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Crystallization and preliminary X-ray crystallographic studies of Drep-3, a DFF-related protein from *Drosophila melanogaster*

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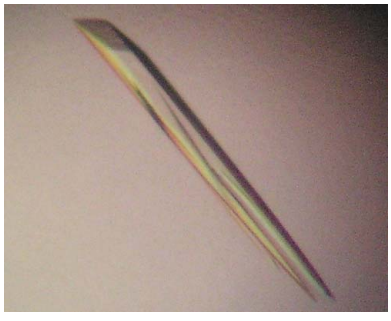

During apoptosis, DNA fragmentation is mainly mediated by the caspase-activated DFF40 nuclease. DFF40 exists as a heterodimeric complex with its inhibitor DFF45. Upon apoptosis induction, DFF45 is cleaved by caspases to allow DFF40 activation. Drep-3 is a recently identified regulator of the DFF40 system in *Drosophila melanogaster*. Here, Drep-3 was expressed with a C-terminal His tag in *Escherichia coli* and the protein was purified to homogeneity. Multi-angle light-scattering analysis showed that Drep-3 is a homotetramer in solution. Native and selenomethionine-substituted Drep-3 proteins were crystallized at 293 K and X-ray diffraction data were collected to 2.8 and 3.0 Å resolution, respectively. The crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 56.9, b = 125.4, c = 168.7 Å. The asymmetric unit is estimated to contain one homotetramer.

1. Introduction

Apoptosis is a process by which cells can be removed in response to various stimuli and plays pivotal roles in embryonic development, immune responses and tissue homeostasis (Jacobson *et al.*, 1997). DNA fragmentation is one of the well known biochemical features of apoptotic cell death. It is mainly controlled by the heterodimeric DNA-fragmentation factor complex DFF40–DFF45 (also known as the CAD–ICAD complex). DFF40 is a caspase-activated endonuclease that can cleave naked and chromosomal DNA, whereas DFF45 is an inhibitor that can suppress the nuclease activity of DFF40. DFF45, which is also a molecular chaperone, assists the correct folding of DFF40 (Liu *et al.*, 1997, 1999).

When effector caspases such as caspase-3 are activated by apoptotic stimuli, they cleave DFF45, leading to dissociation of DFF45 from its binding partner DFF40. The freed DFF40 then becomes active, moves to the nucleus and cleaves chromosomal DNAs (Liu *et al.*, 1997; Sakahira *et al.*, 1998). The N-terminal regions of DFF40 and DFF45 contain a conserved cell-death-inducing DFF45-like effector domain (CIDE-N) of around 80 residues that is involved in homotypic interactions between CIDE-N domain-containing proteins. The interaction between DFF40 and DFF45 also takes place through this CIDE-N domain.

Apoptotic DNA fragmentation is conserved among different species including *Drosophila melanogaster* and several homologous proteins of DFF40–DFF45 have been identified. Unlike in mammals, apoptotic DNA fragmentation in *Drosophila* is controlled by four DFF-related proteins known as Drep-1 (dICAD), Drep-2, Drep-3 and Drep-4 (dCAD) (Inohara & Nunez, 1999). All four proteins have a conserved CIDE-N domain that is important for CIDE-N–CIDE-N interactions. Drep-1 and Drep-4 are DFF45 and DFF40 homologues, respectively (Mukae *et al.*, 2000). Drep-2 and Drep-3 are regulators of Drep-1.

Despite the highly conserved apoptotic machinery, the molecular mechanism of apoptotic DNA fragmentation in *Drosophila* has not been well elucidated. In addition, even though structures of several isolated CIDE-N domains are available (Lugovskoy *et al.*, 1999; Zhou *et al.*, 2001), there have been no reports on any structures of intact proteins with CIDE-N domains. It is therefore unclear how CIDE-N acts in the context of full-length proteins. To understand the

controlling mechanism of apoptotic DNA fragmentation through DFF-related proteins in *Drosophila*, we expressed, purified and crystallized the full-length Drep-3 protein. Interestingly, unlike other DFF-related proteins, which have their CIDE-N domains at the N-terminal region, the CIDE-N domain of Drep-3 is in the middle of its sequence. An atomic structure of Drep-3 should enable us to further understand the controlling mechanism of apoptotic DNA fragmentation in *D. melanogaster*.

