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addenda and errata

Expression, purification and preliminary X-ray crystallographic analysis of the chicken MHC class I molecule YF1*7.1. Addendum

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Expression, purification and preliminary X-ray crystallographic analysis of the chicken MHC class I molecule YF1*7.1

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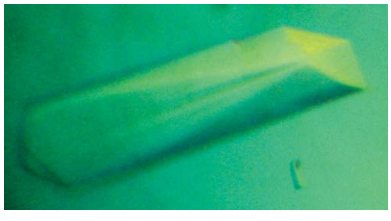

*YF1*7.1* is an allele of a polymorphic major histocompatibility complex (MHC) class I-like locus within the chicken *Y* gene complex. With the aim of understanding the possible role of the YF1*7.1 molecule in antigen presentation, the complex of YF1*7.1 heavy chain and β_2-microglobulin was reconstituted and purified without a peptide. Crystals diffracted synchrotron radiation to 1.32 Å resolution and belonged to the monoclinic space group $P2_1$. The phase problem was solved by molecular replacement. A detailed examination of the structure may provide insight into the type of ligand that could be bound by the YF1*7.1 molecule.

1. Introduction

The chicken *Y* system (previously known as *Rfp-Y*) was initially detected as an independently segregating polymorphic major histocompatibility complex (MHC)-like locus (Briles *et al.*, 1993). The *Y* and the *B* (the chicken MHC) systems are two genetically unlinked gene clusters that map to the same microchromosome (Briles *et al.*, 1993; Miller *et al.*, 1994, 1996; Fillon *et al.*, 1996). Comparable to the situation in the *B* cluster, several MHC genes map to the *Y* cluster, including at least two class I heavy chain loci (*YF1* and *YF2*) and three class IIβ genes (*YLB1*, *YLB2* and *YLB3*) (Zoorob *et al.*, 1993). The sequence identity between the Y class I proteins is about 93%, while values of about 63% are obtained when comparing them with the chicken B system class I proteins (BF). The sequence identity to the human HLA-A antigen, which like BF molecules displays peptides (Madden, 1995; Afanassieff *et al.*, 2001; Wallny *et al.*, 2006; Koch *et al.*, 2007), is still about 49%, but there is only a very limited sequence identity (∼20%) to mammalian and chicken MHC class I-like CD1 molecules (Miller *et al.*, 2005; Salomonsen *et al.*, 2005; Zajonc *et al.*, 2008).

YF transcripts are ubiquitously distributed in nearly all organs of both adult as well as embryonic chickens and are translated to yield a mature heavy chain that is associated with β_2-microglobulin (β_2m; Afanassieff *et al.*, 2001). YF class I antigens are dynamically expressed at levels comparable to but independently of BF class I molecules on erythrocytes, lymphocytes, granulocytes, monocytes and thrombocytes within the spleen of birds before and after hatching (Hunt *et al.*, 2006). These findings revealed that the expression of YF antigens is not restricted, as is observed for the products of many nonclassical class I genes in mammals (Shawar *et al.*, 1994). YF molecules could thus be more closely related to typical class I antigens than to nonclassical class I molecules, as already suggested by the close sequence identity between the two proteins.

YF1 antigens exhibit a number of substitutions of binding-groove residues that are usually conserved in the case of 'classical' class I proteins, indicating that YF molecules might present ligands that are different from the peptides bound by classical BF molecules. However, this remains speculative as ligands for YF molecules have so far not been identified. In addition, although the *Y* system genes have been associated with allograft rejection (Bacon & Witter, 1995; Pharr *et al.*, 1996; Thoraval *et al.*, 2003), resistance to Marek's disease virus (MDV; Wakenell *et al.*, 1996; Pharr *et al.*, 1997) and susceptibility to Rous sarcoma virus (RSV; LePage *et al.*, 2000; van der Laan *et al.*,

2004; Praharaj *et al.*, 2004), there is as yet no definitive proof for these associations and the identity of a cellular receptor for YF antigens is equally uncertain.

