Complex assembly, crystallization and preliminary X-ray crystallographic analysis of the chicken CD8 $\alpha\alpha$ –BF2*0401 complex

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In the process of antigen presentation, the MHCI–CD8 complex is important for immune signal transduction by the activation of cytotoxic T cells. Here, the expression, purification, crystallization and X-ray analysis of the complex of the chicken MHC class I molecule BF2*0401 and CD8 $\alpha\alpha$ (CD8 $\alpha\alpha$ –BF2*0401) are reported. This complex was verified by SDS–PAGE analysis of a CD8 $\alpha\alpha$ –BF2*0401 crystal, which showed three bands corresponding to the molecular weights of BF2*0401, β_2 -microglobulin and CD8 α , respectively. The crystal of CD8 $\alpha\alpha$ –BF2*0401 diffracted to 2.8 Å resolution and belonged to space group $P2_1$, with unit-cell parameters $a = 90.6$, $b = 90.8$, $c = 94.9$ Å, $\beta = 98^\circ$. The Matthews coefficient and solvent content were calculated to be 2.88 Å³ Da^{−1} and ~57.3%, respectively.

1. Introduction

During cellular immunity, antigen presentation by MHC class I molecules is the first step in the activation of cytotoxic T cells (CTLs). In this process, only with the engagement of CD8 are MHC class I molecules capable of presenting peptides to T-cell receptors (TCRs; Neefjes *et al.*, 2011). The CD8 $\alpha\alpha$ –MHC class I molecule complex not only enhances the interaction between TCRs and MHCI peptides but also improves their binding sensitivity (Holler & Kranz, 2003). To date, only three structures of mammalian MHC class I alleles in complex with CD8 $\alpha\alpha$ have been determined (Gao *et al.*, 1997; Kern *et al.*, 1998; Shi *et al.*, 2011). All known CD8 $\alpha\alpha$ –MHCI structures show that the CD8 $\alpha\alpha$ homodimer uses all six complementarity-determining region (CDR)-like loops to bind the CD loop of the MHCI $\alpha 3$ domain in an antibody-like manner. However, the structure of the CD8 $\alpha\alpha$ –MHCI complex in non-mammals such as chickens remains unknown.

Chickens are presently being assailed by emerging infectious pathogens such as highly pathogenic avian influenza virus subtype H5N1, which can threaten human health. In chicken immunity, a single dominantly expressed class I allele has been reported to be associated with pathogen resistance (Koch *et al.*, 2007). The chicken MHC class I molecule BF2*2101 has been reported to confer resistance to Marek's disease, and its antiviral capacity is related to effective antigen presentation by the MHC and an induced strong CTL response (Wallny *et al.*, 2006; Garcia-Camacho *et al.*, 2003). Hence, it is essential for us to have a good understanding of chicken immunity, especially of the CD8 $\alpha\alpha$ –MHCI structure-based CTL responses.

In this study, the crystallization and preliminary crystallographic analysis of the CD8 $\alpha\alpha$ –BF2*0401 complex are reported for the first time. The results will contribute to understanding the structural characteristics of CD8 $\alpha\alpha$ –MHCI in bird species.

2. Materials and methods

2.1. Macromolecule production

A DNA fragment encoding the chicken CD8 α mature peptide residues 1–118 (GenBank AY528647) was cloned from cDNA synthesized from mRNA extracted from the spleen of a specific pathogen-free (SPF) chicken. The purified CD8 α segment was ligated

into pET-21a(+) vector (Novagen, Merck KGaA, Darmstadt, Germany) and expressed in *Escherichia coli* strain BL21 (DE3) (Table 1). The recombinant protein was expressed as inclusion bodies. The bacteria were harvested and lysed by sonication, and the inclusion bodies were washed three times with washing buffer and dissolved in guanidinium chloride (Gua-HCl) buffer to a concentration of 30 mg ml⁻¹ (Sun *et al.*, 2013). 3 ml chicken CD8 α inclusion bodies was then added gradually to 500 ml refolding buffer (100 mM Tris-HCl pH 8.0, 2 mM EDTA, 400 mM L-arginine-HCl, 0.5 mM oxidized glutathione, 5 mM reduced glutathione) and incubated at 277 K for 8 h. The soluble protein was then concentrated and purified by chromatography on a Superdex 200 16/60 HiLoad (GE Healthcare) size-exclusion column (Zhang *et al.*, 2010).

