Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray analysis of the C/EBPβ C-terminal region in complex with DNA

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Received 4 October 2000
Accepted 1 March 2001

The C-terminal fragment (residues 259–345) of human C/EBPβ, a basic region leucine zipper transcriptional regulatory factor which includes the minimal DNA-binding domain, was crystallized in complex with a 16 bp DNA fragment from the *tom*-1 promoter. The crystals were in the form of a parallelepiped belonging to space group $C222_1$, had unit-cell parameters a = 100.7 (2), b = 113.5 (1), c = 74.4 (1) Å and diffracted to a resolution of 2.1 Å. Moreover, truncation of nine residues from the C-terminus not conserved among C/EBP family members yielded isomorphous crystals that diffracted to a resolution of 1.8 Å or better. Truncation of 14 residues from the N-terminus of the C-terminal fragment produced well shaped crystals in the form of hexagonal bipyramids, however; unfortunately, they were unstable and diffracted poorly.

1. Introduction

CAAT/enhancer binding proteins (C/EBPs), which belong to the basic region leucine zipper (bZip) protein family (for a review, see Hurst, 1994), are a group of transcriptional regulatory factors under the control of integrated metabolic processes (for a review, see Lekstrom-Himes & Xanthopoulos, 1998). The basic region is responsible for DNA recognition, while the leucine zipper mediates protein–protein dimerization (Agre *et al.*, 1989). Thus far, six C/EBP family members have been identified: C/EBPα, β, γ, δ, ε and ζ. C/EBPs, like most transcriptional regulatory factors, are modular proteins consisting of a highly conserved C-terminal DNA-binding domain, an N-terminal *trans*-activation domain and central regulatory domains (Williams *et al.*, 1995; Kowenz-Leutz & Leutz, 1999). They are capable of binding to DNA containing the consensus site T(T/G)NNGNAA(T/G) (Akira *et al.*, 1990), forming homo- or heterodimers, and regulate target-gene expression by various means. Phosphorylation of C/EBPβ, for example, derepresses its *trans*-activation domain (Kowenz-Leutz *et al.*, 1994), while DNA recognition can be inhibited by heterodimerization with CHOP, which contains a non-functional basic region (Ron & Habener, 1992). When coexpressed with other myeloid transcriptional factors, such as Ets, acute myelogenous leukemia 1/core binding factor β subunit (AML1/CBFβ), c-Myb or NF-κB, C/EBPs synergistically activate the myeloid-specific genes. This synergy is mediated by direct interaction between these other factors and the bZip regions of C/EBPs (McNagny *et al.*, 1998; Zhang *et al.*, 1996; Mink *et al.*, 1996; Stain *et al.*, 1993). How C/EBP bZip regions, which have a continuous α-helical structure on DNA binding, are able to recognize proteins with different surface properties remains unclear.

Digestion of the DNA-bound form of C/EBPβ (also called a nuclear factor for interleukin-6 expression; NF-IL6) with trypsin yields a stable domain containing residues 266–345, termed the tryptic core domain (TCD). Digestion with endoprotease Asp-N produces a slightly smaller domain [NF-IL6 bZip domain (NFBD); residues 272–345] (Brasier & Kumar, 1994) that forms comparatively unstable DNA complexes having a 15-fold faster off-rate than the TCD (Brasier & Kumar, 1994). Here, we report the crystallization of the C-terminal portion of C/EBPβ in complex with DNA. The two C/EBPβ constructs that yielded high-resolution crystals (residues 259–345 and 259–336) both contain a conserved N-terminal lysine-rich region in addition to TCD. Comparison of the high-resolution crystal structure of C/EBPβ–DNA with that of yeast transcriptional factor GCN4–DNA (Keller *et al.*, 1995) may provide insights into the variations in their DNA-binding specificities. The detailed structure of the C/EBPβ–DNA complex may also help us to understand the mechanism by which the N-terminal extension increases the DNA-binding affinity of C/EBPβ and reveal the unique surface properties of C/EBPβ that enable it to interact with a variety of regulatory proteins.

Table 1
Crystal parameters and data-collection statistics.

Crystal type	C/EBPβ_{VC}–DNA	C/EBPβ_{VE}–DNA
Unit-cell parameters (Å)		
a	100.7 (2)	100.9 (1)
b	113.5 (1)	112.5 (1)
c	74.4 (1)	74.35 (8)
Temperature (K)	100	110
Space group	$C222_1$	$C222_1$
Z	8	8
Solvent content (%)	64.2	66.0
Beamline	BL45XU	BL44B2
Wavelength (Å)	1.02	0.6
Resolution (Å)	20–2.1	20–1.7
Observations	144634	179384
Unique reflections	24863	44759
R_{merge}†	0.063	0.052
Completeness (%)	99.2	95.8
$I/\sigma(I)$	26.4	21.1
Mosaicity (°)	0.39	0.35

† $R_{merge} = \sum|I_j - \langle I_j\rangle|/\sum\langle I_j\rangle$, where I_j is the intensity of reflection *j* and $\langle I_j\rangle$ is the average intensity of reflection *j*.