We have employed X-ray crystallography to shed light on the structural basis of the antigen-presenting properties of the YF1*7.1 antigen. A close examination of the molecule, in particular its ligand-binding site, may provide hints regarding the chemical nature of YF ligands and could thus help to find a function for the avian YF antigens.

2. Materials and methods

2.1. Protein preparation

The cDNA sequences encoding the extracellular domains of YF1*7.1 (residues 22–294 of the signal peptide-containing protein) and chicken β_2m (residues 21–120 of the signal peptide-containing protein) were cloned into the vector pMAL-p4x, expressed as a maltose-binding protein (MBP) fusion construct and purified using the pMAL purification system (New England Biolabs, Germany) with a number of modifications. A 1 l LB culture of *Escherichia coli* TB1 transformed with the YF1*7.1 or the β_2m construct was grown to an OD_{600} of 0.5. Protein expression was induced by adding 0.4 m*M* isopropyl β-D-1-thiogalactopyranoside and cells were incubated for 4 h at 303 K in the case of YF1*7.1 and ∼20 h at 298 K in the case of β_2m. They were harvested by centrifugation at 4500*g* for 10 min at 277 K.

For YF1*7.1, the cell pellet was resuspended in 30 ml lysis buffer [25% saccharose, 1 m*M* EDTA, 50 m*M* Tris–HCl pH 8.0 and 30 µ*M* phenylmethanesulfonyl fluoride (PMSF)] and frozen at 253 K. For preparation of inclusion bodies, the sample was thawed and incubated for 30 min on ice in the presence of 1.7 mg lysozyme. 1 µg ml^{-1} DNase I, 10 µ*M* $MgCl_2$ and 1 µ*M* $MnCl_2$ (all final concentrations) were then added and the cells were broken by sonication on ice for 3 min. The inclusion bodies released from the cells were collected by centrifugation at 10 000*g* for 10 min at 277 K and resuspended in 30 ml detergent buffer [200 m*M* NaCl, 1% deoxycholate, 1%(*v*/*v*) Nonidet-P40, 2 m*M* EDTA, 20 m*M* Tris–HCl pH 8.0 and 2 m*M* dithiothreitol (DTT)]. The sample was then subjected to sonication and centrifugation as above and the pellet was resuspended in 30 ml Triton buffer [100 m*M* NaCl, 1 m*M* EDTA, 0.5%(*v*/*v*) Triton X-100, 50 m*M* Tris–HCl pH 8.0 and 2 m*M* DTT], which was followed by another round of sonication and centrifugation. The pellet was washed three times with Triton buffer as before but without sonication. After the final washing step, the pellet was resuspended in 12 ml inclusion-body buffer (20 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl and 10 m*M* DTT) and the protein was concentrated by centrifugation at 10 000*g* for 10 min at 277 K, resuspended in 3 ml urea buffer [50%(*w*/*v*) urea, 50 m*M* NaCl and 20 m*M* Tris–HCl pH 7.5] and shaken for 30 min at room temperature. After centrifugation at 10 000*g* for 20 min at room temperature, the supernatant was collected.

In the case of β_2m, the MBP-fusion protein was extracted from the periplasm according to the pMAL purification protocol. The cell pellet obtained by centrifugation was resuspended and incubated in 100 ml buffer containing 30 m*M* Tris–HCl pH 8.0, 20% sucrose, 1 m*M* EDTA and 0.3 m*M* PMSF on a shaker for 10 min. After incubation, the sample was centrifuged at 8000*g* for 10 min at 277 K and the pellet was resuspended in 100 ml chilled 5 m*M* $MgSO_4$ followed by incubation for 10 min in an ice bath. The sample was then centrifuged at 8000*g* for 10 min at 277 K; the supernatant was collected and filtered through a 0.22 µm filter membrane (Millipore). The filtered sample was applied onto a 10 ml self-packed amylose resin (New England Biolabs, Germany) column, washed with 100 ml column buffer (20 m*M* Tris–HCl pH 7.5, 200 m*M* NaCl, 1 m*M* EDTA) and eluted with 30 ml column buffer containing 10 m*M* maltose. The eluate was collected in 1 ml fractions and those containing protein (as determined by spectrophotometry at 280 nm) were pooled.

