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Crystallization and preliminary X-ray diffraction analysis of a recombinant cysteine-free mutant of crmA

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CrmA is an unusual serpin that has a reactive-center loop one residue shorter than other members of the superfamily. Most interestingly, crmA has inhibitory activity against both cysteine and serine proteinases involved in the regulation of cell apoptosis. The three-dimensional structure of crmA will give insight into the mechanism that this serpin employs to inhibit both cysteine and the serine proteinases, as well as help to explain the significance of the shorter reactive-center loop. The monodisperse cysteine-free mutant of crmA was crystallized in the presence of phosphate salts. Crystals diffract to 2.90 Å and belong to space group $P2_12_12_1$, with unit-cell parameters a = 42.67, b = 93.15, c = 101.63 Å.

1. Introduction

Serpins are a superfamily of proteins that include many serine proteinase inhibitors which inhibit their target proteinases through a major conformational change in which the reactive-center loop, upon cleavage by target proteinase, is inserted into β-sheet A (Stratikos & Gettins, 1999). Although the cowpox virus cytokine response modifier A protein (crmA) is a member of the serpin superfamily (Ray *et al.*, 1992; Komiyama *et al.*, 1994), it inhibits both cysteine and serine proteinases.

Interestingly, all known target enzymes of crmA, among which are granzyme B (Quan *et al.*, 1995), interleukin-1β converting enzyme (ICE; Ray *et al.*, 1992; Komiyama *et al.*, 1994) and caspase-4, -5, -8, -9 and -10 (Garcia-Calvo *et al.*, 1998), are involved in the regulation of activation-induced programmed cell death (APCD). For instance, it has been demonstrated that crmA inhibits cytotoxic T-lymphocyte-mediated killing (Tewari & Dixit, 1995; Zhou *et al.*, 1997) and abrogates an interleukin-1β response to infection (Ray *et al.*, 1992; Komiyama *et al.*, 1994). CrmA is thus used by the virus to efficiently combat host immune surveillance by inhibiting both serine and cysteine proteinases involved in regulation of inflammatory and apoptotic processes.

As a serpin, crmA is unusual not only by having 'cross-class' inhibitory properties, but also by having a reactive-center loop one residue shorter than all other members of the superfamily. This is mechanistically significant, since the reactive-center loop inserts into β-sheet A as part of the inhibitory mechanism and its precise length is thought to be critical for successful inhibition. The three-dimensional structure of crmA will show the consequences of the shorter reactive-center loop for interaction with target proteinases. Additionally, the structure will give insight into both the inhibitory mechanism of serine proteinases as well as the mechanism of 'cross-class' interaction between crmA and cysteine proteinases. Finally, crmA is the smallest serpin known and its structure will represent the minimal structural core required for serpin function.

In this paper, we report the crystallization and preliminary crystallographic studies of the recombinant cysteine-free mutant of crmA from cowpox virus. Crystals of crmA have been obtained and a complete native data set has been collected to 2.9 Å.

2. Materials and methods

2.1. Protein expression and purification.

The cysteine-free mutant of crmA was constructed by a series of site-directed mutagenesis changes of the wild-type crmA using the Quick-Change Kit (Stratagene). Primers for site-directed mutagenesis were constructed so that all cysteines were replaced by serines. Mutations were confirmed by DNA sequencing. The protein was expressed in the form of inclusion bodies in *Escherichia coli* at 310 K using the pQE-60 expression system (Quiagen). Inclusion bodies were isolated and denatured using a high concentration of guanidine. The protein was refolded by dilution in phosphate buffer as previously described for the serpin α_1-proteinase inhibitor (Stratikos & Gettins, 1998). Refolded protein was loaded onto a DEAE–Sephacel column and eluted with a linear 0–1 *M* NaCl gradient in Na_2HPO_4 buffer pH 6.50. Fractions containing crmA were pooled and dialyzed overnight against 20 m*M* Tris pH 8.00, 20 m*M*

Table 1
Data-collection and processing statistics.

Values in parentheses correspond to the highest resolution shell (3–2.9 Å).