2. Experimental procedures

2.1. Expression, purification and sample preparation

DNA-binding domains of human C/EBPβ (residues 259–345, 259–336 and 273–336, designated C/EBPβ_{VC}, C/EBPβ_{VE} and C/EBPβ_{EE}, respectively) were overexpressed in *Escherichia coli* BL21 (DE3) using a T7 expression system. The bacterial cells containing the overexpressed proteins were harvested, lysed using a French press and centrifuged. The resultant supernatants containing the target proteins were purified through three column-chromatographic steps using phosphocellulose (P11, Whatman International Ltd, England), CM cellulose (CM52, Whatman International Ltd, England) and Superdex 75 (Amersham Pharmacia Biotech, USA) columns.

DNA fragments 5′-AATGTGGCGCAATCCT-3′ and 5′-TAGGATTGCGCCACAT-3′ (BEX Co., Tokyo) were purified by reverse-phase HPLC (Wako Pure Chemical Industries Ltd, Osaka) and annealed. The double-stranded oligonucleotides obtained were separated from the single-stranded material using hydroxyapatite (Bio-Rad Laboratories, CA, USA) column chromatography. Aliquots of the concentrated double-stranded DNA solution were then added to the C/EBPβ solution (10 m*M* DTT and 0.5 m*M* NaN_3 pH 6.8), yielding a final DNA:protein ratio of 1.2:1.0.

2.2. Crystallization

Crystallization trials were conducted using the sitting-drop vapour-diffusion method in 24-well plates at a temperature of 297 K. The initial screening carried out with Natrix, a crystallization reagent kit for nucleic acids (Hampton Research; Scott *et al.*, 1995), produced crystals in 1–30 d under several conditions. The best crystals of C/EBPβ_{VC}–DNA and C/EBPβ_{VE}–DNA were in the form of parallelepipeds and grew in 1–3 d when 2 µl drops of 10 mg ml^{-1} protein–DNA solution were mixed with 2 µl reservoir solution and equilibrated against 0.5 ml of reservoir solution numbers 9 [0.1 *M* KCl, 0.01 *M* $MgCl_2$, 10%(*v*/*v*) PEG 400 in 0.05 *M* MES buffer at pH 6.0] and 26 [0.2 *M* KCl, 0.1 *M* magnesium acetate, 10%(*v*/*v*) PEG 8000 in 0.05 *M* sodium cacodylate buffer at pH 6.5], respectively. Crystals of C/EBPβ_{EE}–DNA grew in the form of hexagonal bipyramids under Natrix condition 26. The size and shape of the crystals were enlarged and improved by diluting the reservoir solution by 30%, which reduced the growth rate from 1–2 d to over 10 d.

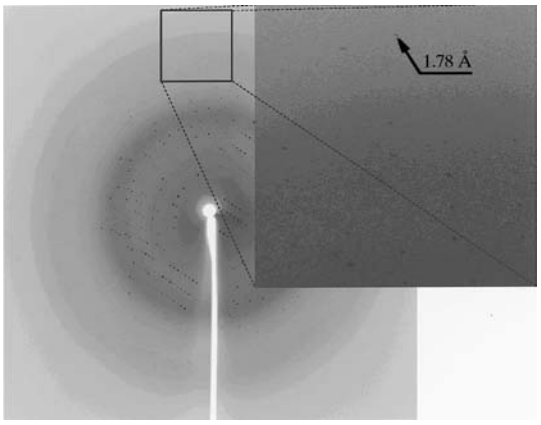

Figure 1
Diffraction pattern of the C/EBPβ_{VE}–DNA crystal; the rotation range is 1.0° and the exposure time is 8 min. The image was obtained using an R-AXIS IV imaging plate at beamline BL44B2 in SPring-8. The wavelength of X-rays was 0.6 Å and the distance from the crystal to the imaging plate was 400 mm.

