

Insights from the crystal structure of the chicken CREB3 bZIP suggest that members of the CREB3 subfamily transcription factors may be activated in response to oxidative stress

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Received 26 November 2018; Accepted 14 January 2019

DOI: 10.1002/pro.3573

Published online 17 January 2019 proteinscience.org

Abstract: cAMP response element binding Protein 3 (CREB3) is an endoplasmic reticulum (ER) membrane-bound transcription factor, which belongs to the basic leucine zipper (bZIP) superfamily of eukaryotic transcription factors. CREB3 plays a role in the ER-stress induced unfolded protein response (UPR) and is a multifunctional cellular factor implicated in a number of biological processes including cell proliferation and migration, tumor suppression, and immune-related gene expression. To gain structural insights into the transcription factor, we determined the crystal structure of the conserved bZIP domain of chicken CREB3 (chCREB3) to a resolution of 3.95 Å. The X-ray structure provides evidence that chCREB3 can form a stable homodimer. The chCREB3 bZIP has a structured, pre-formed DNA binding region, even in the absence of DNA, a feature that could potentially enhance both the DNA binding specificity and affinity of chCREB3. Significantly, the homodimeric bZIP possesses an intermolecular disulfide bond that connects equivalent cysteine residues of the parallel helices in the leucine zipper region. This disulfide bond in the hydrophobic core of the bZIP may increase the stability of the homodimer under oxidizing conditions. Moreover, sequence alignment of bZIP sequences from chicken, human, and mouse reveals that only members of the CREB3 subfamily contain this cysteine residue, indicating that it could act as a

Abbreviations: α , Alpha.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Wellcome Trust 075491/Z/04 204703/Z/16/Z; Grant sponsor: Diamond Light Source.

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redox-sensor. Taken together, these results suggest that the activity of these transcription factors may be redox-regulated and they may be activated in response to oxidative stress.

Keywords: CREB3; bZIP; crystallographic structure; homodimeric bZIP unbound to DNA

Introduction

cAMP response element binding Protein 3 (CREB3) is an endoplasmic reticulum (ER) membrane-bound transcription factor, which plays a role in the ER-stress induced unfolded protein response (UPR).¹ It has been shown to be involved in the ER-associated degradation (ERAD) pathway, which is regulated by the UPR.¹⁻⁴ CREB3 also plays a role in Golgi-stress response,⁴ cell proliferation and migration,^{5,6} tumor suppression,⁷ and inflammatory gene expression^{8,9} and has been shown to be a cellular target of viruses such as Herpes Simplex Virus (HSV), Hepatitis C Virus (HCV), and Human Immunodeficiency Virus (HIV).^{7,10,11}

CREB3 belongs to the CREB3 subfamily of the basic leucine zipper (bZIP) superfamily of transcription factors.¹² bZIP transcription factors bind target DNA sites as homodimers or heterodimers and are characterized by the conserved bZIP domain, composed of a basic region that recognizes a specific DNA sequence and a leucine zipper region that facilitates dimerization.^{1,13} CREB3 is activated by regulated intramembrane proteolysis (RIP) in response to various stimuli including ER- and Golgi-stress.^{1,14} Upon stimulation, the ER-bound factor is transported to the Golgi apparatus where it is sequentially cleaved by Site 1 protease (S1P) and Site 2 protease (S2P) to liberate the N-terminal fragment, comprising the transcription activation and bZIP domains. The released N-terminal fragment translocates to the nucleus to activate transcription of target genes.^{1,14} The sequences recognized by CREB3 include the c-AMP response element (CRE), ER stress responsive element II (ERSE-II), and unfolded protein response (UPR) element (UPRE).¹

Although CREB3 is an important, multifunctional cellular factor, no crystal structures are available for CREB3. We here report the crystal structure of the homodimeric chicken CREB3 (chCREB3) bZIP in the DNA-free form, determined to a resolution of 3.95 Å. Insights from the structure of the chCREB3 bZIP suggest that members of the CREB3 subfamily of bZIP transcription factors have a putative redox-sensitive cysteine in the leucine zipper region, hence the activity of these transcription factors may be redox-regulated.