The YF1*7.1-MBP and β_2m-MBP fusion proteins were mixed at a molar ratio of 1:1 (total volume of ∼10 ml) and reconstituted by adding the mixture to 500 ml of a buffer containing 400 m*M* arginine–HCl, 2 m*M* EDTA, 5 m*M* reduced glutathione, 0.5 m*M* oxidized glutathione, 100 m*M* Tris–HCl pH 7.5 as described by Garboczi *et al.* (1992). Notably, no peptide ligand was added to the mixture. After 2 d incubation at 277 K, the YF1*7.1-MBP–β_2m-MBP complex was collected and concentrated using an Amicon Ultra-15 protein centrifugal filter with 10 kDa cutoff (Millipore) before purification by gel-filtration chromatography using a Superdex 200 16/60 column on an ÄKTA FPLC instrument (GE Healthcare, Germany).

The purified YF1*7.1-MBP–β_2m-MBP complex was then cleaved with protease factor Xa (New England Biolabs, Germany) by incubation at 296 K for 48 h to remove the fused MBP at a complex:protease ratio of 100:1(*w*:*w*). The YF1*7.1–β_2m complex was separated from MBP using a 10 ml self-packed amylose resin column followed by purification on a Superdex 75 column using an ÄKTA FPLC instrument. The purities of the protein complexes were assessed by SDS–PAGE. The highly pure YF1*7.1–β_2m complex was used for crystallization trials at a concentration of 13 mg ml^{-1} in a buffer containing 50 m*M* NaCl and 20 m*M* HEPES pH 7.5. The concentration of the protein complex was determined by measuring the absorption at a wavelength of 280 nm, assuming an absorption coefficient of 1.92.

2.2. Crystallization and data collection

Initial crystallization trials using the sitting-drop vapour-diffusion technique were set up at 293 K (300 nl protein solution plus 300 nl reservoir solution equilibrated against 85 µl reservoir solution) using a Robbins Hydra II Plus One crystallization robot. The pHClear, Classics, JCSG+, Protein Complex, PEGs and PEGs II crystal screens from Qiagen were used for initial crystallization trials. Conditions producing protein crystals were optimized in a larger volume (1 µl protein solution plus 1 µl reservoir solution equilibrated against 500 µl reservoir solution) prepared manually using the hanging-drop technique. Typically, crystals appeared in 1 d and reached their maximal size after 3 d. The final crystallization conditions were 0.2 *M* ammonium acetate, 0.1 *M* sodium acetate pH 5.0, 20%(*w*/*v*) polyethylene glycol (PEG) 4000. Before flash-cooling in liquid nitrogen, crystals were briefly soaked in a cryosolution containing 0.2 *M* ammonium acetate, 0.1 *M* sodium acetate pH 5.0, 20%(*w*/*v*) PEG 4000 and 25%(*v*/*v*) glycerol.

Two X-ray diffraction data sets were collected from the same crystal at 100 K on beamline 14.1 at the BESSY II synchrotron facility in Berlin, Germany using a wavelength of 0.91841 Å. The beamline was equipped with an MX225 CCD mosaic detector (Rayonics LLC). The crystal of YF1*7.1–β_2m belonged to space group $P12_11$ and diffracted to a maximal resolution of 1.32 Å. A high-resolution data set consisting of 170 images was collected using an exposure time of 10 s per image, an oscillation range of 1° and a crystal-to-detector distance of 131.4 mm. To complete the overloaded reflections from the first data set, a second data set containing the same number of images was collected using an exposure time of 1 s, an oscillation range of 1° and a crystal-to-detector distance of 288.7 mm. The two

Table 1
Data-collection statistics for the YF1*7.1–β_2m complex.

Values in parentheses are for the highest resolution shell.

