysis occurred during the purification. The initial cell lysate contained a 38 kDa band (Figure 1B, lane 1), which corresponds to the full-length fusion protein, whereas the eluate (lane 5) lacked this band and instead contained a band at approximately 35 kDa. Based on the change in molecular weight of these observed bands, proteolytic cleavage does not correspond to release of the 10 kDa ATF5 bZIP protein. Subsequent attempts to specifically cleave the GST tag from ATF5 using Factor Xa were unfruitful, as demonstrated by the lack of a band near the expected molecular weight for the full-length ATF5 bZIP domain or a partially cleaved 7 kDa fragment (Figure 1B, lane 7). Moreover, the intensity of the band at 35 kDa did not decrease to an appreciable extent following incubation with Factor Xa (Figure 1B, lane 7), suggesting that access to the cleavage motif may be blocked. After completing the chromatographic separation, the GSTrap resin was washed with 1% SDS to remove any residual proteins from the column. This fraction contained a large amount of GST-ATF5 (Figure 1B, lane 8), which suggests the protein had aggregated on the column or formed large oligomers that were not effectively competed off the resin during the elution step.

Most of the expressed fusion protein was located in the insoluble cellular fraction (Figure 1B, lane 9), suggesting that the ATF5 bZIP protein may be poorly soluble or prone to aggregation. Aggregate formation in solution also could limit access to the Factor Xa cleavage site, resulting in retention of the apparent higher molecular weight bands. Because much of the GST-tagged ATF5 was insoluble following cell lysis and efforts to cleave the tag from the soluble protein were unsuccessful, an alternative approach was devised to obtain ATF5. Because GST (not fused to ATF5) is present in the soluble portion of the cell lysate, we expected that untagged ATF5 would appear in the pellet. Many proteins that are directed to inclusion bodies can be recovered from the insoluble pellet using a denaturation and refolding procedure [18]. We decided to investigate whether improved yields of the ATF5 bZIP protein could be achieved by purifying an untagged version from this insoluble fraction.

Expression and refolding of untagged ATF5

Analysis of the SDS-PAGE data revealed that a high level of expression of the untagged form of ATF5 was achieved from this construct (Figure 2A). Anomalous behavior was observed for this protein when evaluated using SDS-PAGE analysis. The protein runs more slowly than would be expected, likely due to its basicity and overall highly positive charge (pI=10.07). This behavior has been observed with ATF5 before and with other highly basic proteins [17, 24–26]. To confirm the band at approximately 12 kDa corresponds to ATF5, mass spectrometric data was collected (Figure 3). The MS spectrum shows a peak at 9333.7 u, which provides confirmation that the product obtained has the expected mass calculated for ATF5 bZIP.

The cells containing untagged ATF5 were lysed and centrifuged using the same conditions and procedure as described above. SDS-PAGE revealed that the majority of ATF5 protein is contained in the pellet following lysis. ATF5 was extracted efficiently from the insoluble pellet when incubated with a high concentration of the denaturant guanidine and the reducing agent DTT (data not shown). The protein refolded as the denatured solution was diluted drop wise into a large volume of non-denaturing buffer. Samples from each step in the purification process could not be analyzed by SDS-PAGE, because this method is incompatible with the use of

guanidine HCl. There was some precipitation or turbidity observed during the refolding step, which could be due to the presence of lipids and/or membrane proteins that did not refold upon dilution. This insoluble material was successfully pelleted by centrifugation following refolding, and the clarified supernatant was decanted and dialyzed. The dialysis step, which facilitated a 50-fold reduction in the guanidine concentration, resulted in some visible precipitation. The precipitate was removed by centrifugation prior to concentration of the final material. Initial attempts at refolding were conducted in Tris buffer at pH 7.5. Under these solutions conditions, the protein was found to be highly prone to aggregation as detected by visual observation of large amounts of precipitation. Performing the refolding step in phosphate buffer at pH 6.5, however, allowed for greater protein stability and increased solution concentrations to be achieved. More extensive studies are underway to determine the importance of the pH and buffer choice in protein solubility and stability, which will be reported separately.