	$P2_12_12_1$ crystal form
Unit-cell parameters (Å)	a = 42.67, b = 93.15, c = 101.63
Crystal-to-detector distance (mm)	200
Maximum resolution (Å)	2.90
Rotation for each exposure (°)	0.5
Time for each image (s)	10
Total rotation for data set (°)	180
Number of measured reflections	318152
Number of unique reflections	9406
$R_{\rm merge}$† (%)	11.2 (42)
Multiplicity	5.8 (5.7)
Completeness (%)	99.9 (99.9)
$I > 3\sigma(I)$ (%)	82.2 (55.3)
Observable data (%)	98.1 (93.8)
Average $I/\sigma(I)$	15.5 (4.9)

† $R_{\rm merge} = \sum\sum|I(h)_i - \langle I(h)\rangle|/\sum\langle I(h)\rangle$; $I(h)$ is the observed intensity of the ith measurement of reflection h and $\langle I(h)\rangle$ is the mean intensity of reflection h calculated after loading and scaling.

NaCl and were then loaded onto a high-performance Q-Sepharose column (Pharmacia). The protein eluted as a single peak with a linear gradient of 0.25–0.45 *M* NaCl in 20 m*M* Tris pH 8.00. The purity and homogeneity of the sample was confirmed both by 10% SDS–PAGE (Laemmli, 1970) and a 10% native gel (Bowen *et al.*, 1997). The activity of the refolded and purified cysteine-free mutant of crmA was tested by continuous kinetic assay using the fluorogenic substrate acetyl-YVAD-7-amido-4-methylcoumarin (Bachem). Assays were performed in 0.1 *M* Na HEPES pH 7.5, 0.1 m*M* EDTA, 5 m*M* DTT at 298 K (Tesch *et al.*, 2000). Purified crmA was dialyzed twice against 50 m*M* Tris pH 7.40, 20 m*M* NaCl and was concentrated to 20 mg ml^{-1} using an Ultrafree-4 centrifugal filter unit (Millipore). The protein was flash-frozen in dry ice and was kept at 203 K until use.

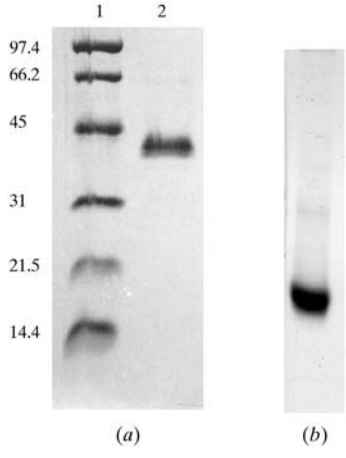

Figure 1
Analysis of the cysteine-free mutant of crmA upon purification on a high-performance Q-Sepharose column (linear gradient of 0.25–0.45 *M* NaCl with 20 m*M* Tris pH 8.00). (*a*) 10% SDS–PAGE. The cysteine-free mutant of crmA has been purified to homogeneity (lane 2). Molecular-weight markers were loaded in lane 1 (numbers correspond to molecular weights in kDa). (*b*) 10% native gel demonstrates that the purified crmA is in monomeric form.

2.2. Dynamic light-scattering experiments

Measurements of the molecular weight and hydration radius of the cysteine-free mutant of crmA were carried out by dynamic light scattering (DynaPro-801WIN, Protein Solutions). The protein was at a concentration of 3 mg ml^{-1} in 50 m*M* Tris pH 7.40, 20 m*M* NaCl. The radius of hydration was calculated according to a standard curve for proteins of 24–110 kDa, while the molecular weight was calculated from a standard curve for globular proteins.

2.3. Crystallization

Crystallization experiments were carried out at constant temperature of 293 K using the hanging-drop vapour-diffusion method. All precipitant solutions were filtered prior to use and all contained 0.02%(*w*/*v*) sodium azide. The initial protein concentration was 20 mg ml^{-1} in 50 m*M* Tris pH 7.40, 20 m*M* NaCl. Drops were prepared by mixing equal volumes of the protein solution with the well solution. The mixture was allowed to equilibrate against 1 ml reservoir solution.

2.4. X-ray diffraction analysis

Crystals were harvested from drops, soaked for 15–30 s in mineral oil (Sigma), which served as a cryoprotectant, mounted on nylon loops (Hampton Research) and flash-frozen in liquid N_2. X-ray diffraction data were collected at liquid-N_2 temperature on the BioCars synchrotron beamline X-ray source (Advanced Photon Source, Argonne National Laboratories) at a wavelength of 1 Å using a CCD-Q4 detector. The data were indexed with *DENZO* (Otwinowski & Minor, 1997) and scaled and reduced with *SCALEPACK* (Otwinowski & Minor, 1997).