Results and Discussion

chCREB3 bZIP forms a stable homodimer with a structured DNA-binding region

We identified the bZIP domain of chCREB3 (Residues 211–274) using the Uniprot database ([\[uniprot.org\]\(http://www.uniprot.org\)\). For crystallization studies, we cloned the region encompassing Residues 206–280 of chCREB3 into the pOPINF expression vector,¹⁵ which harbors an N-terminal His6-3C cleavable tag \(MAHHHHHHSSGLEVLFFQGP\). We expressed the His-tagged protein \(with a predicted molecular weight of ~11.1 kDa\) in *Escherichia coli* Rosetta 2\(DE3\) pLysS cells and purified it by affinity chromatography followed by size exclusion chromatography \(SEC\). The SEC elution profile displayed three unresolved peaks \[Fig. 1\(A\)\]. The bZIP protein was observed in all fractions under the peaks, as determined by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis \(SDS-PAGE\) \[Fig. 1\(A\)\]. To determine the composition of the fractions, we assessed selected samples without boiling by non-reducing SDS-PAGE. These fractions consisted predominantly of dimeric species \[Fig. 1\(A\)\]. These results suggested the presence of a potential inter-molecular disulfide bridge between chCREB3 bZIP monomers, promoted by the cysteine residue \(Cys 246\) in the leucine zipper region. We pooled all fractions containing the protein and performed crystallization trials without target DNA and yielded diffraction-quality crystals that allowed the structure determination.](http://www.</p></div><div data-bbox=)

We determined the crystal structure of the homodimeric chCREB3 bZIP, in the DNA-free form, to a resolution of 3.95 Å [Fig. 1(B)], by molecular replacement. There are three and a half dimers per asymmetric unit (with one dimer sitting on a crystallographic three-fold axis). The structure reveals chCREB3 is a stable homodimer even in the absence of DNA. The chCREB3 bZIP forms a continuous α -helix in which the C-terminal leucine zipper region forms a parallel coiled-coil dimerization interface. The N-terminal basic region of most bZIP transcription factors is largely disordered in the absence of DNA.¹⁶ However, the chCREB3 bZIP possesses a structured N-terminal basic region even when unbound to target DNA. This feature has been previously observed in the crystal structure of the heterodimer formed by the bZIP domains of activating transcription factor (ATF) 4 and CCAAT box/enhancer-binding protein β (C/EBP β).¹⁷ In this structure, the basic region of C/EBP β is disordered, whereas the basic region of ATF4 adopts a stable helical conformation. Podust et al. proposed that the presence of a structured α -helical DNA-binding region could act to augment the DNA-binding specificity of a bZIP transcription factor. The basic region of most bZIP proteins is largely unfolded in the DNA-unbound state.¹⁶ Upon binding to DNA, the unfolded basic region becomes α -helical, bringing into position the DNA-binding residues that are essential