2. Materials and methods

2.1. Expression and purification

The construct for expression of full-length Drep-3 (1–266) was made as follows. The cDNA of full-length Drep-3 was used as a template for the polymerase chain reaction (PCR) and the plasmid vector pOKD5 (Dzivenu *et al.*, 2004) was used to add a His tag at the carboxy-terminus for affinity purification. PCR products were digested with *Nde*I and *Xho*I (NEB) restriction enzymes and ligated into pOKD5. This vector construction adds eight residues (LEHHHHHH) to the C-terminus of Drep-3.

The resulting plasmid was transformed into BL21 (DE3) *Escherichia coli* competent cells. Expression was induced overnight at 293 K using 0.5 m*M* isopropyl β-D-thiogalactopyranoside (IPTG). The bacteria were collected, resuspended and lysed by sonication in lysis buffer containing 50 m*M* sodium phosphate pH 7.4, 400 m*M* NaCl, 20 m*M* imidazole and 1 m*M* Tris(2-carboxyethyl) phosphine (TCEP). The bacterial lysate was then centrifuged at 16 000 rev min^{-1} for 1 h at 277 K. The supernatant fraction was applied onto a gravity-flow column (Bio-Rad) packed with Ni–NTA affinity resin (Qiagen). The unbound bacterial proteins were removed from the column using washing buffer containing 20 m*M* sodium phosphate pH 8.0, 400 m*M* NaCl, 1 m*M* TCEP, 50 m*M* imidazole and 10% glycerol. The C-terminally His-tagged Drep-3 was eluted from the column using elution buffer containing 50 m*M* sodium phosphate pH 8.0, 500 m*M* NaCl, 300 m*M* imidazole and 1 m*M* TCEP. The protein was further purified by Q-Sepharose and Superdex 200 gel-filtration columns (Pharmacia). The latter was pre-equilibrated with a solution of 20 m*M* sodium HEPES pH 7.4, 0.2 m*M* $CaCl_2$ and 20 m*M* KCl.

Drep-3 was concentrated to 15 mg ml^{-1} using a YM10 ultrafiltration kit (Amicon) for crystallization trials. The selenomethionine-substituted Drep-3 was expressed in the methionine-auxotrophic strain B834 (Novagen) grown in minimal media supplemented with seleno-L-methionine (Sigma) and other nutrients. It was purified using the same procedure as used for the native protein.

2.2. MALS

The molecular weight of purified Drep-3 was determined by static multi-angle light scattering (MALS). The protein was injected onto a Superdex 200 HR 10/30 gel-filtration column equilibrated with a buffer containing 20 m*M* sodium HEPES pH 7.5, 50 m*M* NaCl and 2 m*M* $CaCl_2$. The purification system was coupled to a Mini-DAWN EOS three-angle light-scattering detector and an Optilab DSP refractive-index detector (Wyatt Technology). Collected data were analyzed using the program *ASTRA*, yielding the molecular weight and mass distribution (polydispersity) of the sample.

2.3. Crystallization and data collection

Crystallization conditions were initially screened at 293 K by the hanging-drop vapor-diffusion method using screening kits from Hampton Research (Crystal Screens I and II, Natrix, MembFac, SaltRX) and from deCODE Biostructures (Wizard I and II). Crystals were grown on a siliconized cover slip by equilibrating a mixture containing 1 µl protein solution and 1 µl reservoir solution against 0.5 ml reservoir solution. The initial hit was condition No. 45 of Wizard II, consisting of 1.26 *M* ammonium sulfate and 100 m*M* MES pH 6.0. The optimized crystallization condition was 0.9 *M* ammonium sulfate and 100 m*M* sodium HEPES pH 7.0. Crystals grew to maximum dimensions of 0.1 × 0.1 × 0.6 mm in two months. The selenomethionine-substituted Drep-3 crystals were grown under a similar condition containing 1.1 *M* ammonium sulfate and 100 m*M* sodium HEPES pH 7.0.

For diffraction experiments, crystals were transiently soaked in a solution corresponding to the reservoir plus 20%(*v*/*v*) ethylene glycol. The crystals were flash-frozen at 110 K using a nitrogen stream (Oxford Cryosystems). Diffraction data sets were collected at beamline NE-CAT (24ID) at the Advanced Photon Source (APS).

