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Complex assembly, crystallization and preliminary X-ray crystallographic studies of duck MHC class I molecule

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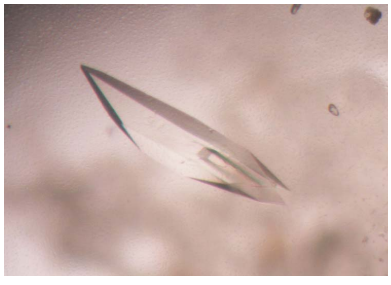
In order to understand the biological properties of the immune systems of waterfowl and to establish a system for structural studies of duck class I major histocompatibility complex (DuMHC I), a complex of DuMHC I with duck β_2-microglobulin (Duβ_2m) and the peptide AEIEDLIF (AF8) derived from H5N1 NP residues 251–258 was assembled. The complex was crystallized; the crystals belonged to space group $C222_1$, with unit-cell parameters a = 54.7, b = 72.4, c = 102.2 Å, and diffracted to 2.3 Å resolution. Matthews coefficient calculation and initial structure determination by molecular replacement showed that the crystals did not contain the whole DuMHC I complex, but instead contained the DuMHC I α3 domain and a Duβ2m molecule (DuMHC I α3+β2m). Another complex of DuMHC I with the peptide IDWFDGKE derived from a chicken fusion protein also generated the same results. The stable structure of DuMHC I α3+β2m may reflect some unique characteristics of DuMHC I and pave the way for novel MHC structure-related studies in the future.

1. Introduction

The largest waterfowl-breeding industry in China involves the duck, with a population of up to 20–30 billion per year. Moreover, wild ducks, as well as other aquatic birds, are a natural reservoir of influenza type A viruses and play an important role in the ecology and propagation of these viruses. Virus representatives of all 16 haemagglutinin (HA) and all nine neuraminidase (NA) subtypes have been isolated from waterfowl (Sturm-Ramirez *et al.*, 2005). Previous studies have shown that the H5N1 avian influenza virus (AIV) only causes asymptomatic or low-pathogenic infections in ducks (Perkins & Swayne, 2002). However, more and more evidence supports the facts that ducks not only show high susceptibility to H5N1 but also transmit the virus to other avian and mammalian hosts, including humans, and can cause outbreaks of severe disease (Perkins & Swayne, 2002; Zhou *et al.*, 2006). Therefore, further investigations into the immune system of ducks is needed, especially regarding the cellular immune response.

Major histocompatibility complex (MHC) class I molecules are critical for immune defences against viruses. These proteins present antigenic peptides to specific T-cell receptors (TCRs) on $CD8^+$ T cells, resulting in the activation of cytotoxic lymphocytes (CTL) and the subsequent lysis of target cells (Bjorkman & Parham, 1990; Garboczi *et al.*, 1996). The MHC I complex contains a heavy chain and a light chain. The heavy chain is comprised of α1, α2 and α3 domains, all of which have an approximate molecular weight of 11 kDa. The light chain (also called β_2-microglobulin; β_2m) is a member of the immunoglobulin superfamily with a molecular weight of 12 kDa. The reported crystal structures of MHC I reveal that the α1 and α2 domains together form a peptide-binding groove, with two α-helices at the top and an eight-stranded β-sheet at the bottom. The α3 domain noncovalently associates with β_2m beneath the α1/α2 domains (Saper *et al.*, 1991; Koch *et al.*, 2007).



Little was known about the duck MHC class I molecule (DuMHC I) until its cDNA sequence was reported in 2004 (Xia *et al.*, 2004). Further studies have demonstrated that ducks predominantly express one MHC class I gene (Moon *et al.*, 2005). However, structural analysis of DuMHC I has not yet been reported. In this article, we describe the expression, refolding and crystallization of the duck MHC class I molecule in order to obtain a better understanding of the biological properties of the waterfowl immune system. The diffraction data that we obtained from the MHC I crystal unveiled some unique characteristics of the duck MHC class I molecule which deserve further attention.

2. Material and methods

2.1. Preparation of DuMHC I and Duβ_2m as inclusion bodies

The expression vectors pET21a(+)-*DuMHC I* (GenBank accession No. AB115245, residues 1–270) and pET21a(+)-*Duβ_2m* (GenBank accession No. AB246408, residues 1–98) were constructed previously in the laboratory and transformed into *Escherichia coli* strain BL21 (DE3). The recombinant proteins were both expressed as inclusion bodies. The bacteria were harvested and suspended in cold phosphate-buffered saline (PBS). After sonication, the sample was centrifuged at 20 000*g* and the pellets were washed three times with a solution consisting of 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT and 0.5% Triton X-100. DuMHC I and Duβ_2m inclusion bodies were dissolved in guanidinium chloride (Gua–HCl) buffer [6 *M* Gua–HCl, 50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 10 m*M* EDTA, 10%(*v*/*v*) glycerine, 10 m*M* DTT].

