Complex assembly, crystallization and preliminary
X-ray crystallographic analysis of the bovine
CD8 $\alpha\alpha$ –BoLA-2*02201 complexZhenbao Wang,^a Rong Chen,^a
Mansoor Tariq,^a Bo Jiang,^a
Zhaosan Chen^a and Chun Xia^{a,b,*}^aDepartment of Microbiology and Immunology,
College of Veterinary Medicine, China
Agricultural University, Beijing 100193,
People's Republic of China, and ^bKey Laboratory
of Animal Epidemiology and Zoonosis, Ministry
of Agriculture, Beijing, People's Republic of
China

Correspondence e-mail: xiachun@cau.edu.cn

Received 19 March 2014

Accepted 17 April 2014

In order to clarify the structural characteristics of the bovine MHC class I molecule (BoLA-I) complexed with CD8 $\alpha\alpha$ (CD8 $\alpha\alpha$ –BoLA-I), bovine CD8 $\alpha\alpha$, BoLA-I (BoLA-2*02201) and β_2m were expressed and purified, and were then assembled with a peptide derived from *Foot-and-mouth disease virus* (FMDV-VP1_{YY9}) and crystallized. The crystal diffracted to 1.7 Å resolution and belonged to space group $P2_1$, with unit-cell parameters $a = 53.9$, $b = 103.8$, $c = 61.8$ Å, $\alpha = \gamma = 90$, $\beta = 96^\circ$. The asymmetric unit contained one complex, with a Matthews coefficient of 2.41 Å³ Da⁻¹ and a solvent content of 48.9%. The rotation-function Z -score and translation-function Z -score for molecular replacement were 3.4 and 8.9, respectively. In addition, SDS–PAGE analysis of CD8 $\alpha\alpha$ –BoLA-I crystals showed three bands corresponding to the molecular weights of BoLA-I heavy chain, β_2m and CD8 α . The structure of the CD8 $\alpha\alpha$ –BoLA-I complex should be helpful in obtaining insight into the interaction between bovine CD8 $\alpha\alpha$ and MHC class I molecules. Structure determination of BoLA-2*02201–FMDV-VP1_{YY9} will be useful in the design of vaccines for foot-and-mouth disease.

1. Introduction

The CD8 $\alpha\alpha$ homodimer is essential as a co-receptor for major histocompatibility complex (MHC) class I molecules in the activation of the cytotoxic T-lymphocyte (CTL) response and the clearance of virally infected cells (Rudolph *et al.*, 2006; Cheroutre & Lambolez, 2008). The first structure of CD8 $\alpha\alpha$ bound to a human MHC class I molecule was reported by Gao *et al.* (1997), and proved that the residues of CD8 $\alpha 1$ make more interactions with MHC class I than those of CD8 $\alpha 2$ (Gao *et al.*, 1997). Comparison of the three known CD8 $\alpha\alpha$ –MHC class I complexes (human CD8 $\alpha\alpha$ –HLA-A2, CD8 $\alpha\alpha$ –HLA-A24 and mouse CD8 $\alpha\alpha$ –H-2K^b) revealed some species-specific features, such as different complementarity-determining region (CDR)-like loops of CD8 $\alpha\alpha$ in human and mouse, which mediate key interactions with MHC class I in an antibody-like manner (Kern *et al.*, 1998; Shi *et al.*, 2011; Li *et al.*, 2013). However, it is still unknown whether these structural characteristics appear in other mammals.

The bovine MHC class I molecule is termed bovine leukocyte antigen (BoLA-I). To date, only two structures of BoLA-I (BoLA-2*01801 and BoLA-6*01301) have been determined (Li *et al.*, 2011; Macdonald *et al.*, 2010). The structure of BoLA-2*01801 from the BoLA-A11 haplotype revealed an unusually large A pocket and a polymorphic isoleucine 73 that allow the MHC groove to adopt different conformations of the *Rinderpest virus* peptide (Li *et al.*, 2011). BoLA-6*01301 from the BoLA-A18 haplotype demonstrated that the peptide of *Theileria parva* was presented in a highly unusual conformation (Macdonald *et al.*, 2010). BoLA-2*02201 from the A31 haplotype confirmed that a stronger MHC class I-restricted CD8⁺ T-cell response was induced after *Foot-and-mouth disease virus* (FMDV) infection. The result suggested that the BoLA-2*02201 allele plays an important role in eliminating FMDV (Guzman *et al.*, 2010). To understand the structural basis of the complex between bovine CD8 $\alpha\alpha$ and BoLA-2*02201 (CD8 $\alpha\alpha$ –BoLA-2*02201), and for further study of FMDV vaccines in cattle, in this article we report