Using phosphate buffer, our scheme for obtaining untagged ATF5 from the insoluble cellular fraction yielded approximately 8 mg protein from a 1 L culture, as determined by an A₂₈₀ absorption reading (Table 1). Densitometry was performed to quantify the total amount of ATF5 produced and the percent purity of the final product (Figure 2B). Approximately 45 mg of total protein were present in the whole cell lysate derived from 1 L culture, and 20% (or 9 mg) of this corresponds to ATF5 bZIP (Table 1). The final yield of purified ATF5 bZIP was 8 mg, which in relation to the total amount produced is 89%. This methodology not only allowed for a very high yield of purified product, but using these conditions concentrations of ATF5 in excess of 0.2 mM (or 2 mg/mL) could be achieved, which permits study of the protein using multi-dimensional NMR analysis.

Mass Spectrometric Analyses

Analysis of a non-reduced sample of ATF5 indicated the presence of both monomeric (average theoretical mass = 9333.4) and covalently-linked dimeric (average theoretical mass = 16664.8) species (Figure 3A). Reduction of ATF5 by DTT resulted in loss of signal from the dimer and a concomitant increase in signal from the monomer (Figure 3B). SDS-PAGE performed in the absence of DTT or any reducing agent further confirmed the presence of a mixture of monomeric and dimeric species in the refolded ATF5 sample (Figure 3C). Addition of DTT to the sample followed by SDS-PAGE produced a single band corresponding exclusively to the monomer. The apparent molecular weight of the dimer is 24 kDa, which is consistent with the migration of the monomeric species at approximately 12 kDa. The SDS-PAGE results indicate an intermolecular disulfide bond forms readily in solution. Densitometry performed on the SDS-PAGE gel shown in Figure 3 reveals a ratio of 1.0:1.7 monomer:dimer.

NMR Analysis

Purified ¹⁵N-labeled ATF5 bZIP was examined using two-dimensional, heteronuclear NMR to verify that the protein can be detected by the spectrometer and is not aggregated when prepared at high concentration, as this would impede further structural investigations using solution NMR. A spectrum of the reduced ATF5 sample was acquired using the ¹⁵N-HSQC experiment, because further evaluation of protein structure using NMR often relies on HSQC-based experiments. The ¹⁵N-HSQC selectively detects NH pairs and correlates each ¹H to the directly attached ¹⁵N. This spectrum is considered a “fingerprint” of the protein, because chemical shifts reflect the unique environment encountered by each nucleus in the backbone in an organized structure. A well-behaved globular protein will have good signal dispersion, typically ranging from 6 to 12 ppm on the ¹H axis and 100 to 140 ppm on the ¹⁵N. This dispersion results from the presence of stable structural features that confine the amide groups of the constituent amino acids to unique chemical environments within the folded protein. As such, more disordered proteins typically have peaks clustered more closely around random coil

values (~8.3 ppm in ^1H), because on average the nuclei experience more similar chemical environments in solution than in the structured protein [27–31]. The HSQC spectra of proteins composed of only a single helix, like the ATF5 bZIP monomer, are difficult to interpret without additional data, because in a standard alpha helix, all the amides are in similar environments. Their environment, however, can be altered upon dimerization, and the presence of peaks in more unusual positions can imply coiled-coil formation [16,32–36].

The ^1H - ^{15}N HSQC spectrum of reduced ATF5 suggests that the protein is predominantly in a monomeric, homogeneous state under the conditions used (Figure 4). Limited chemical shift dispersion is observed in the spectrum, as the majority of peaks appear between 7.9 and 8.5 ppm on the ^1H axis (see black peaks in Figure 4). The narrow range of peak positions is consistent with the presence of a species that is composed of a single helix and/or is unstructured [27–31]. Side chain NH_2 moieties from Asn and Gln residues produce a pair of ^1H peaks corresponding to a single ^{15}N value, which are observed at approximately 112 ppm. In the spectrum of ATF5, all the side chain amides have nearly equivalent chemical shifts to each other and the free amino acids, suggesting all are equally solution exposed. While this single NMR spectrum of ATF5 cannot be used to distinguish regions of α -helix from random coil, it does indicate that the protein exists largely in a homogeneous state. Increased signal dispersion is, however, observed closer to the noise (see red peaks in Figure 4). The peaks highlighted in red are visible only at a 10-fold lower signal-to-noise ratio than the peaks shown in black. The red peaks near 111 ppm on the ^{15}N axis and also those at larger ^{15}N values between 8.3 and 7.4 ppm on the ^1H axis are characteristic of higher order structure. Among these peaks is a pair that corresponds to an NH_2 side chain (111.1 ppm ^{15}N , 6.75 and 7.46 ppm ^1H). The presence of these peaks indicates that the environment around one of the Asn or Gln side chains is altered in the more structured state by participating in a protein-protein interaction. Overall, the red peaks constitute only a small percentage of the total peak intensity (less than 10%) in the ATF5 spectrum, suggesting that even at 0.2 mM concentration a minimal amount of higher-order structure is present.