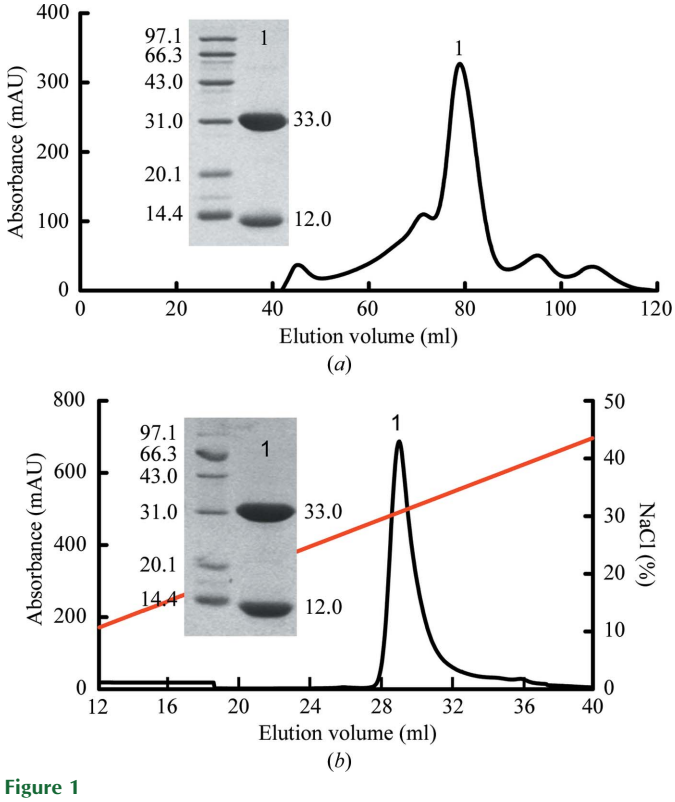


Figure 1
Purification of the refolded complex of DuMHC I heavy chain with Duβ_2m and AF8 peptide by FPLC Superdex 200 16/60 HiLoad gel-filtration and Resource Q anion-exchange chromatography (GE Healthcare). (*a*) Gel-filtration profile of the refolded products. Peak 1 represents the correctly refolded complex (45 kDa). Inset: reduced SDS–PAGE gel (15%) for peak 1. The left column contains molecular-weight markers (kDa). (*b*) Results of further purification of the refolded products by anion exchange; the peak was eluted at an NaCl concentration of 28.5–34.0%. Insert: reduced SDS–PAGE gel (15%) of corresponding purified protein.

2.2. Refolding of the DuMHC I complex

The preparation of the DuMHC I–AF8–β_2m complex was carried out essentially as described previously by Garboczi *et al.* (1992) with modifications introduced in our laboratory (Zhou *et al.*, 2004; Chu *et al.*, 2005). The synthetically prepared H5N1-derived peptide AF8 (AEIEDLIF) was dissolved in dimethyl sulfoxide (DMSO). DuMHC I, Duβ_2m and AF8 in a 1:1:3 molar ratio were refolded using the gradual solution method. After incubation for 48 h at 277 K, the remaining soluble portion of the complex was concentrated and then purified by chromatography on a Superdex 200 16/60 HiLoad (GE Healthcare) size-exclusion column followed by Resource Q (GE Healthcare) anion-exchange chromatography under nonreducing conditions.

2.3. Crystallization of the DuMHC I complex

The purified protein complex (45 kDa) was dialyzed against crystallization buffer (10 m*M* Tris–HCl pH 8.0, 10 m*M* NaCl) and concentrated to 15 mg ml^{-1}. Crystallization trials were set up with PEG/Ion screen (Hampton Research) at 291 K using the hanging-drop method. Three drops containing equal volumes (1 µl each) of protein solution (at 5, 10 and 15 mg ml^{-1}) and reservoir crystallization buffer were placed over a well containing 200 µl reservoir solution using a VDX plate (HR3-142; Hampton Research). Crystals were obtained in 5–7 d using solution No. 14 (0.2 *M* potassium thiocyanate, 12% PEG 3350 pH 7.0). The crystals obtained using a protein concentration of 10 mg ml^{-1} were suitable for data collection.

2.4. Data collection and processing

Data collection was performed in-house using a Rigaku MicroMax007 rotating-anode X-ray generator operated at 40 kV and 20 mA (Cu $K\alpha$; λ = 1.5418 Å) and equipped with an R-AXIS VII^{++} image-plate detector. The crystals were soaked for 20–30 s in reservoir solution supplemented with 20% glycerol as a cryoprotectant and then flash-cooled directly in liquid nitrogen. A complete data set was collected to 2.3 Å resolution. Data were indexed and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The Matthews coefficient and solvent content were calculated with *MATTHEWS_COEF* (Matthews, 1968; Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

3.1. Strategy of peptide design

The peptide-binding motif of duck MHC class I (DuMHC I) is not very clear. As both duck and chicken belong to the avian family, we tested the known peptide IE8 (IDWFDGKE) and a predicted peptide AF8 (AEIEDLIF from H5N1 NP residues 251–258) based on the peptide-binding motif of the chicken MHC class I molecule (Wallny *et al.*, 2006). Both peptides worked beautifully in refolding DuMHC I.