The pET-21a plasmids of BF2*0401 (GenBank AM282693, residues 1–270 of the mature protein) and chicken β_2 -microglobulin (β_2 m; GenBank AB178590, residues 1–98 of the mature protein) were kept in our laboratory (Zhang *et al.*, 2012). Peptide IE8 (IDWFDGKE) was synthesized and purified by HPLC (Scilight-Peptide). The extraction of BF2*0401 and chicken β_2 m inclusion bodies and the preparation of refolded BF2*0401 complex were performed essentially as described previously (Sun *et al.*, 2013;

Garboczi *et al.*, 1996). The peptide IE8 dissolved in dimethyl sulfoxide (DMSO) and the BF2*0401 and β_2 m inclusion bodies were then refolded in a 3:1:1 molar ratio using the dilution method. After refolding, the pBF2*0401 complex was purified by Superdex 200 16/60 HiLoad (GE Healthcare) and Resource Q anion-exchange (GE Healthcare) chromatography (Zhang *et al.*, 2012).

2.2. Crystallization of the CD8 α –BF2*0401 complex

The purified chicken CD8 α and pBF2*0401 were concentrated to 20 mg ml⁻¹ and mixed in a 1:1 molar ratio at 277 K overnight. The

Table 1

Chicken CD8 α –BF2*0401 production information.

Source organism	<i>Gallus gallus</i>
DNA source	Cloning from cDNA
Forward primer	ACGCATATGCCCGAGGCCAGCGTAACAG
Reverse primer	CCGCTCGAGGAAGAAGCGGGTGTCCCGAG
Cloning vector	pMD18T
Expression vector	pET-21a(+)
Expression host	<i>E. coli</i>
Complete amino-acid sequence of the construct produced	
Chicken CD8 α	AQQRNTMEARFLNRNMKHPQEGQPLECMPFNIDNGVSWIRQ-DKDGKLFHIVYISPLSRATFPNERTSSQFEGSKQGSSFRLL-VVKNFRAQDQGTGYFCIANINQMLYFSSGQPAFF
BF2*0401	ELHTLRVIRTAMTDPGPGQPWFVTVGVYDGLFVHYNSTARRYV-PRTEWIAANTDQYWDGQTIGQLNEQINRENLRGIRRRYN-QTGGSHTVQWMFGCDILEDTIRGYRQSAYDGRDFIALDKD-MKTFTAAPVAPTRKRKWESEPERWKNYLEETCWEVLRRL-YVEYKGAELGRRERPEVRVWGKEADGILTLSCRAHGFYPRP-IVVSWLKDGAVRGQDAHSGGIVPNGDTYHTVTTIDAQPGD-GDKYQCRVEHASLPQGLYSW
Chicken β_2 m	DLTPKVQVYSRFPASAGTKNVLNCFAGFHPKISITLMKDGVP-MEAGQYSDMSFNDWTFQRLVHADFTSSGSGTYACKVEHET-LKEPQVYKWDPEF

Table 2

Crystallization conditions.

Method	Sitting-drop vapour diffusion
Plate type	VDX plate
Temperature (K)	291
Protein concentration (mg ml ⁻¹)	5, 10
Buffer composition of protein solution	20 mM Tris-HCl pH 8.0, 50 mM NaCl
Composition of reservoir solution	0.2 M ammonium sulfate, 0.1 M Tris pH 8.5, 25% (w/v) polyethylene glycol 3350
Volume and ratio of drop	1:1
Volume of reservoir (μ l)	160

Figure 1

Purification of the soluble chicken CD8 α and pBF2*0401 proteins by Superdex 200 16/60 HiLoad gel-filtration and Resource Q anion-exchange chromatography (GE Healthcare). (a) The profile peak represents the chicken CD8 α homodimer (~28 kDa). Inset, reduced SDS-PAGE gel (15%) for the peak. Lane M contains molecular-mass markers (labelled in kDa). (b) Gel-filtration profile of the pBF2*0401 complex. Peaks 1, 2 and 3 correspond to the aggregated BF2*0401 heavy chain, the BF2*0401 complex (~43 kDa) and excess chicken β_2 m, respectively. Inset, reduced SDS-PAGE gel (15%) for peaks 1, 2 and 3. Lane M contains molecular-mass markers (labelled in kDa). (c) Anion-exchange chromatography profile of the refolded pBF2*0401 complex, which was eluted at a NaCl concentration of 10–15%. Inset, reduced SDS-PAGE gel (15%) for the peak. Lane M contains molecular-mass markers (labelled in kDa).

Figure 2
Photograph of the crystal used for diffraction analysis.

Figure 3
SDS-PAGE for identification of the chicken CD8α-BF2*0401 crystal (lane S). Lane M contains molecular-mass markers (labelled in kDa).

Figure 4
Diffraction pattern of the chicken CD8α-BF2*0401 complex; spots corresponding to high-resolution diffraction are highlighted in the box.

