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Protein expression, crystallization and preliminary X-ray crystallographic analysis of chicken interferon-γ receptor α chain

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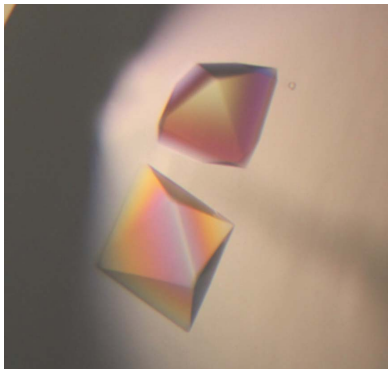


The activity of interferon-γ (IFN-γ) relies on signal transduction, which is triggered by combination with the receptors interferon-γ receptor α chain (IFNGR1) and β chain (IFNGR2). Native recombinant chicken IFNGR1 (chIFNGR1; residues 25–237) was overexpressed in *Escherichia coli*, purified by refolding and crystallized using the vapour-diffusion technique. The crystals belonged to space group $P6_522$, with unit-cell parameters $a = b = 64.1$, $c = 216.3$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. The Matthews coefficient and solvent content were calculated as 2.67 Å^3 Da^{-1} and 53.97%, respectively. X-ray diffraction data for chIFNGR1 were collected to 2.0 Å resolution at a synchrotron source.

1. Introduction

Interferon-γ (IFN-γ) is recognized as a key component in immunosurveillance, immunoregulation and defence against viruses (Staeheli, 1990; Kerr & Stark, 1992; Platanias, 2005; Sadler & Williams, 2008) and is produced by natural killer (NK) cells (Perussia, 1991) and T cells (Blackett *et al.*, 1989; Sad *et al.*, 1995). IFN-γ has two receptors: interferon-γ receptor α chain (IFNGR1) and β chain (IFNGR2) (Bach *et al.*, 1997). Both IFNGR1 and IFNGR2 belong to the class II cytokine receptor family, which consists of 11 characterized receptors, and are used for signalling by members of the interleukin 10 (IL-10) and interferon (IFN) ligand families (Kotenko *et al.*, 2003). This class of receptors bind the associated ligands in the small angle of a V formed by the two Ig-like folds (Bazan, 1990; Thoreau *et al.*, 1991). In order to determine the ligand–receptor interactions and crystallization characteristics of chicken IFN-γ (chIFN-γ) and chicken interferon-γ receptor α chain (chIFNGR1), chIFNGR1 was cloned and identified using the rapid amplification of cDNA ends (RACE) method. The chIFNGR1 protein was expressed in *Escherichia coli* and was further characterized by mass spectrometry and circular-dichroism (CD) spectroscopy (Han *et al.*, 2008).

The biological activity of IFN-γ relies on binding to its two receptors. In the case of human IFN-γ, binding of IFN-γ to IFNGR1 and IFNGR2 is necessary in the IFN-γ signal transduction pathway. It has been reported that IFN-γ possesses high binding affinity ($K_d = 10^{-8}$–10^{-10} M) for IFNGR1 (Marsters *et al.*, 1995; Walter & Nagabhushan, 1995; Green *et al.*, 1998). The IFN-γ dimer can also directly interact with IFNGR1 (Ealick *et al.*, 1991; Fountoulakis *et al.*, 1992; Chène *et al.*, 1995), as shown by the solution of the crystal structure of their complex (Walter *et al.*, 1995; Randal & Kossiakoff, 1998; Acebrón *et al.*, 2009). Furthermore, specific interaction of IFN-γ with its receptors is crucial in the IFN-γ/signal transducer and activator of transcription (STAT) signalling pathway cascade. Firstly, a symmetrical tetramer consisting of two IFNGR1 subunits and two IFNGR2 subunits binds to an IFN-γ homodimer; the intracellular domains of the receptor subunits then become close and combine with Janus tyrosine kinases (JAKs) and STAT. Finally, these large complexes enter the nuclei of the target cells, thus propagating signal transduction (Farrar *et al.*, 1991; Hashimoto *et al.*, 1994; Greenlund *et al.*, 1995; Sakatsume *et al.*, 1995; Bach *et al.*, 1996; Kaplan *et al.*, 1996; Kotenko *et al.*, 2003). Therefore, understanding the crystallographic properties of chIFNGR1 could help in determination of the structure

of chicken IFNGR1, which could pave the way to solution of the structure of a complex consisting of chicken IFN-γ and chIFNGR1 and also help in determining the signalling pathway of chicken IFN-γ.

Here, we report the cloning of chIFNGR1 and its expression in prokaryotic cells as inclusion bodies; after gel-filtration purification, native crystals of chIFNGR1 were obtained. Diffraction data were collected to 2.0 Å resolution from crystals cryoprotected with glycerol.