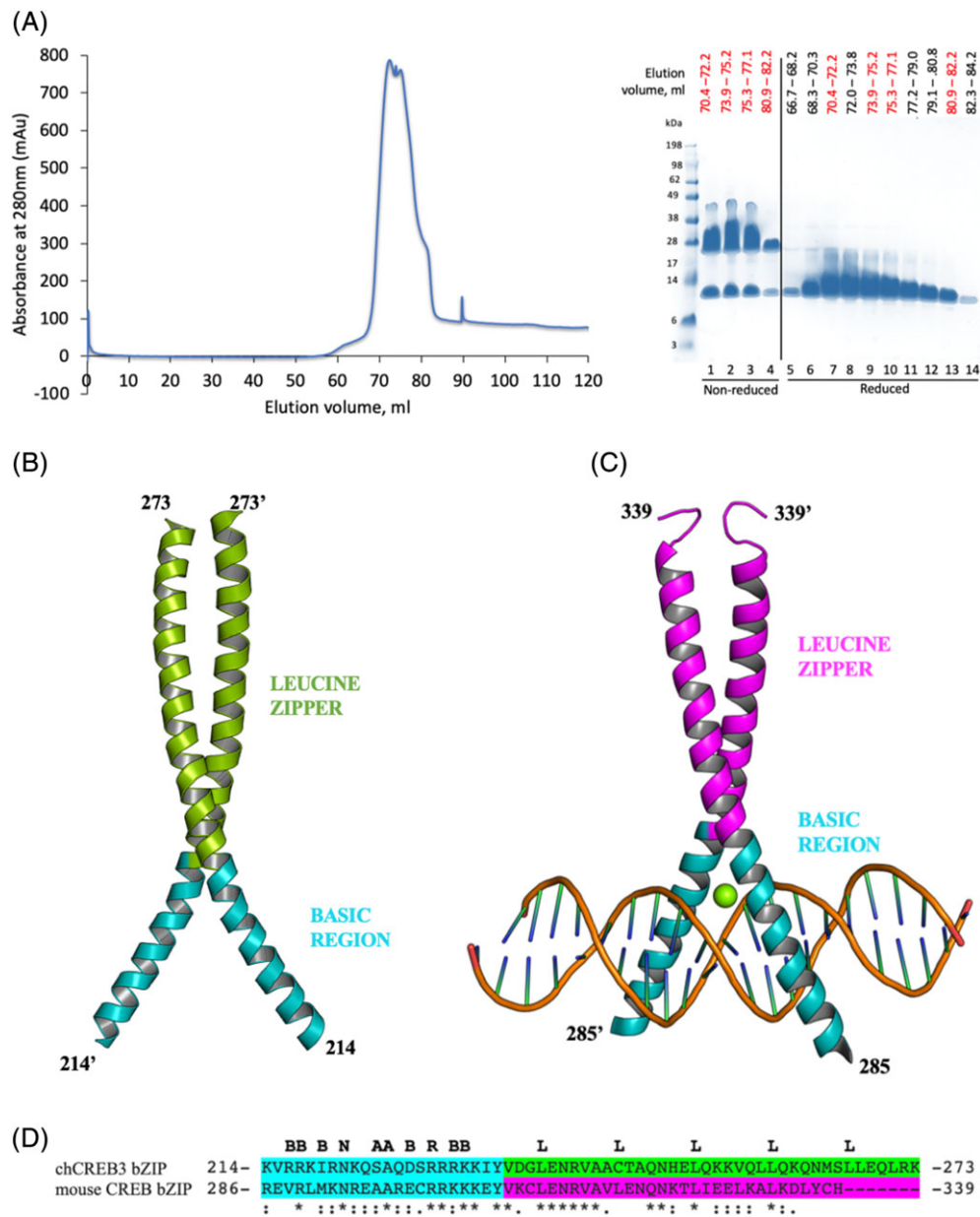


Figure 1. The bZIP domain of chCREB3 forms a stable homodimer with a structured basic region even in the absence of DNA. (A) *Left:* SEC chromatogram of the His tagged chCREB3 bZIP (~11.1 kDa) analyzed on a HiLoad 16/60 Superdex 75 column (GE Healthcare) in 50 mM Tris-HCl pH 8, 150 mM NaCl. *Right:* selected samples under the peaks were analyzed by non-reducing SDS-PAGE (Lanes 1–4). All fractions containing protein were analyzed by reducing SDS-PAGE (Lanes 5–14). The Coomassie blue-stained gel is shown here. Samples analyzed by both reducing and non-reducing SDS-PAGE are labeled red. Samples were resolved on a 10% Bis-Tris gel (Invitrogen), which was run in 1× 2-(N-morpholino) ethanesulfonic acid (MES) buffer (Invitrogen), alongside the SeeBlue[®] Plus2 Prestained Protein Standard, Invitrogen. (B) Cartoon representation of the chCREB3 bZIP homodimer showing the structured basic region (cyan) and the leucine zipper region (green). (C) Structure of the CREB bZIP bound to the somatostatin CRE and a hexahydrated Mg²⁺ ion (PDB code: 1DH3) (D) Amino acid sequence alignment of chCREB3 (cyan/green) and mouse CREB (cyan/magenta) bZIP sequences. The basic region residues are highlighted in cyan and the leucine zipper residues are highlighted in green or magenta. The consensus sequence is shown above the sequence (B = any basic residue; N = asparagine; A = alanine; R = arginine; L = leucine). An * (asterisk) indicates positions which have a single, fully conserved residue. The bZIP domain of mouse CREB shares 36% amino acid sequence identity with the bZIP domain of chCREB3.