Table 3
Data-collection statistics for chicken CD8α-BF2*0401.

Values in parentheses are for the outer shell.	
Diffraction source	BL17U, SSRF
Wavelength (Å)	0.97972
Temperature (K)	100
Detector	ADSC Q315
Crystal-to-detector distance (mm)	300
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	0.8
Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 90.6, b = 90.8, c = 94.9,$ $\alpha = 90.0, \beta = 98.6, \gamma = 90.0$
Mosaicity (°)	1.0
Resolution range (Å)	50.00–2.80 (2.90–2.80)
Total No. of reflections	163307
No. of unique reflections	37579
Completeness (%)	99.8 (100.0)
Multiplicity	4.3 (4.5)
$\langle I/\sigma(I) \rangle$	14.140 (2.630)
R_{merge} (%)†	10.5 (61.3)
Overall B factor from Wilson plot (Å ²)	52.44

† $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl) \rangle$ is the average intensity from multiple measurements.

extinction coefficients of chicken CD8α and pBF2*0401 were estimated to be 0.73 and 2.36 mg ml^{−1} at 280 nm, respectively, using *ProtParam* (<http://web.expasy.org/protparam/>). The protein concentration was determined using the BCA kit (Novagen, Germany). The CD8α-BF2*0401 complex was then concentrated to 5 and 10 mg ml^{−1} and mixed with reservoir buffer in a 1:1 ratio. Finally, the complex protein was crystallized by the sitting-drop vapour-diffusion method at 291 K. After 15 d, crystals of the chicken CD8α-BF2*0401 complex were obtained (Table 2). The complex crystals were picked out from the crystallization drops and washed three times with the reservoir solution for SDS-PAGE analysis.

2.3. Data collection and processing

The crystal was first soaked in a cryoprotectant containing 17%(v/v) glycerol for a few seconds and then flash-cooled in liquid nitrogen. Diffraction data were collected on beamline BL17U at the Shanghai Synchrotron Radiation Facility (SSRF; Shanghai, People's Republic of China) at a wavelength of 0.97972 Å using an ADSC

Q315 CCD detector. The collected intensities were indexed, integrated, corrected for absorption, scaled and merged using *HKL-2000* (Otwinowski & Minor, 1997; Table 3).

3. Results and discussion

Multiple amino-acid sequence alignments between the chicken proteins and their mammalian counterparts (PDB entries 1akj, 1bqh and 3qzw; Gao *et al.*, 1997; Kern *et al.*, 1998; Shi *et al.*, 2011) were performed using the *DNAMAN* program. The alignment results showed that the chicken BF2*0401 molecule has 41.7, 42.8 and 42.8% identity to the HLA-A*0201, HLA-A*2402 and H-2K^b molecules, respectively, while the chicken CD8 α molecule shares 21.7 and 17.9% sequence identity with human and mouse CD8 α , respectively.

After refolding *in vitro*, approximately 10–12% yields of soluble chicken CD8 α and pBF2*0401 proteins were obtained. The soluble chicken CD8 α protein was purified by Superdex 200 16/60 HiLoad size-exclusion chromatography (Fig. 1*a*). SDS–PAGE analysis of chicken CD8 α showed one clear band at about 13.6 kDa (see inset in Fig. 1). According to the reference elution profile of the column, this shows that chicken CD8 α exists in a homodimeric form ($\sim$ 28 kDa). The refolded pBF2*0401 complex was also first purified by Superdex 200 16/60 HiLoad size-exclusion chromatography (GE Healthcare; Fig. 1*b*). The refolded protein (peak 2) was further purified by Resource Q anion-exchange chromatography. A single and specific elution peak appeared when the NaCl concentration of the buffer was 10–15% (Fig. 1*c*). SDS–PAGE analysis showed two clear bands corresponding to the expected molecular weights of BF2*0401 ($\sim$ 32 kDa) and chicken β_2 m ($\sim$ 11 kDa) (see inset in Fig. 1).

On initial crystallization screening, crystals appeared after 15 d (Fig. 2). SDS–PAGE analysis of the crystals displayed three clear bands corresponding to BF2*0401, CD8 α and β_2 m (Fig. 3). In addition, the CD8 α –BF2*0401 complex crystal displayed a good diffraction pattern (Fig. 4). The Matthews coefficient value V_M was 2.88 Å³ Da^{−1}, with a calculated solvent content of $\sim$ 57.3%.

Solution of the CD8 α –BF2*0401 complex crystal structure will reveal specific structural features of the CD8 α –MHCI complex in chickens and will contribute to a better understanding of the details of the interaction between CD8 α and MHCI in birds.

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