Table 1CD8 α –BoLA-2*02201 production information.

Source organism	<i>B. taurus</i>
DNA source	Synthesized according to the sequences in GenBank (BoLA-2*02201, X80935; bovine β_2 m, X69084.1; bovine CD8 α , NM_174015.1)
Expression vector	pET-21a
Expression host	<i>E. coli</i> strain BL21 (DE3)
Complete amino-acid sequences of the constructs produced	
BoLA-2*02201	GSLSRLYFHTAVSRPGLGEPFRFISVGYVDDTQFVRFDSDSANPR- EEPAPWMEQEGPEYWEQTRIVKDTAQSRVGLNLTGRGY- NQSEAGSHTLQMYGCDVGPDRLLRGYEQDAYDGRDIYAL- NEDLRSWTAADTAQITKRREAAGAAERLRNYLEGTQVEG- LRRYLENGKDALLRADPPKAHVTHPVSREVTLRCWALGF- YPPEISLTWQREGEDQTQDMELVETRPSSGDGTFQKWAALVV- PSGEEQRYTCRVQHEGLQEPLTLRWE
Bovine β_2 m	IQRPPIQVYSRHPEDGKPNYLCYVYGFHPPQIEIDLKNGE- KIKSEQSDLSFSKDWFSYLLSHAEFTPNKQDQYSCRVKHVT- LEQPRIVKWRDL
Bovine CD8 α	GGSLFRMSPTQKELRGKVELQCELLQSGMATGCSWLRHIPG- DDPRPTFLMYLSAQRVKLAEGLDPRHISGAKVSGTKFQLTL- SSFLQEDQGYFCSVVSNSILYFSNFVPVFLPAKPATT

Table 2

Crystallization conditions.

Method	Sitting drop
Plate type	48-well VDX plate
Temperature (K)	291
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	20 mM Tris–HCl pH 8.0, 50 mM NaCl
Composition of reservoir solution	20% (w/v) polyethylene glycol 10 000, 0.1 M HEPES–NaOH pH 7.5
Volume and ratio of drop	2 μ l, 1:1 CD8 α –BoLA-2*02201 and reservoir solutions
Volume of reservoir (μ l)	150

the refolding, purification, assembly and crystallization of the CD8 α –BoLA-2*02201 complex.

2. Materials and methods

2.1. Macromolecule production

The gene fragment encoding the BoLA-2*02201 allele mature peptide residues 19–293 (extracellular domain) from *Bos taurus* was synthesized by Shanghai Invitrogen Life Technologies according to the sequence in GenBank (accession No. X80935). The BoLA-2*02201 gene was ligated into the pET-21a expression vector (Novagen, Merck KGaA, Darmstadt, Germany) and transformed into *Escherichia coli* strain BL21 (DE3). The expression plasmids pET-21a/bovine β_2 microglobulin (b β_2 m; residues 1–98; GenBank accession No. X69084.1) and pET-21a/bovine CD8 α (bCD8 α ; residues 24–145; GenBank accession No. NM_174015.1) were constructed previously in our laboratory and were also transformed into *E. coli* strain BL21 (DE3) (Table 1). These proteins were expressed as inclusion bodies. The bacteria were harvested and lysed by sonication (Yao *et al.*, 2012). The samples were centrifuged for 10 min at 11 000 rev min⁻¹ and the pellets were washed three times with a solution consisting of 20 mM Tris–HCl pH 8.0, 1 mM DTT, 1 mM EDTA, 100 mM NaCl, 0.5% Triton X-100. Finally, the BoLA-2*02201, b β_2 m and bCD8 α inclusion bodies were dissolved in guanidinium chloride (Gua–HCl) buffer [6 M Gua–HCl, 50 mM Tris–HCl pH 8.0, 100 mM NaCl, 10 mM EDTA, 10 mM DTT, 10% (v/v) glycerine] to a concentration of 30 mg ml⁻¹.