2. Materials and methods

2.1. Cloning of chIFNGR1

The chIFNGR1 gene (GenBank accession No. NP_001123859.1) was obtained by the rapid amplification of cDNA ends (RACE) method as described previously (Han *et al.*, 2008). The gene encoding chIFNGR1 extracellular region (residues 25–237) was amplified using the primers 5′-CGC**GGATCC**GAGCGTCTTCCCGCAGTGCCT-3′ (forward) and 5′-CCG**CTCGAG**TCAAGCCTGCGTGATAGGA-AC-3′ (reverse) (AuGCT Corporation, Beijing, People's Republic of China) containing sites for restriction endonucleases *Bam*HI and *Xho*I (shown in bold), respectively. The primers were then subcloned into pET-21a (Novagen) with a stop codon at the C-terminus. The recombinant plasmid chIFNGR1/pET-21a was transformed into DH5α (Novagen) for plasmid amplification and identified by PCR, double enzyme digestion and DNA sequencing (AuGCT Corporation).

2.2. Expression and purification

ChIFNGR1/pET-21a was expressed in *Escherichia coli* Rosetta (DE3) (Novagen). The cells were grown in LB at 310 K with 100 mg ml^{-1} ampicillin until the OD_{600} reached 0.8. Induction was performed by the addition of 1 m*M* IPTG followed by growth at 295 K for a further 5 h. Cells containing inclusion bodies were harvested by centrifugation and resuspended in ice-cold washing buffer consisting of 20 m*M* Tris–HCl, 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT, 0.5% Triton X-100 pH 8.0. The cell pellet was disrupted using an ultrasonic cell crusher (Scientz Biotechnology, Ningbo, People's Republic of China) to obtain the purified inclusion bodies.

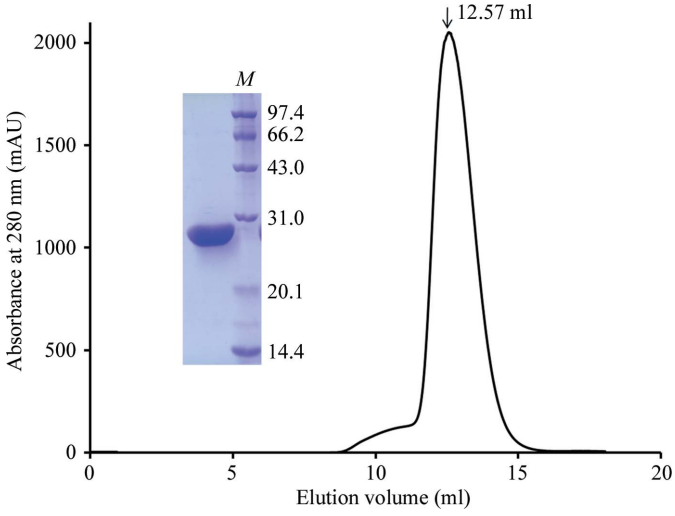
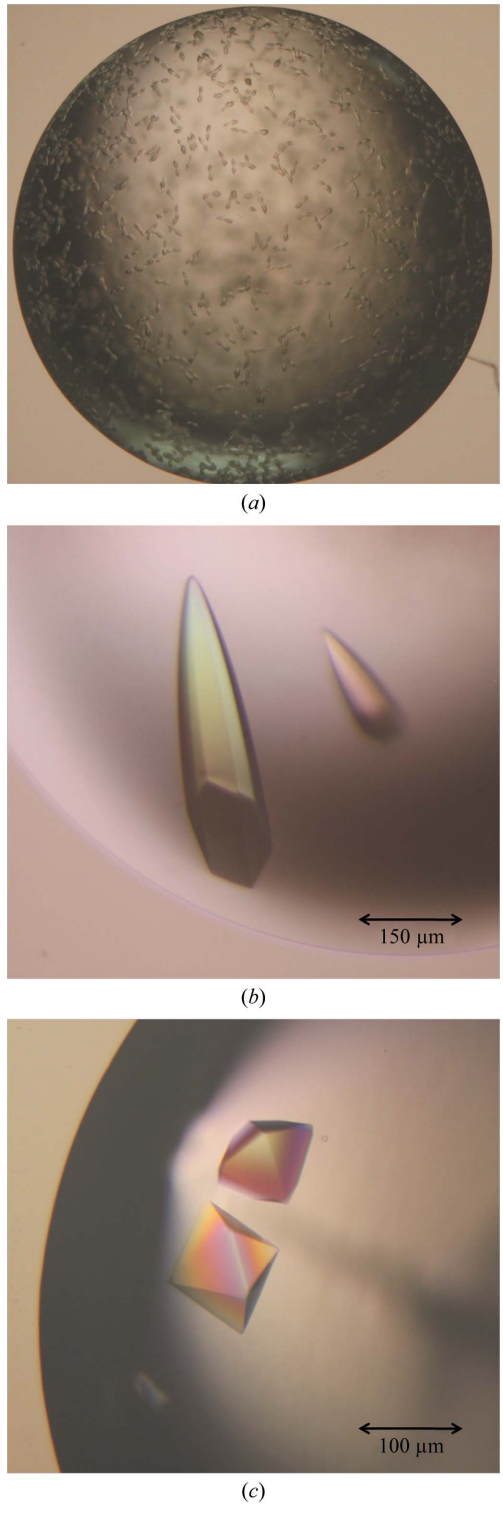