for sequence-specific interactions with target DNA.¹² Since structural disorder imparts plasticity, permitting more than one conformation to be adopted,¹⁸ disorder in the basic region of bZIP domains suggests that the bZIPs can adapt to interact with a broad range of sequences.¹⁷ In contrast, a well-defined

conformation would restrict interactions with those sequences that are not the specific target site, consequently imposing more stringent binding specificity.¹⁷ In support of this hypothesis, the crystal structure of the heterodimeric bZIP C/EBP β :ATF4 unbound to DNA reveals that most of the conserved DNA-binding

residues in the basic region of ATF4 are oriented such that they are in position for specific DNA (CRE) binding. This heterodimeric bZIP was also shown to bind the ATF-binding site (CRE element) with high affinity, but not the canonical C/EBP β DNA site (CCAAT box DNA),¹⁷ implying that the structured DNA binding region of ATF4 might be determining the binding specificity of this heterodimer.

In addition to imposing specificity, possessing an ordered DNA-binding region could also enhance the DNA-binding affinity of bZIP proteins. Previous studies have revealed an increase in helical content of bZIP proteins correlates with increased DNA-binding affinity.^{19–21} It has also been proposed that a high intrinsic helicity in the bZIP basic region could increase the overall stability of the bound protein–DNA complex, leading to low dissociation rates.²² The converse could be predicted for sequences with low intrinsic helicity.²² Taken together, this structural feature of the bZIP suggests that chCREB3 may interact with its specific target site, CRE element, with high affinity. Interestingly, the basic region of the chCREB3 bZIP adopts a similar conformation to the CRE–DNA bound homodimeric bZIP CREB [Fig. 1(B); PDB code: 1DH3].²³ The bZIP domain of CREB harbors three cysteine residues (Cys300, Cys310, and Cys337), one in the basic region and two in the leucine zipper region. For structural studies, these cysteine residues were mutated to Serine (Ser) to improve protein solubility.^{23–27} These serine mutations, however, have been demonstrated to not alter DNA binding activity of the bZIP.²⁴ The root-mean-square deviation (RMSD) between the DNA-unbound chCREB3 bZIP dimers (three copies non-crystallographic dimers in an asymmetric unit) and the DNA-bound CREB bZIP dimer was found to range between 0.9 and 1.4 Å. The angular separation of the basic regions in the chCREB3 homodimers (44°, 52°, and 61°) also overlapped the angular separation of the basic regions in the homodimeric bZIP CREB bound to DNA (48.0°).

Putative redox-sensitive cysteine in the leucine zipper region of the bZIP

A striking feature of the structure of the homodimeric chCREB3 bZIP is the presence of a disulfide bond in the hydrophobic core of the dimer interface (Fig. 2). bZIP family members form dimers through their characteristic leucine zipper helices which consist of seven-residue repeats of amino acids (denoted **a–b–c–d–e–f–g**). Hydrophobic residues occur preferentially in the **a** and **d** positions, with a variety of possible amino acids in the **a** positions and usually leucine in the **d** position.²⁸ Hydrophobic interactions by leucine and other hydrophobic amino acids in **a** and **d** positions in the helix form the hydrophobic core of the bZIP dimer, facilitating dimerization.²⁸ Interestingly, a cysteine residue (Cys 246) occupies the **d** position of the first heptad of the chCREB3 bZIP and forms a disulfide bond

with the cysteine from its dimeric partner. Difference electron density maps, where Cys 246 has been omitted from the refinement, show strong density features for the disulfide [Fig. 2(C)]. Mutational studies have demonstrated that amino acids in Positions **a** and **d** regulate leucine zipper oligomerization, dimerization stability, and specificity.²⁸ This cysteine residue replacing the consensus leucine at this position, by forming a covalent bond, likely enhances the dimer stability.