Two components of the complex were prepared. First of all, the BoLA-2*02201–FMDV-VP1_{YY9} complex was prepared essentially as described previously by Garboczi *et al.* (1992) with some modifications (Yao *et al.*, 2012). Briefly, the peptide FMDV-VP1_{YY9} (YRMKRAETY) derived from FMDV structural protein VP1

Table 3Data-collection statistics for CD8 α –BoLA-2*02201.

Values in parentheses are for the outer shell.

Diffraction source	BL17U, SSRF
Wavelength (Å)	0.97916
Temperature (K)	100
Detector	ADSC 315 CCD
Crystal-to-detector distance (mm)	200
Rotation range per image (°)	1
Total rotation range (°)	200
Exposure time per image (s)	0.4
Space group	<i>P</i> ₂ ₁
Unit-cell parameters (Å, °)	<i>a</i> = 53.9, <i>b</i> = 103.8, <i>c</i> = 61.8, α = 90.0, β = 96.0, γ = 90.0
RFZ score	3.4
TFZ score	8.9
$ E^2 - 1 $	0.738
<i>L</i>	0.498
Mosaicity (°)	0.2
Resolution range (Å)	50.00–1.70 (1.76–1.70)
Total No. of reflections	267729
No. of unique reflections	71354
Completeness (%)	96.2 (94.9)
Multiplicity	3.8 (3.7)
$\langle I/\sigma(I) \rangle$	23.9 (2.9)
<i>R</i> _{merge} [†] (%)	5.6 (47.9)
Overall <i>B</i> factor from Wilson plot (Å ²)	28.9

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

(FMDV-VP1) residues 902–910 was synthesized and purified by HPLC (Scilight-Peptide). The peptide FMDV-VP1_{YY9} dissolved in dimethyl sulfoxide (DMSO) and the BoLA-2*02201 and b β_2 m inclusion bodies were then refolded in a 3:1:1 molar ratio using the dilution method. After incubation for 24 h at 277 K, the protein was concentrated and purified by chromatography on a Superdex 200 16/60 HiLoad (GE Healthcare) size-exclusion column followed by Resource Q (GE Healthcare) anion-exchange chromatography. Secondly, a 3 ml solution of the bovine CD8 α inclusion bodies was refolded by the same method and purified by chromatography on a Superdex 200 16/60 HiLoad column.

2.2. Crystallization

The purified BoLA-2*02201 (~43 kDa) and bovine CD8 α (~27.2 kDa) proteins were concentrated to 20 and 10 mg ml⁻¹, respectively, in the same buffer consisting of 20 mM Tris–HCl pH 8.0, 50 mM NaCl. The proteins were mixed in a 1:1 molar ratio and incubated for 12 h at 277 K. Finally, the complex was diluted to 5 and 10 mg ml⁻¹ for crystallization. Crystallization trials were carried out by the sitting-drop method using Crystal Screen 2 (Hampton Research, Riverside, California, USA) at 291 K (Yao *et al.*, 2012). Drops were prepared by mixing 1 μ l protein solution with 1 μ l reservoir solution and were equilibrated against 150 μ l of the same reservoir solution in 48-well VDX plates (Hampton Research). After 10 d, the best crystals were obtained using Crystal Screen 2 condition No. 38 [20% (w/v) polyethylene glycol 10 000, 0.1 M HEPES–NaOH pH 7.5] with a protein concentration of 10 mg ml⁻¹ (Table 2). One crystal was picked out, rinsed thoroughly with the reservoir solution and analysed by SDS–PAGE (15%).