2.3. Data collection

The diffractions of crystals mounted in glass capillaries were initially checked at room temperature (293 K) on a MacScience machine equipped with a DIP2030 imaging plate using Cu $K\alpha$ radiation filtered with a 0.15 mm nickel filter and focused with a double mirror. X-rays were generated by a rotating anode with a focus size of 0.2 × 2 mm (collimator 0.3 mm), powered by a M06XHF[22]-Fine generator operated at 50 kV and 50 mA. Crystals of C/EBPβ_{VC}–DNA and C/EBPβ_{VE}–DNA diffracted well, with the resolution ranging from 3.2 to 2.4 Å for the former and to 2.0 Å for the latter; by contrast, the best C/EBPβ_{EE}–DNA crystal diffracted to a resolution of 6 Å. Complete data sets for crystals of C/EBPβ_{VC}–DNA and C/EBPβ_{VE}–DNA were collected at cryotemperatures using 300 × 300 mm R-AXIS IV imaging plates and synchrotron radiation at SPring-8. This was accomplished by soaking the crystals in cryoprotectant solutions while gradually increasing the concentration of cryoprotectants over a period of 5–10 min before flash-cooling them in a stream of cold nitrogen gas. The cryoprotectant solution for C/EBPβ_{VC}–DNA crystals consisted of 0.08 *M* KCl, 8 m*M* $MgCl_2$, 8%(*v*/*v*) PEG 400 and 20%(*v*/*v*) MPD in 0.04 *M* MES buffer at pH 6.0; that for C/EBPβ_{VE}–DNA crystals consisted of 0.2 *M* KCl, 0.1 *M* magnesium acetate, 14%(*w*/*v*) PEG 8000 and 24%(*v*/*v*) PEG 400 in 0.05 *M* sodium cacodylate buffer at pH 6.5. The diffraction data were indexed, integrated and scaled using *DENZO* (Otwinowski, 1993) and *SCALEPACK* (Otwinowski & Minor, 1997) software. Details of the data-collection procedure, crystal parameters and data-processing statistics are summarized in Table 1.

2.4. Design of DNA fragment

Since the present study was our first determination of the crystal structures of protein–DNA complexes, we performed a comprehensive survey of the protein–DNA complex structures determined at a resolution of 2.4 Å or higher. We found only 16 structures with accessible coordinates in the Protein Data Bank at the time of our search. These structures could be classified into two groups: in one (seven structures) the DNA molecules were packed as monomers, dimers or tetramers, while in the second (nine structures) the DNAs were paired with overhanging bases and formed continuous double-stranded B-DNA pseudo-helices that contained either a whole number of helical turns or a whole number of turns plus a half turn. In the crystal, the latter type of DNA fragments, which are contiguous in the DNA pseudo-helix, are related by crystallographic or pseudo-twofold screw symmetry. Although the DNA fragments were bent or distorted in most protein–DNA complexes, the introduction of twofold screw symmetry did not further strain the DNA, which persisted if the crystals contained only fragments related by translational symmetry (*i.e.* with a whole number

of helical turns). On the basis of these insights, we designed a DNA fragment for the crystallization of the C/EBPβ–DNA complex:

AATGTGGCGCAATCCT
TACACCGCGTTAGGAT .

This fragment contains one and a half helical turns with overhanging A and T bases, found at the C/EBPβ binding site of the *tom*-1 promoter (Burk *et al.*, 1997). While our current work was in progress, a similar strategy for the selection of DNA fragment was applied for the crystallization of MATα2–MCM1–DNA ternary complex (Tan *et al.*, 2000).

3. Results and discussion

Only one type of DNA fragment enabled us to obtain high-resolution crystals of C/EBPβ–DNA. C/EBPβ_{VE}–DNA crystals (Fig. 1) diffracted beyond a resolution of 1.8 Å, making it possible to accurately determine their structure using the full data set collected under cryoconditions from only one crystal. The crystal was orthorhombic, belonging to space group $C222_1$. The solvent-content calculation (Matthews, 1968) suggests that one molecule of C/EBPβ_{VE}–DNA could be located in an asymmetric unit, assuming the solvent content of the crystal to be 66%.

The unit-cell parameters of the C/EBPβ_{VC}–DNA complex were nearly identical to those of C/EBPβ_{VE}–DNA, indicating that the packing modes of the molecules in these crystals were nearly identical. Nevertheless, the resolution of C/EBPβ_{VC}–DNA crystals was slightly lower, probably owing to high fluctuations or steric hindrance at the C-terminal end of bZip introduced by the addition of nine residues. Our failure to obtain high-resolution crystals of C/EBPβ_{EE}–DNA may be related to its lower stability caused by the absence of the N-terminal extension of bZip (Brasier & Kumar, 1994).

We used the molecular-replacement method to determine the crystal structures of C/EBPβ_{VE}–DNA and C/EBPβ_{VC}–DNA using the DNA and basic region coordinates from the 2.2 Å resolution crystal structure of yeast transcriptional factor GCN4 (Keller *et al.*, 1995) as a search model. The refined structures will be reported elsewhere.

We thank S. Akira for NF-IL6 cDNA, M. Yamamoto and T. Kumasaka for assistance with diffraction data collection at beamline BL45XU, S. Ishii for helpful discussions and generous support and T. Kato and M. Hosaka for generous support. This research was supported in part by CREST (Core Research for Evolutional Science and Technology) of the Japan Science and Technology Corporation (JST) to KO.

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