This is the first time a disulfide bond has been observed in a crystal structure of a bZIP domain, although previous studies have reported evidence for the presence of an intermolecular disulfide bond in the leucine zipper region of ATF4 and ATF5 bZIP homodimers. These proteins possess a cysteine residue at Position **a** of the first heptad in the bZIP region. Circular dichroism (CD) and nuclear magnetic resonance (NMR) studies of ATF4 and ATF5 bZIPs have indicated that in the absence of DNA, these bZIPs are only partially structured, are predominantly monomeric, and are not capable of forming stable homodimers, even in the presence of a disulfide bond.^{17,29} In contrast, our study reveals the bZIP domain of chCREB3 that has a cysteine residue at the first **d** position is able to form a stable homodimer, even in the absence of DNA. Taken together, these results suggest that an intermolecular disulfide bond at the first **d** position may play a role in promoting homodimerization and enhancing dimer stability. Moreover, Podust et al.¹⁷ and Ciaccio and Laurence²⁹ reported that the disulfide bond formation increased the α -helical content of the bZIP domains of ATF4 and ATF5 and suggested that an intermolecular disulfide bond could act to stabilize a coiled-coil. Based on these findings, this disulfide bond in the chCREB3 bZIP may be important for extending the stabilizing α -helical conformation from the leucine zipper region to the basic region.^{30–37}

Multiple sequence alignment analysis of bZIP domain sequences of chicken, human, and mouse bZIP transcription factors reveals that only members of the CREB3 subfamily (CREB3, CREB3L2, and CREB3L3 of the chicken CREB3 subfamily, CREB3L2, CREB3L3, and CREB3L4 of the human CREB3 subfamily and CREB3L2, CREB3L3, and CREB3L4 of the mouse CREB3 subfamily) contain a cysteine residue at Position **d** of the first heptad (Fig. S1). These results suggest that this cysteine, which is unique for the CREB3 subfamily, could act as a redox-sensor; hence, the activity of these transcription factors may be redox-regulated. Several transcription factors have been reported to contain redox-sensitive cysteine residues at their DNA-binding sites, which affect their DNA-binding ability.³⁸ In most cases, oxidation of critical cysteine residues negatively regulates the DNA-binding activity of these transcription factors.³⁸ Examples of factors whose DNA-binding activity is negatively regulated by oxidation include