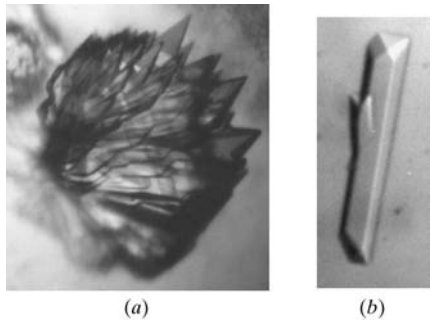

Figure 2
(*a*) A crystal cluster and (*b*) a single crystal of the cysteine-free mutant of crmA (same scale). Crystals were formed over a month by the hanging-drop vapour-diffusion method when drops were equilibrated against 1 ml 0.1 *M* Na HEPES pH 7.50, 1.6 *M* Na/KH_2PO_4. The crystal in (*b*) was 150 µm long.

3. Results and discussion

Initial crystallization attempts were carried out with the wild-type crmA, which contains nine cysteines but no disulfides. However, dynamic light-scattering experiments showed that the sample was polydisperse even in the presence of high concentrations of (−)-1,4-dithio-L-threitol. A cysteine-free mutant of crmA was therefore prepared by a series of site-directed mutagenesis changes and the protein was expressed and purified to homogeneity (Fig. 1). Dynamic light-scattering experiments confirmed that the sample was monodisperse, with a polydispersity parameter less than 20% of the radius of hydration and a calculated molecular weight of 41 kDa (Protein Solutions). Kinetic studies of the refolded cysteine-free mutant with ICE demonstrated that the mutant crmA had the same inhibitory activity as wild-type crmA (Tesch *et al.*, in preparation). However, a secondary peak from the last chromatographic step contained oligomers of higher order.

The sparse-matrix screening protocol (Jancarik & Kim, 1991) was used as a starting point in determining the optimal conditions for crystal growth at a constant temperature of 293 K. After a month, showers of haystack needles grew from a light precipitate in a drop that was equilibrated against 0.1 *M* Na HEPES pH 7.50, 1.6 *M* Na/KH_2PO_4. However, the needles subsequently dissolved and were transformed into crystal clusters (Fig. 2*a*) and large single crystals (Fig. 2*b*) after a week. Dimensions of single crystals were 0.2 × 0.06 × 0.05 mm. These were frozen in liquid N_2 in the presence of mineral oil as a cryoprotectant. The choice of cryoprotectant was severely limited because of the high salt concentrations, as well as crystal instability in the presence of (±)-2-methyl-2,4-pentenediol, glycerol and various polyethylene glycols. Smaller single crystals were washed twice in mother liquor and macroseeded into fresh drops that were pre-

equilibrated against 0.1 *M* Na HEPES pH 7.50, 1.6 *M* Na/KH_2PO_4 for 2 d. Over the course of two weeks, single crystals grew to maximum dimensions of 0.2 × 0.06 × 0.04 mm. Crystals were harvested from drops, soaked in mineral oil for 15–30 s and flash-frozen in liquid N_2. The mean size of the crystals was 0.15 × 0.06 × 0.04 mm. A complete data set was collected from one frozen crystal in two scans at the BioCars synchrotron beamline (Advanced Photon Source, Argonne National Laboratories). These crystals belong to the orthorhombic space group $P2_12_12_1$, have unit-cell parameters $a = 42.67$, $b = 93.15$, $c = 101.63$ Å and diffract to 2.9 Å. Data collection and processing statistics are presented in Table 1.

The calculated packing parameter V_M (Matthews, 1968) was 2.65 $Å^3\ Da^{-1}$ for this crystalline form with one molecule per asymmetric unit, suggesting that there is 54% solvent content in the crystal. CrmA shares 25–38% primary structure identity with other members of the serpin superfamily; therefore, the method of molecular replacement for the phase calculation is being pursued with several search models.

The successful crystallization and data collection is the first step in determining the three-dimensional structure of the smallest serpin crmA. This is the first crystallization of a serpin that is either viral or a cysteine proteinase inhibitor. Solution of the structure will give insight into the mechanism of 'cross-class' inhibition of cysteine proteinases by crmA as well as the mechanism of inhibition of serine proteinases. Finally, it will be a valuable tool for investigating the molecular mechanism of apoptosis.

The plasmid pQE-60-crmA, used both for mutagenesis and expression of the crmA, was kindly provided by Dr David Ucker, Department of Microbiology and Immunology, College of Medicine, University of Illinois at Chicago. Mutagenesis of the cysteine residues to serine side chains was carried out by Jill Bayliss. This work was supported by grants from the National Institutes of Health (GM47522 to KV and HL49234 to PGWG) and by funds from the Vice Chancellor for Research, University of Illinois at Chicago. MS is the recipient of a University fellowship from the University of Illinois at Chicago.

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