Circular Dichroism Analysis

CD was performed on ATF5 bZIP in order to determine the secondary structure. Analysis of the CD spectrum of the purified bZIP domain of ATF5 shows the presence of α -helical structure, evident in the double minima absorption observed at both 208 and 222 nm (Figure 5). CD data can be used to quantify the relative amounts of secondary structure in a protein. A simple calculation using the following equation can be performed to estimate the α -helical content:

$$[\theta]_{222} = -30,300f_H - 2340,$$

where $[\theta]_{222}$ is the mean residue ellipticity at 222nm and f_H is the fraction of α -helical content in the protein [37,38]. This equation has been applied to other bZIP domains as a means of approximating the percent of alpha helix composing the structure [39]. Using this method, the calculated percent helix in ATF5 bZIP is 27%. The estimate equates to the involvement of 21 residues per monomer in helical structure. A more accurate quantitative analysis requires the inclusion of the data from 190 to 260 nm. Absorption data for this entire range could not be obtained for ATF5. Data between 190 and 200 nm is not shown because measurement in this region is unreliable under the conditions required for ATF5 analysis. Absorption from buffer components interferes with the data collection, and rigorous quantitative analysis cannot be performed because signal from the ATF5 protein cannot be deconvoluted.

DNA Binding Analysis

The DNA binding activity of ATF5 was examined using an electromobility shift assay (Figure 6). Because it was previously reported that ATF5 binds to the CRE motif, we incubated the

purified bZIP domain with fluorescently labeled double-stranded oligonucleotide DNA containing this motif to verify the protein functions as expected [17]. A shift in the electrophoretic mobility of the CRE DNA is observed in the presence of ATF5. As expected the DNA: protein complex migrates more slowly (Figure 6, lane 2) than that of the DNA alone (lane 1). Addition of 100-fold excess unlabeled CRE DNA results in restoration of the band corresponding to unbound labeled CRE (lane 3). This indicates that binding is reversible because unlabeled CRE effectively competes with labeled CRE for ATF5 binding.

Discussion

The results presented here show for the first time that the bZIP domain of ATF5 can be expressed and purified in sufficient quantity, concentration and homogeneity to facilitate assay development and structural analyses. Our refolding procedure for isolating ATF5 is simple and involves limited introduction of surfaces that could initiate aggregation or adsorption events, which is important because the protein appears prone to aggregation. The procedure is efficient, resulting in a very high yield of soluble protein in which the final product is sufficiently pure to permit spectroscopic or structural analysis.