	Data set 1†	Data set 2†	Merged data set
Space group	$P12_11$		
Unit-cell parameters (Å, °)	a = 52.80, b = 55.47, c = 63.84, α = 90.00, β = 96.85, γ = 90.00		
Solvent content (%)	40.4		
Matthews coefficient‡ (Å^3 Da^{-1})	2.1		
Wavelength (Å)	0.91841	0.91841	0.91841
Resolution (Å)	19.11–1.32 (1.32–1.35)	19.75–2.50 (2.50–2.56)	19.75–1.32 (1.32–1.35)
R_{merge}§	0.035 (0.462)	0.026 (0.051)	0.044 (0.461)
$R_{r.i.m.}$¶	0.041 (0.542)	0.031 (0.060)	0.049 (0.541)
$R_{p.i.m.}$¶	0.022 (0.286)	0.016 (0.032)	0.022 (0.286)
Unique reflections	82130 (5825)	12580 (929)	82457 (5836)
$I/\sigma(I)$	20.60 (2.95)	38.35 (23.88)	20.22 (2.99)
Completeness (%)	95.4 (92.5)	97.5 (97.7)	95.9 (92.8)
Redundancy	3.62 (3.60)	3.60 (3.62)	4.15 (3.60)

† Data sets 1 and 2 correspond to the high- and low-resolution data set, respectively. ‡ According to Matthews (1968). § $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$. ¶ According to Weiss (2001).

data sets were integrated, scaled and merged using the *XDS* software package (Kabsch, 1993).

The molecular-replacement solution was obtained using the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994) program *MOLREP* (Vagin & Teplyakov, 1997). Refinement was performed using *REFMAC*5 (Murshudov *et al.*, 1997).

3. Results and discussion

Highly pure YF1*7.1–β_2m heterodimer was obtained following the expression of both chains in *E. coli* and a multistep purification and reconstitution procedure as described in §2 (see also Fig. 1). The YF1*7.1–β_2m complex could be reconstituted without a peptide ligand, thus distinguishing YF from other classical MHC class I molecules, in which a peptide is essential to stabilize the cell surface-expressed heterotrimer. Crystallization attempts at 293 K yielded several initial crystallization conditions which all contained PEG as the precipitant. After optimizing the conditions, well ordered crystals with typical dimensions of 250 × 50 × 30 µm (Fig. 2) could be obtained using 0.2 *M* ammonium acetate, 20%(*w*/*v*) PEG 4000 and 0.1 *M* sodium acetate pH 5.0. The crystals diffracted X-rays to a maximal resolution of 1.3 Å using synchrotron radiation (Fig. 3) and belonged to the monoclinic space group $P12_11$ (crystallographic data and X-ray data-collection statistics are summarized in Table 1). Assuming the presence of one YF1*7.1–β_2m complex in the asymmetric unit, the corresponding Matthews coefficient and solvent content (Matthews, 1968) were calculated to be 2.1 Å^3 Da^{-1} and 40%, respectively, based on a molecular mass M_r of 45 000 for the YF complex.

Initial phases could be obtained by the molecular-replacement approach using a peptide-stripped and water-stripped model of BF2*2101 as the search model (PDB code 3bev; Koch *et al.*, 2007). The electron-density map calculated from the initial phases showed one YF1*7.1–β_2m heterodimer in the asymmetric unit. After 20 cycles of restrained refinement, R_{work} and R_{free} values of 0.36 and 0.39, respectively, could be obtained within the resolution range