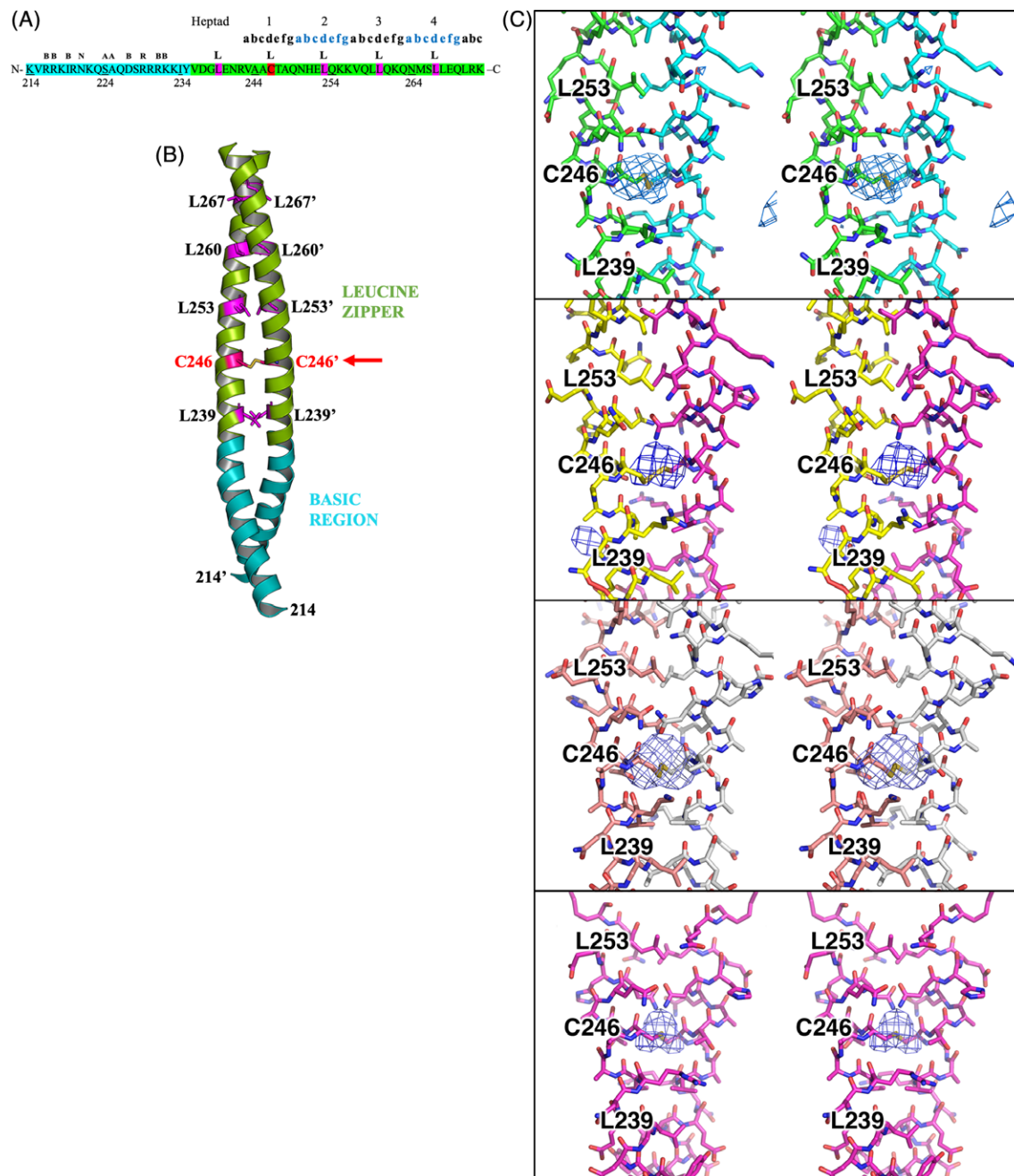


Figure 2. chCREB3 has a disulfide bond in the hydrophobic core of the bZIP. (A) The amino acid sequence of the chCREB3 bZIP composed of heptad repeats with the seven positions of the heptads labeled **a**, **b**, **c**, **d**, **e**, **f**, and **g**. The consensus sequence is shown above the sequence (B = any basic residue; N = asparagine; A = alanine; R = arginine; L = leucine). The leucine residues in Position **d** of the heptads are highlighted in pink. The cysteine residue (C246) at Position **d** of the first heptad is highlighted in red. (B) The main chains of the two peptide chains of the chCREB3 bZIP are represented as ribbons. The side chains of the leucine residues and the cysteine participating in the dimer association are represented as stick models. The position of the intermolecular disulfide bond is indicated by a red arrow. (C) Stereo figures of the $F_o - F_c$ difference electron density maps at 3.95 Å resolution, contoured at 3σ , for the four chCREB3 bZIP dimers, where Residue Cys 246 has been removed from the model, and crystallographic refinement has been rerun using the initial model. Maps are drawn with a radius of 10 Å centered around Cys 246. The final panel shows the dimer sitting on the crystallographic two-fold.

the heterodimeric bZIP transcription factor Fos/Jun, the nuclear factor kappa B (NF- κ B), and hypoxia-inducible factor-1 α (HIF-1 α).^{39–42} In contrast, an example of a transcription factor whose DNA-binding activity is up-regulated by oxidative stress is HoxB5, a member of the Hox family of proteins.^{40,43} Oxidative

conditions trigger the dimerization of HoxB5, a structural reorganization required for the cooperative binding of this factor to tandem DNA target sites.^{40,43} A critical cysteine residue (Cys 232) located in the conserved homeobox encoded DNA-binding domain is essential for this redox regulation.^{40,43} The critical

cysteine of the chCREB3 bZIP is located downstream of the DNA-binding region and takes part in dimerization. Hence, as observed for HoxB5, the oxidation of this cysteine is likely to upregulate the DNA-binding activity of chCREB3 by promoting dimerization and structural changes required for DNA binding.