Most reports of bZIP proteins indicate that these domains are unstructured or weakly helical and monomeric in solution and their helicity is stabilized by DNA binding via coiled-coil formation. An exception to this was reported in two separate studies on GCN4 that provided evidence that monomeric and dimeric species can be helical in solution and both are able to bind DNA [40]. Our data most closely resembles that of GCN4. The CD results for reduced ATF5 indicate approximately one-third of the bZIP domain is alpha helical in solution in the absence of DNA, while the NMR spectrum lacks the dispersion typically associated with coiled-coil formation. In the expressed protein, approximately 45% of the sequence constitutes the basic DNA binding segment while the other 55% is expected to compose the leucine zipper. The DNA binding region is expected to be in a random coil conformation in the absence of DNA. The remaining residues typically are helical in the coiled-coil form, which equates to 43 residues in our construct. Our CD data shows that approximately half of the predicted zipper region (21 residues) is helical in solution. The comparatively low helical content likely results because the last two typically conserved Leu positions in the zipper region are occupied by valine residues in ATF5. Approximately 90% of the peaks in the NMR spectrum (black in Figure 4) are within a narrow range, which is consistent with the presence of a monomer. If ATF5 exists predominantly as a coiled-coil dimer, the chemical shifts in the NMR spectrum would be expected to appear in a broader range than is observed. The fact that the NMR data was acquired at a 10-fold greater protein concentration negates the possibility that there is any coiled-coil in the CD sample and indicates that the monomer is helical. The subset of more dispersed peaks in the NMR spectrum, which have weaker intensity, suggest that only a minor amount of homodimer is present at the higher protein concentration. An additional recent study of GCN4 provides good evidence to support this conclusion, as it identified the presence of an intermediate form of the GCN4 protein (x-form) that is monomeric yet retains helical structure in the absence of DNA [16]. This study further describes a subset of more distributed NMR peaks, which reflect the coiled-coil structure. The black peaks in our spectrum of ATF5 are consistent with ATF5 being in the x-form, while the red peaks resemble those observed for the coiled-coil form in the GCN4 study. The pair of NH₂ peaks shown in red further lends support to the idea that these peaks reflect dimer formation, because in other bZIP domains a conserved Asn is embedded in the dimer interface [10]. This residue is in the **a** position in the helix, which directly participates in the “knob and hole” packing arrangement composing the coiled-coil [41]. It is most likely that the shifted NH₂ peaks correspond to the analogous position in ATF5, which is occupied by Asn245. Peak assignment and structure determination will be required to test this hypothesis.

The bZIP family proteins are known to homo- and heterodimerize selectively [7–13]. It is this selective dimerization that helps impart DNA binding specificity to these transcription factors. In many cases, bZIP proteins have been shown to be unstructured in the absence of DNA but adopt structure in the DNA-bound form [7,13,39,42–44]. While ATF5 appears to lack higher order structure in solution, because ATF5 can bind specifically to the CRE DNA motif in the absence of another protein, DNA binding may induce formation of a coiled-coil homodimer. ATF4, the closest homolog of ATF5 at 74% sequence homology, has been shown to homo- and heterodimerize [45]. Currently, a high-resolution structure of a homodimer of ATF4 or ATF5 is not available, but a crystal structure of a heterodimer involving ATF4 has been reported [40]. Based on strong sequence identity, this structure can be used to inform our understanding until a structure of ATF5 is completed. In the reported structure ATF4 forms a heterodimeric coiled-coil with CCAAT Enhancer-binding Protein β (C/EBP β). The ATF4 helix is unusually straight, and heterodimerization is accomplished because C/EBP β is able to wrap around ATF4. The rod-like conformation observed for ATF4 in the heterodimeric complex seems incompatible with homodimer formation, suggesting an alternative structure must be employed by the homodimer to accomplish DNA binding.

Disulfide bond formation commonly is considered to be an artifact of *in vitro* experiments, and this may be the case in our studies. However, in recent years data has been accumulating to support that oxidation and reduction of many proteins regulates their activity *in vivo* [46–52]. Furthermore, it has been previously reported that DNA binding of the Fos/Jun bZIP heterodimer is modulated by redox regulation of a conserved cysteine residue [53]. Examination of the ATF4 structure reveals the Cys involved in homodimeric cross-linking is positioned at the interface of the coiled-coil structure. A cysteine is present in the analogous position in the bZIP domain of ATF5, and this is the only Cys present in either protein as expressed. In the ATF4-C/EBP β structure paper the authors noted the need to use a mutant of the ATF4 protein in which the cysteine residue in the center of the bZIP domain was mutated to alanine in order to prevent precipitation and obtain the crystal structure of the heterodimer. Likewise, inclusion of reducing agent during refolding helped minimize precipitation of ATF5. This difference in solubility indicates that cross-linking alters the structure of these transcription factors. Our solution studies show that the physical association of ATF5 monomers is weak, with little coiled-coil structure observed even at high protein concentration. If the homodimer has a function *in vivo*, then obviously cross-linking would greatly stabilize the interaction. In ATF5 the intermolecular disulfide bond forms readily in the absence of reducing agents and is slowly reversible with the addition of 1000-fold excess of the thiol-modulating compound DTT. Because ATF5 is upregulated in response to oxidative stress [54], it is tempting to speculate that this intermolecular disulfide bond may influence DNA binding and/or have functional significance in transcriptional regulation. The research presented here provides a basis for conducting a more extensive investigation into how this disulfide linkage affects the structure of the ATF5 dimer and may influence its interaction with DNA.