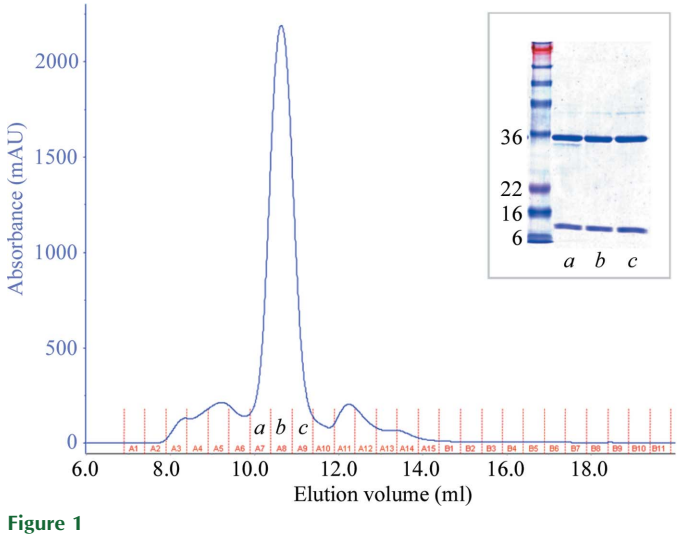

Figure 1
Assessment of the quality of the purified YF1*7.1–β_2m complex using size-exclusion chromatography and SDS–PAGE under reducing conditions (inset). The blue curve represents the sample absorbance at 280 nm; numbered red fields indicate the eluted fractions. The single symmetrical peak indicates the purity and homogeneity of the sample. Inset: SDS–PAGE under reducing conditions with fractions collected from FPLC. The two bands observed in each of lanes *a*, *b* and *c* represent YF1*7.1 heavy chain (31.4 kDa) and β_2m (11.6 kDa), respectively. Molecular-weight markers (kDa) are shown in the left lane.

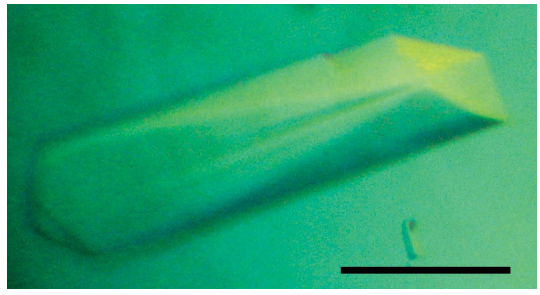

Figure 2
Photograph of the YF1*7.1–β_2m crystal used for the diffraction analyses. The scale bar indicates 100 µm.

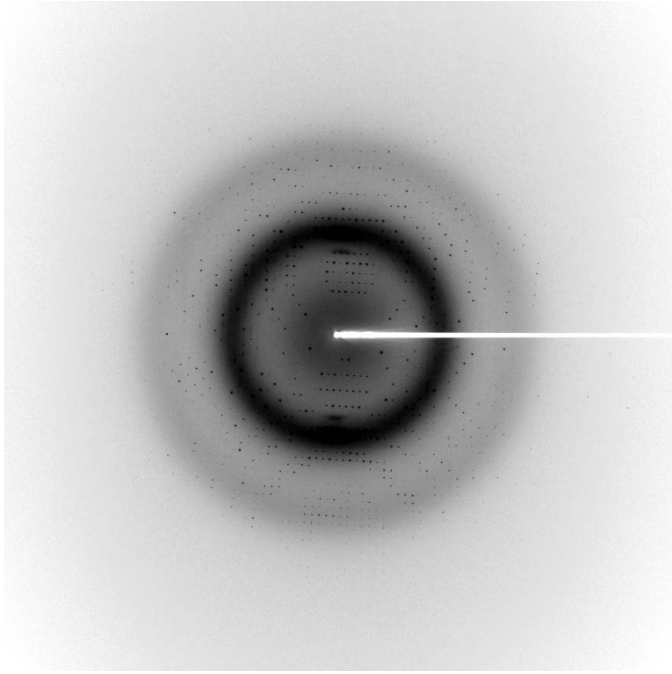

Figure 3
Diffraction pattern of the YF1*7.1–β_2m crystal.

19.75–1.32 Å with a correlation coefficient of 0.841, indicating that the molecular-replacement solution was correct.

Following the recent determination of the structures of two BF-2*2101–peptide complexes (Koch *et al.*, 2007), the YF1*7.1 structure will constitute the second example of a classical MHC molecule from a nonmammalian species. However, the obvious absence of a peptide ligand from its binding groove, confirmed after the initial refinement steps, dramatically distinguishes the YF1*7.1 molecule from the BF-2*2101 antigen. A detailed investigation of the structure of the YF1*7.1 complex is currently in progress and may shed light on the type of ligand(s) that could be bound by the molecule. This might in turn allow us to obtain insight into the cellular interaction partners of YF-bearing cells.

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