2.3. Data collection and processing

Diffraction data were collected on beamline BL17U at Shanghai Synchrotron Radiation Facility (SSRF; Shanghai, People's Republic of China) at a wavelength of 1.07212 Å using an ADSC 315 CCD detector. The crystals were first soaked in reservoir solution with 15% glycerol as a cryoprotectant for several seconds and were then

flash-cooled in a stream of gaseous nitrogen at 100 K (Parkin & Hope, 1998). The collected intensities were indexed, integrated,

corrected for absorption, scaled and merged using *HKL-2000* (Otwinowski & Minor, 1997; Table 3).

Figure 1
Elution profiles of purification of the refolded BoLA-2*02201 and CD8αα recorded on an ÄKTApurifier FPLC system (GE Healthcare) at 280 nm wavelength. (a) Gel-filtration profile of the folded products. Peak 1 represents the heavy chain of BoLA-2*02201, peak 2 represents the folded complex BoLA-2*0220 and peak 3 represents bovine β₂m. Inset, reduced SDS-PAGE gel (15%) for each peak. Lane M contains molecular-weight markers (labelled in kDa). (b) Results of further purification of the folded BoLA-2*02201 by anion-exchange chromatography. The complex was eluted at a NaCl concentration of 11.0–14.0%. Inset, reduced SDS-PAGE gel (15%) for peak 1. (c) Gel-filtration profile of the folded bovine CD8αα products. Peak 1 represents the bovine CD8αα homodimer. Inset, reduced SDS-PAGE gel (15%) for peak 1.

3. Results and discussion

The peptide FMDV-VP1_{YY9} was complexed with BoLA-2*02201-β₂m. The refolding experiment resulted in an approximately 15% yield of the folded MHC class I molecule (~43 kDa). The complex was purified by Superdex 200 16/60 HiLoad size-exclusion chromatography and Resource Q anion-exchange chromatography (Fig. 1). The elution profile showed three peaks according to molecular weight: folded complex (43 kDa; peak 2), aggregated products (peak 1) and bovine β₂m (peak 3) (Fig. 1a). The collected complex (peak 2) was further purified by Resource Q anion-exchange chromatography and eluted at a NaCl concentration of 11.0–14.0% (Fig. 1b). Bovine CD8αα was purified by Superdex 200 16/60 HiLoad size-exclusion chromatography (Fig. 1c). Peak 1 represents the folded CD8αα homodimer (27.2 kDa; peak 1).

After mixing and incubation, the CD8αα-BoLA-2*02201 complex was diluted to 5 and 10 mg ml⁻¹ for crystal screening. Crystals appeared after 10 d using Crystal Screen 2 condition No. 38

Figure 2
(a) Diffracting crystal of the bovine CD8αα-BoLA-I complex. (b) SDS-PAGE gel (15%) analysis of the crystal. Lane M contains molecular-weight markers (labelled in kDa).

Figure 3

Diffraction pattern of the CD8 α -BoLA-I complex. Spots corresponding to diffraction to high resolution are highlighted in the box.

[20% (w/v) polyethylene glycol 10 000, 0.1 M HEPES-NaOH pH 7.5] using a concentration of 10 mg ml⁻¹ at 291 K (Fig. 2a). A crystal was picked from the VDX plate and analysed by SDS-PAGE (Fig. 2b). The SDS-PAGE gel contained three bands corresponding to the molecular weights of BoLA-I heavy chain, b β ₂m and CD8 α . The crystal used for data collection diffracted to 1.7 Å resolution and belonged to space group $P2_1$, with unit-cell parameters $a = 53.9$, $b = 103.8$, $c = 61.8$ Å (Fig. 3). The space group was assigned based on systematic absences and molecular replacement. The asymmetric unit contained one complex, with a Matthews coefficient of 2.41 Å³ Da⁻¹ and a solvent content of 48.9% (Kantardjieff & Rupp, 2003; Matthews, 1968). Selected data statistics are shown in Table 3. The content of the CD8 α -BoLA-I crystal was confirmed by the rotation-function Z-score (RFZ) and translation function Z-score (TFZ) for molecular replacement (RFZ score 3.4, TFZ score 8.9). A packing

diagram of the complex is shown in Fig. 4. There are two CD8 α -BoLA-I complexes in the unit cell.