Figure 1
Refolded chIFNGR1 was purified by gel-filtration chromatography. The purity and molecular weight of chIFNGR1 (24 kDa) were determined by 15% SDS–PAGE after gel-filtration chromatography purification (inset). Lane *M*, molecular-weight marker (labelled in kDa).

To refold the protein, the inclusion bodies were dissolved (~30 mg ml^{-1}) in 6 *M* guanidine–HCl (with 50 m*M* Tris–HCl,



(*a*)

(*b*)

(*c*)

Figure 2
Crystals of chIFNGR1. (*a*) Initial clusters of chIFNGR1 crystals. (*b*) Single crystals of chIFNGR1 were obtained after optimization in 20%(*v*/*v*) polyethylene glycol 5000 monomethyl ether, 0.1 *M* HEPES, 0.5 *M* sodium carbonate pH 7.0. Only the crystals with a sword-like shape were able to diffract X-rays to 2.0 Å resolution. (*c*) Cubic-shaped single crystals of chIFNGR1 were obtained using 1.26 *M* sodium phosphate monobasic monohydrate, 0.14 *M* potassium phosphate dibasic pH 5.6 but diffracted X-rays to less than 4 Å resolution.

100 m*M* NaCl, 10 m*M* EDTA, 10% glycerol, 10 m*M* DTT pH 8.0) and the insoluble material was separated by centrifugation at 10 000*g* for 15 min at 277 K. Subsequently, 3–5 ml of the supernatant was dropped into 0.5–1 l refolding buffer consisting of 100 m*M* Tris, 400 m*M* L-arginine, 2 m*M* EDTA, 5 m*M* reduced glutathione, 0.5 m*M* oxidized glutathione pH 8.0 using the needle of a 1 ml injector. Finally, the refolded product was concentrated using an Amicon Stirred Cell 8400 (Millipore) and the buffer of the prepared sample was exchanged to gel-filtration buffer consisting of 20 m*M* Tris–HCl, 50 m*M* NaCl pH 8.0. The sample was then centrifuged at 20 000*g* for 15 min prior to further analysis.

The refolded chIFNGR1 was initially purified using a Resource Q anion-exchange column (6 ml; GE Healthcare) and was then applied onto a HiLoad 16/60 Superdex 75 column (GE Healthcare) on an ÄKTAexplorer system (GE Healthcare) to separate misfolded aggregates and impurities. The column was pre-equilibrated with gel-filtration buffer (as described above). The purified chIFNGR1 protein was concentrated and its concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific). The protein solution was then used for screening of crystallization conditions.

2.3. Crystallization

The ChIFNGR1 protein was concentrated to 13 mg ml^{-1} by centrifugation with an Amicon Ultra-15 centrifugal filter device (10 kDa cutoff, Millipore). The protein buffer was exchanged to 10 m*M* Tris–HCl, 10 m*M* NaCl pH 8.0. Screening of crystallization conditions was carried out by mixing 1 µl protein solution and 1 µl reservoir solution in 16-well Linbro plates (Tianyu, Tianjin, People's Republic of China) at 291 K. Crystallization screening kits I and II (XtalQuest Inc., Beijing, People's Republic of China) and Index (Hampton Research, California, USA) were used for crystallization trials.