Based on the results of this study, we hypothesize that chCREB3 and other members of the CREB3 subfamily may be able to form stable or weakly associated homodimers depending on the oxidation state of the cell. During non-oxidizing conditions, lacking a disulfide bond, these bZIP factors may form weakly associated homodimers in the absence of DNA. We predict these weakly bound dimers to possess a largely unfolded DNA-binding region and the flexibility may enable them to bind a range of sequences, although the interaction with these sites may be weak. Under oxidizing conditions, the formation of a disulfide bond may enhance dimer stability and induce structural changes to the basic DNA-binding region of these bZIP factors, influencing target DNA interactions. These structural changes may augment DNA-binding specificity and affinity and could act to potentiate the activity of these factors. Further experiments will need to be performed to determine if the disulfide bond is indeed essential for promoting homodimerization stability and whether it is required for inducing structural changes to the basic DNA-binding region of the bZIP domain of chCREB3.

Materials and Methods

Protein expression and purification

The gene encoding the bZIP domain of chCREB3 (Residues 206–280) was cloned into the pOPINF expression vector,¹⁵ which harbors an *N-terminal His6-3C* cleavable tag (MAHHHHHHSS GLEVLFGQP). For protein expression, competent *E. coli* Rosetta 2(DE3) pLysS cells were transformed with the expression plasmid. Cultures were grown to an OD₆₀₀ of 0.5–0.6 at 37°C, induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown for 3 h at 37°C. For protein extraction, the pellets were resuspended in 50 mM Tris-hydrochloride (HCl) pH 8, 150 mM NaCl, 0.1% Tween 20, 1× Roche Complete EDTA free protease inhibitors and lysed by sonication. His-tagged protein was purified using Nickel–nitrilotriacetic acid (Ni-NTA) agarose and eluted with buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, and 300 mM Imidazole. The protein was further purified by size exclusion chromatography (SEC) using a Superdex 75 column (GE Healthcare) in 50 mM Tris-HCl, pH 8, 150 mM NaCl. All fractions containing the bZIP domain of chCREB3 were pooled and concentrated and used for crystallization.

Protein analysis

For denaturing SDS-PAGE, protein samples were mixed with 4× SDS sample loading buffer (250 mM

Tris pH 6.8, 8% w/v SDS, 0.02% w/v bromophenol blue, 40% v/v glycerol, 8% β-mercaptoethanol (BME)], heated to 99°C for 10 min, and spun down before loading. Samples were resolved on a 10% Bis-Tris gels (Invitrogen), which were run in 1× MES buffer (Invitrogen) using the NuPage Novex gel system (Invitrogen). Ten microliter of samples were loaded per well alongside the SeeBlue® Plus2 Pre-stained Protein Standard (3–198 kDa), Invitrogen.

Crystallization, data collection, and structure determination

The bZIP domain of chCREB3 was crystallized using the sitting-drop vapor-diffusion method in 96-well plates (CrystalQuick, Greiner Bio-One, Germany).⁴⁴ Drops were prepared using 100 nL protein solution mixed with 100 nL reservoir solution and were equilibrated against 100 μL precipitant solution. The concentration of the protein was 44 mg mL⁻¹. Clusters consisting of thin, rod-shaped crystals were obtained at 4°C with the reservoir condition 50% v/v 2-methyl-2,4-pentanediol (MPD), 100 mM Tri-sodium citrate pH 5.6, 10 mM magnesium chloride (MORPHEUS screen).⁴⁵ After optimization, diffraction-quality crystals were obtained from a drop containing 43% v/v 2-methyl-2,4-pentanediol