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Figure 1.

(a) Coomassie stained SDS-PAGE showing expression of GST-ATF5 bZIP. Lane 1: *E. Coli* lysate pre-IPTG induction, Lane 2: *E. Coli* lysate post-IPTG induction, Lane 3: molecular weight marker (Bio-Rad Precision Plus Unstained MW Standard). (b) Coomassie stained SDS-PAGE gel showing fractions from the purification of GST-ATF5 bZIP using GSTrap affinity chromatography. Lane 1: supernatant following cell lysis, Lane 2: column flow-through, Lane 3: rinse fraction, Lane 4: elution fraction #1, Lane 5: elution fraction #2, Lane 6: elution fraction #3, Lane 7: Eluate after dialysis, reaction with Factor Xa and incubation with GSTrap beads, Lane 8: GSTrap beads containing residual protein post GST removal, Fraction 9: insoluble pellet after cell lysis, Fraction 10: molecular weight marker (Bio-Rad Precision Plus Unstained MW Standard).

Figure 2.

(a) Coomassie stained SDS-PAGE showing expression of ATF5 bZIP. Lane 1: *E. Coli* lysate pre-IPTG induction, Lane 2: *E. Coli* lysate post-IPTG induction, Lane 3: molecular weight marker (Bio-Rad Precision Plus Unstained MW Standard). (b) Coomassie stained SDS-PAGE gel showing lysis fractions and purified ATF5 bZIP. Lane 1: molecular weight marker (Bio-Rad Precision Plus Unstained MW Standard), Lane 2: lysis supernatant, Lane 3: insoluble pellet after lysis, Lane 4: purified ATF5 bZIP domain.

Figure 3.

(a) Transformed mass spectrum of non-reduced ATF5 bZIP domain. (b) Transformed mass spectrum of reduced ATF5 bZIP domain. (c) Coomassie stained SDS-PAGE gel of purified ATF5 bZIP. Lane 1: molecular weight marker (Bio-Rad Precision Plus Unstained MW Standard), Lane 2: reduced sample of ATF5, Lane 3: Non-reduced sample of ATF5. Note: The non-reduced sample was run on the same gel as the reduced sample but with several lanes in between the two to prevent diffusion of the reducing agent into the non-reduced lane. The intermediate lanes were removed from the figure above and the two sample lanes were placed adjacent one another to allow for better comparison.

Figure 4. 2D ^1H - ^{15}N HSQC spectrum of 0.2 mM ^{15}N -labeled ATF5 in 50 mM sodium phosphate, pH 6.5, containing 100 mM NaCl and 5 mM DTT. Peaks in black are observed at S/N ratio = 1000:1. Peaks in red were overlaid onto the spectrum in black and are observed only at signal/noise ratio = 100:1.

Figure 5. Circular dichroism absorption spectrum of the bZIP domain of ATF5 at 20 μ M concentration in 50 mM sodium phosphate, pH 6.5, containing 100 mM NaCl and 5 mM DTT. The double minimum at 208 and 222 nm indicates α -helix is present in ATF5.

Figure 6.

Native PAGE showing the electrophoretic mobility of fluorescently tagged CRE DNA in the absence and presence of ATF5. Lane 1: dsCRE DNA, Lane 2: dsCRE DNA in the presence of ATF5, Lane 3: dsCRE DNA in the presence of ATF5 and 100-fold molar excess of unlabeled dsCRE DNA.

Table 1

Total protein (mg) Whole Cell Lysate	Amount Expressed ATF5 (mg)	Final Amount Purified ATF5 (mg)	Final Yield Protein (%)
45	9	8	89