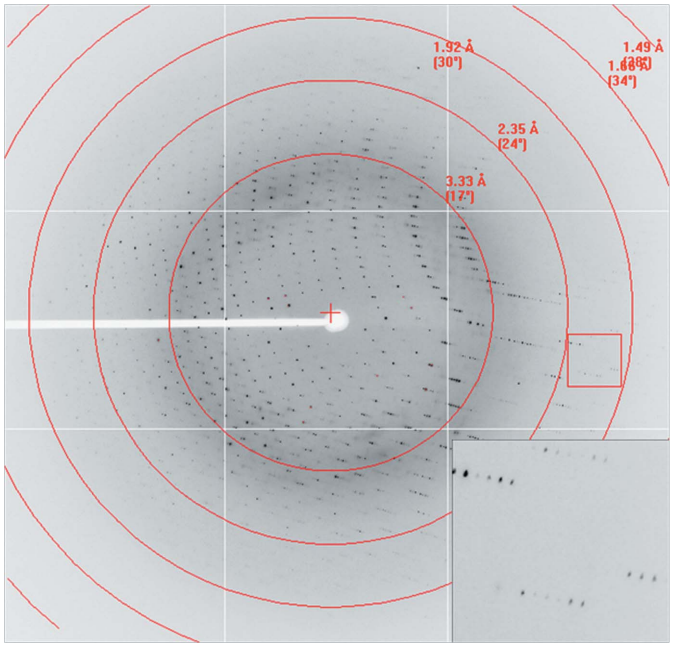


Figure 3
X-ray diffraction pattern of a native chIFNGR1 crystal. The sword-shaped native chIFNGR1 crystal diffracted to a resolution of about 2.0 Å.

Table 1
Crystallographic parameters and data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P6_522$
Unit-cell parameters (Å, °)	$a = b = 64.1$, $c = 216.3$, $\alpha = \beta = 90$, $\gamma = 120$
Wavelength (Å)	0.97908
Resolution (Å)	50.00–2.00 (2.07–2.00)
No. of reflections	361378
Matthews coefficient V_M (Å^3 Da^{-1})	2.67
No. of atoms in the asymmetric unit	1791
No. of residues in the asymmetric unit	220
Solvent content (%)	53.97
Multiplicity	20.8 (19.2)
Completeness (%)	100.0 (96.8)
R_{merge}† (%)	0.469 (0.143)
$\langle I/\sigma(I)\rangle$	20.6 (14.3)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th observation of the unique reflection *hkl* and $\langle I(hkl)\rangle$ is the mean of the intensities of all observations of reflection *hkl*.

2.4. X-ray data collection and processing

Single crystals were transferred to the corresponding reservoir solution containing 20%(*v*/*v*) glycerol and cooled in liquid nitrogen. Data were collected at 100 K on beamline BL17U1 (wavelength 0.98 Å) at Shanghai Synchrotron Radiation Facility (SSRF).

3. Results and discussion

To eliminate interference from a His tag, the extracellular fragment (residues 25–237) of chIFNGR1 was constructed in pET-21a vector with a stop codon at the C-terminus. *E. coli* Rosetta strain was used to compensate for differences in codon usage by the expression of rare codons (Bane *et al.*, 2007). The chIFNGR1 inclusion bodies were extensively washed and refolded in an appropriate buffer and further purified by gel-filtration chromatography. The purity and molecular weight of chIFNGR1 (24 kDa) were determined by 15% SDS–PAGE (Fig. 1).

The initial chIFNGR1 crystals appeared using a solution consisting of 20%(*v*/*v*) polyethylene glycol 5000 monomethyl ether, 0.1 *M* HEPES, 0.5 *M* sodium carbonate pH 7.0 in three weeks at 291 K (Fig. 2*a*). Optimization was carried out by adjusting the pH, salt and precipitant concentration of the reservoir and single crystals of chIFNGR1 with sharper edges and dimensions of about 0.5 × 0.1 × 0.04 mm were obtained in 14 d (Fig. 2*b*). These crystals diffracted to a resolution of 2.0 Å (Fig. 3). During the optimization procedure, cubic-shaped crystals of chIFNGR1 also grew in 1.26 *M* sodium phosphate monobasic monohydrate, 0.14 *M* potassium phosphate dibasic pH 5.6 (Fig. 2*c*). However, diffraction data were only collected to 4.0 Å resolution from these crystals.

The high-resolution data from the chIFNGR1 crystal were processed and scaled using the *HKL*-2000 (Otwinowski & Minor, 1997) and *CCP*4 (Winn *et al.*, 2011) program suites. The crystals of chIFNGR1 belonged to space group $P6_522$, with unit-cell parameters $a = b = 64.1$, $c = 216.3$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. The total number of reflections and the number of atoms and residues in the asymmetric unit were 361 378, 1791 and 220, respectively. The Matthews coefficient and the solvent content were calculated as 2.67 Å^3 Da^{-1} and 53.97%, respectively. The data-processing statistics are given in Table 1.

The crystallization and preliminary X-ray crystallographic studies of chIFNGR1 provide the possibility of solving its structure. It is hoped that the phase can be determined *via* expression and purification of selenomethionine-substituted chIFNGR1 using a modified

methionine-pathway inhibition procedure (Begley *et al*., 2003). The solved structure of chIFNGR1 could form the basis for structure determination of the complex of chicken IFN-γ and chIFNGR1, which could help in determining the signal transduction pathway of chicken IFN-γ.

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