Table I. Data collection and Refinement statistics

<i>Data collection</i>	
Wavelength (Å)	0.98
Space group	C 2 2 2
Unit cell parameters	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	137.90, 167.06, 115.46
<i>α</i> , <i>β</i> , <i>γ</i> (°)	90.00, 90.00, 90.00
Resolution (Å)	51.63–3.95 (4.09–3.95) ^a
CC [1/2]	0.93 (0.76)
<i>R</i> _{meas}	0.30 (1.80)
Total no. of reflections	77770 (5573)
Unique reflections	12046 (887)
Mean [(<i>I</i>)/σ(<i>I</i>)]	3.30 (1.00)
Completeness (%)	100.00 (100.00)
Multiplicity	6.5 (6.3)
<i>Refinement</i>	
Number of reflections	73,881 (5294)
Resolution (Å)	51.63–3.95 (4.09–3.95)
<i>R</i> _{work}	0.26
<i>R</i> _{free}	0.30
No. of non-H atoms	
Protein	3563
Mean <i>B</i> -value (Å ²)	
Protein	184.88
RMSD from ideal values	
Bond lengths (Å)	0.020
Bond angles (°)	2.18
Ramachandran statistics (%)	
Preferred regions	99
Outliers	1

RMSD, root mean square deviation from ideal geometry; CC (1/2), cross-correlation between random half-datasets. *R*_{free} was calculated for a 5% subset of reflections.

^aValues in parentheses correspond to those of the highest resolution shell.

(MPD), 100 mM tri-sodium citrate pH 5.6, 10 mM magnesium chloride. For data collection, crystals of the chCREB3 bZIP were dragged through a drop of the cryoprotectant (MPD)-containing reservoir solution and cooled to liquid nitrogen temperature. Diffraction data were collected at 100 K at Diamond Light Source, beamline I04. A data set extending to a resolution of 3.95 Å was collected on a single crystal of the chCREB3 bZIP covering a 180° of rotation range. The data were auto-processed using Xia2 (Winter et al., 2010). Data processing statistics are summarized in Table I. The crystals belonged to the base-centered orthorhombic Space group C222, with unit cell parameters $a = 137.90$, $b = 167.06$, and $c = 115.46$ Å. The crystals contained seven copies of the chCREB3 bZIP monomer per asymmetric unit, arranged as three and half dimers, with one dimer sitting on crystallographic two-fold axis. Seven copies of the bZIP monomer give a solvent content of approximately 85%.

The structure of the bZIP domain of chCREB3 was solved by molecular replacement with the program PHASER⁴⁶ using a dimer extracted from the CREB bZIP-CRE complex structure (PDB code: 1DH3) after the deletion of the bound duplex DNA. Three dimers were found using PHASER, and the final monomer identified from visualization of 2Fo-Fc and Fo-Fc electron density maps. The bZIP domain of mouse CREB has 36% amino acid sequence identity to the bZIP domain of chCREB3. The structure was refined by iterative rounds of manual model building in COOT⁴⁷ and using BUSTER.⁴⁸ Refinement resulted in R_{work} and R_{free} values of 0.26 and 0.30, respectively. Refinement statistics are summarized in Table I. The geometry of the final model was validated with MOLPROBITY.⁴⁹ Protein coordinates have been deposited in the Protein Data Bank (PDB entry 6IAK). Figures were generated using PyMOL (DeLano WL 2002).

Sequence and structural alignments

bZIP sequences were obtained from the Uniprot database (<http://www.uniprot.org/>). Multiple sequence alignments were performed using Clustal Omega.⁵⁰ Structural alignments were performed using PyMOL (DeLano WL 2002).

Supplementary Material

Amino acid sequence alignment of bZIP sequences (Fig. S1). Stereo image of the unit cell and the contents of the asymmetric units. The bZIP domain molecules in each asymmetric unit are coloured identically (Fig S2).

Acknowledgments

We thank Diamond Light Source for beamtime (proposal mx14744), and the staff of beamline I04 for assistance with crystal testing and data collection. MR is supported by a Wellcome Trust fellowship (204703/Z/16/Z) and J.M.G. by a Wellcome Investigator Award

200835/Z/16/Z. The Wellcome Trust is also acknowledged for providing administrative support (Grant 075491/Z/04). VN is supported by the BBSRC (grant no. BBS/E/I/00007032) and RJO by the Medical Research Council (grant no. MR/K018779/1)

Funding information

Wellcome Trust, Medical Research Council, Biotechnology and Biological Sciences Research Council; Pirbright Institute; University of Oxford.

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