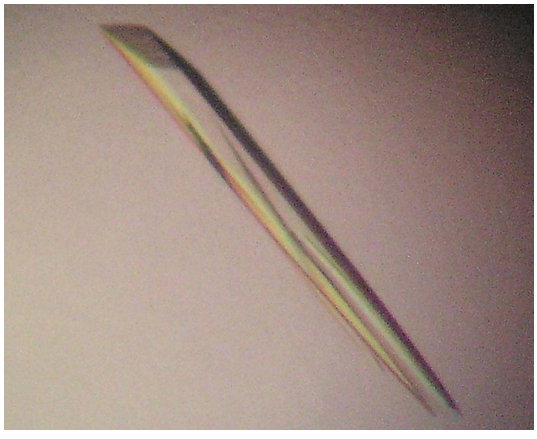

Figure 1
A native Drep-3 crystal grown for two months using ammonium sulfate as the precipitating agent. Its approximate dimensions are 0.1 × 0.1 × 0.6 mm.

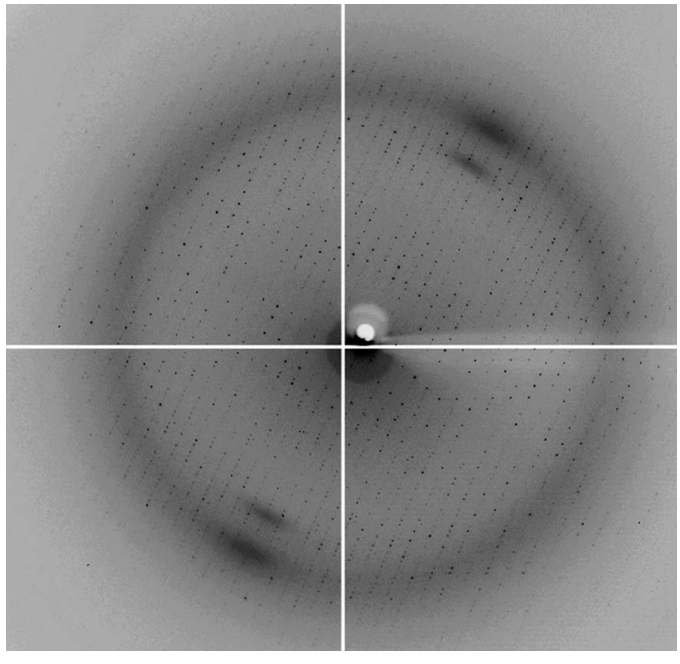

Figure 2
Diffraction image (1° oscillation) of a Drep-3 native crystal.

Table 1
Diffraction data statistics of Drep-3 crystals.

Values in parentheses are for the last resolution shell.

	Native	SeMet
X-ray source	NE-CAT (24ID) at APS	NE-CAT (24ID) at APS
Wavelength (Å)	0.91860	0.97919
Space group	$P2_12_12_1$	$P2_12_12_1$
Unint-cell parameters (Å)	a = 56.9, b = 125.4, c = 168.7	a = 56.4, b = 125.7, c = 168.6
Resolution limits (Å)	50.0–2.8 (2.93–2.80)	50.0–3.0 (3.11–3.00)
Mosaicity (°)	0.3	0.3
No. of observations	232142	228889
No. of unique reflections	37754	45768
Mean $I/\sigma(I)$	23.0 (6.4)	18.4 (2.8)
Completeness (%)	99.5 (99.3)	99.7 (99.2)
R_{sym}	0.084 (0.32)	0.096 (0.30)

The data sets were indexed and processed using *HKL*2000 (Otwinowski & Minor, 1997).

3. Results and discussion

The calculated monomeric molecular weight of Drep-3 including the C-terminal His tag is 30 113 Da. The molecular weight of the intact recombinant Drep-3 was measured by MALS to be 133 900 Da (0.4% error) with a polydispersity of 1.035, indicating that Drep-3 exists as a homotetramer in solution.

Drep-3 crystals are rod-shaped (Fig. 1). Diffraction data sets were collected from native and selenomethionine-substituted crystals to resolution limits of 2.8 and 3.0 Å, respectively, on CCD detectors (Fig. 2). The space group was determined to be $P2_12_12_1$ for both crystals, with unit-cell parameters a = 56.9, b = 125.4, c = 168.7 Å for the native crystal. Assuming the presence of a homotetramer in the crystallographic asymmetric unit, the Matthews coefficient (V_M) was calculated to be 2.5 Å^3 Da^{-1}, which corresponds to a solvent content of 50.9% (Matthews, 1968). Diffraction data statistics for a native and a selenomethionine-substituted Drep-3 crystal are given in Table 1. Current efforts are under way to determine the crystal structure of Drep-3.

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