3.2. Refolding and purification of the DuMHC I complex

DuMHC I could be refolded in the presence of Duβ_2m and peptide AF8. The refolding resulted in a yield of approximately 15% of the correctly folded complex, which could be purified to homogeneity by Superdex 200 16/60 HiLoad size-exclusion chromatography and Resource Q anion-exchange chromatography (Fig. 1). The chroma-

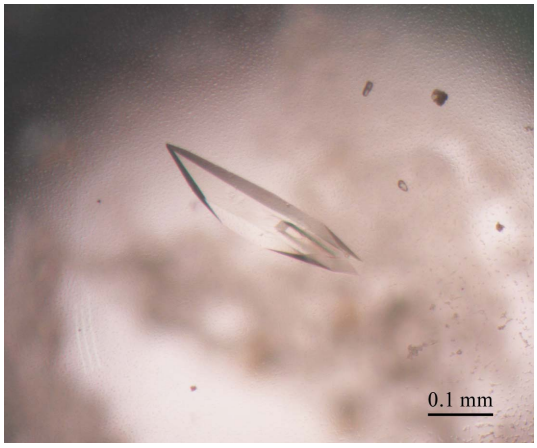


Figure 2
Typical appearance of a crystal in the hanging drop.

tographic profile showed a primary peak corresponding to the expected refolded complex (peak 1, 45 kDa; Fig. 1*a*). The refolded complex was further purified by Resource Q chromatography and eluted at an NaCl concentration of 28.5–34.0% (Fig. 1*b*). Subsequent reducing SDS–PAGE analysis showed two bands corresponding to the expected molecular weights of the heavy chain (33 kDa) and of β_2m (12 kDa).

3.3. Ideal crystals suitable for data collection

After purification and concentration, the DuMHC I complex protein was set up for crystal screening. Ideal crystals appeared in 5–7 d under the initial conditions (Fig. 2). The crystals belonged to space group $C222_1$, with unit-cell parameters a = 54.7, b = 72.4, c = 102.2 Å, and diffracted to 2.3 Å resolution. Data statistics are shown in Table 1.

3.4. Initial structure determination

To determine the number of molecules per asymmetric unit, we calculated the Matthews coefficient, the solvent content and the molecular weight of the molecules in the unit cell. As the molecular weight of the DuMHC I complex is about 45 kDa, we found that the unit cell of the obtained crystal could not accommodate the whole DuMHC I complex.

Further molecular replacement (Collaborative Computational Project, Number 4, 1994) using different models, such as the complete MHC I complex, the heavy chain of MHC I only, β_2m only, the α1 and α2 domains of the heavy chain and the α3 domain of the heavy chain plus β2m, confirmed that the protein in the crystal was not the whole MHC class I molecule but consisted of $\alpha3$+β_2m (23 kDa). Based on this information, the solvent content was about 42% and the Matthews coefficient was 2.11 Å^3 Da^{-1}, which is reasonable. We also prepared DuMHC I protein using another peptide IE8 (IDWF-DGKE), derived from a chicken fusion protein, and obtained the same results.

The initial DuMHC I $\alpha3$+β_2m structure was determined by molecular replacement using *MOLREP* from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994; Lebedev *et al.*, 2008) with the structure of the chicken MHC class I molecule BF2*2101 (PDB code 3bev, with the peptide, α1 and α2 domains excluded; Koch *et al.*, 2007) as the search model. Duβ_2m was located first and the DuMHC I α3 domain was then found in a rotation- and translation-function search with Duβ_2m fixed. The molecular-replacement solution had an initial R factor of 0.441 and a correlation coefficient of 0.603. In terms of the initial R factor and correlation coefficient, solution of the crystal structure of DuMHC I $\alpha3$+$\beta2$m has been achieved.

Table 1
X-ray diffraction data and processing statistics.

Values in parentheses are for the highest resolution shell.

Space group	$C222_1$
Unit-cell parameters (Å)	a = 54.7, b = 72.4, c = 102.2
Resolution range (Å)	50.00–2.30 (2.36–2.30)
Total No. of reflections	92739
No. of unique reflections	12376
Average redundancy	7.5 (7.2)
Completeness (%)	99.8 (98.3)
$R_{\rm merge}$† (%)	7.0 (30.0)
Average $I/\sigma(I)$	35.8 (8.9)

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

Unravelling the structural basis of the molecules involved in cellular immune responses is crucial for functional studies and drug/vaccine design. Although the reason for the degradation of DuMHC I into its subparts ($\alpha3$+β_2m) during crystallization is not clear, the stability of duck MHC I $\alpha3$+β_2m has been proved. After the final structure has been released, the contact characteristics of α3 and β_2m will be available, paving the way for novel MHC-structure related studies in the near future.

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