A multiple amino-acid sequence alignment of bovine CD8 α with other molecules is shown in Supplementary Fig. S1¹. The amino-acid sequence used in the alignment was the extracellular domain of CD8 α . Bovine CD8 α has 57.85, 27.50 and 20.69% identity to the CD8 α molecules from swine, chicken and carp, respectively, and shares 55.37 and 37.70% sequence identity with human and mouse CD8 α , respectively. This suggests that the bovine CD8 α has some differences in the amino acids that interact with the bovine MHC class I molecule (Supplementary Fig. S1).

The first structure of the bovine CD8 α -BoLA-I complex will be helpful in obtaining insight into the immunologically important interactions between bovine CD8 α and MHC class I molecule. In addition, determination of the BoLA-2*02201-FMDV-VP1_{YY9} structure will be useful for designing an epitope vaccine for foot-and-mouth disease.

This work was supported by grants from the National Key Basic Research Program of China (973 Program, grant No. 2013CB835302), the Graduate Student Scientific Research and Innovation Program of China Agricultural University (grant No. 2013YJ005) and the Key National Natural Science Foundation of China (grant No. 31230074). We thank Professor George F. Gao and Dr Jianxun Qi (Institute of Microbiology, Chinese Academy of Sciences) for much help and Shanghai Synchrotron Radiation Facility (SSRF; Shanghai, People's Republic of China) for the diffraction data of the crystals.

References

- Cheroutre, H. & Lambolez, F. (2008). *Immunity*, **28**, 149–159.
- Gao, G. F., Tormo, J., Gerth, U. C., Wyer, J. R., McMichael, A. J., Stuart, D. I., Bell, J. I., Jones, E. Y. & Jakobsen, B. K. (1997). *Nature (London)*, **387**, 630–634.
- Garboczi, D. N., Hung, D. T. & Wiley, D. C. (1992). *Proc. Natl Acad. Sci. USA*, **89**, 3429–3433.
- Guzman, E., Taylor, G., Charleston, B. & Ellis, S. A. (2010). *J. Virol.* **84**, 12375–12384.
- Kantardjieff, K. A. & Rupp, B. (2003). *Protein Sci.* **12**, 1865–1871.

¹ Supporting information has been deposited in the IUCr electronic archive (Reference: RL5069).

Figure 4

Ribbon packing diagram of the CD8 α -BoLA-I complex in the $P2_1$ crystal lattice viewed down the b axis. There are two complexes in the unit cell (shown in blue and magenta). The box depicts the unit cell.

- Kern, P. S., Teng, M.-K., Smolyar, A., Liu, J.-H., Liu, J., Hussey, R. E., Spoerl, R., Chang, H.-C., Reinherz, E. L. & Wang, J.-H. (1998). *Immunity*, **9**, 519–530.
- Li, X., Liu, J., Qi, J., Gao, F., Li, Q., Li, X., Zhang, N., Xia, C. & Gao, G. F. (2011). *J. Virol.* **85**, 6038–6048.
- Li, Y., Yin, Y. & Mariuzza, R. A. (2013). *Front. Immunol.* **4**, 206.
- Macdonald, I. K., Harkiolaki, M., Hunt, L., Connelley, T., Carroll, A. V., MacHugh, N. D., Graham, S. P., Jones, E. Y., Morrison, W. I., Flower, D. R. & Ellis, S. A. (2010). *PLoS Pathog.* **6**, e1001149.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Parkin, S. & Hope, H. (1998). *J. Appl. Cryst.* **31**, 945–953.
- Rudolph, M. G., Stanfield, R. L. & Wilson, I. A. (2006). *Annu. Rev. Immunol.* **24**, 419–466.
- Shi, Y., Qi, J., Iwamoto, A. & Gao, G. F. (2011). *Mol. Immunol.* **48**, 2198–2202.
- Yao, S., Qi, J., Liu, J., Chen, R., Pan, X., Li, X., Gao, F. & Xia, C. (2012). *Acta Cryst. F* **68**, 20–23.