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Crystallization and preliminary crystallographic studies of the metalloglycoprotein esterase A4 using a baculovirus expression system

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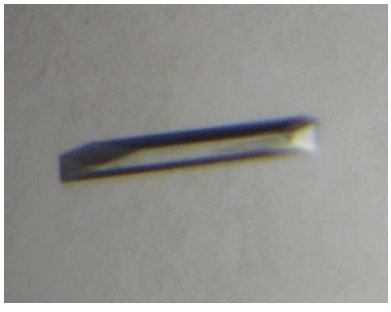

Esterase A4 (EA4) is a timer protein found in diapause eggs of the silkworm *Bombyx mori*. The gene for this metalloglycoprotein was cloned from *B. mori* eggs and expressed using a baculovirus expression system in silkworm pupae. Crystals of the purified protein have been grown that diffract to beyond 2.1 Å resolution at 100 K using synchrotron radiation. The protein crystals belong to space group $P2_1$, with unit-cell parameters a = 47.1, b = 73.9, c = 47.4 Å, β = 104.1°. With one dimer per asymmetric unit, the crystal volume per unit protein weight (V_M) is 2.3 Å^3 Da^{-1} and the solvent content is 47%.

1. Introduction

Esterase A4 (EA4) is a developmental timer in silkworm diapause eggs (Kai *et al.*, 1988*a*,*b*) that plays an important role in regulating the timing of diapause termination of *Bombyx mori* (Kai, Kawai & Doi, 1984; Kai & Nishi, 1976). The length of diapause, the delay in embryonic development, is generally controlled by temperature. To resume development after entering diapause, the eggs must experience low temperature, as they would naturally do in winter. This triggers EA4 to show a burst of ATPase activity a set number of days later (Kai *et al.*, 1986). After diapause eggs have been chilled to 278 K, EA4 ATPase activity appears about two weeks later, triggering the resumption of development (Kai, Kawai & Kaneto, 1984). Purified EA4 chilled to 278 K *in vitro* shows exactly the same time delay as the protein *in vivo* (Kai *et al.*, 1986), suggesting that the protein acts alone as a diapause-duration timer (Kai *et al.*, 1988*b*). EA4 is regulated by a short peptide named PIN (peptidyl inhibitory needle), which is found in several forms with lengths from 28 to 38 amino acids (Isobe *et al.*, 1995). PIN inhibits the ATPase activity. EA4 consists of 156 amino acids and one sugar chain linked to Asn22 (Ti *et al.*, 2006). Mass spectrometry revealed the sugar chain to consist of four sugar residues (Asn22-GlcNAc-GlcNAc-Man-Man; Kurahashi *et al.*, 2002; Pitchayawasin *et al.*, 2004) and the sugar chain was found to be essential for the binding of PIN (Tani *et al.*, 2001). EA4 can be separated from PIN by gel-filtration chromatography, which triggers the protein to hydrolyse ATP after a long time delay. Using purified EA4 *in vitro*, this period is found to depend on temperature. At 318 K the activation of pure EA4 occurred over a similar timescale to that for diapausing eggs: about two weeks. However, at 298 K pure EA4 was rapidly activated and showed maximum enzyme activity within about 7 h (Kai *et al.*, 1999). In order to understand the mechanism of this remarkable protein, we have expressed it as the native glycosylated form using a eukaryotic expression system and crystallized it. Here, we present the crystallization conditions of glycosylated EA4.

2. Material and methods

2.1. cDNA cloning and recombinant virus purification

The total RNA was extracted from diapause eggs of the silkworm *B. mori*. The EA4 gene was amplified by RT-PCR and cloned into pET3b vector. The recombinant vector was identified by restriction-

endonuclease digestion analysis and DNA sequencing. This plasmid was named pET3b-EA4. The cDNA fragment of pET3b-EA4 codes for *B. mori* EA4 without the signal peptide sequence. Using the oligonucleotide primers 5′-GGGGGGGAATTCATGCTGCTTCA-ACTAACATTCCTGGCTGCGATCGCTCTGGCGACGGCTCAT-CATGGCTTCACCACGCCG-3′ and 5′-GGGGGGTCTAGATTAC-TACTATAGAATGCCGATGACACCGCAAGCGAC-3′, a 520 bp fragment encoding *B. mori* EA4 with the signal peptide sequence was amplified by PCR and ligated into the transfer vector pBm31 (Kai *et al.*, 1999). The resultant plasmid was named pBm31-EA4. The recombinant transfer vector pBm31-EA4 was co-transfected with linearized BmNPV virus DNA (Nosan) into BmN4 cells using a lipofectin transfection reagent (Invitrogen). The BmN4 cell line was maintained in TC-100 medium (Nosan) containing 10% FBS (Gibco) at 301 K. The recombinant virus was screened by the plaque-purification method and named BmNPV-EA4. This construct encodes full-length mature (residues 1–156) EA4 protein fused without a spacer to a four-residue N-terminal signal peptide required for high-level expression using the viral expression system. This signal is removed from EA4 during expression.

2.2. Expression and purification of glycosylated *B. mori* EA4

EA4 protein was expressed in silkworm pupae infected with the recombinant virus BmNPV-EA4. 5 µl virus solution was directly injected into the pupae and the infected pupae were stored at 291 K. After 5 d, 100 pupae were crushed in a homogenizer on ice in 140 m*M* NaCl. The purification was carried out using a method based on that used for native EA4 (Kai *et al.*, 1999). The homogenized pupae were centrifuged at 140 000*g* and 277 K. DNA was removed by adding 0.5% polyethleneimine pH 7.0 to the supernatant on ice. The centrifuged supernatant was heated to 358 K for 15 min. An ammonium sulfate cut of the heat-stable supernatant was obtained using 50% and 80% saturated ammonium sulfate (final concentrations). After the addition of solid ammonium sulfate, the pH value of the supernatant was adjusted to pH 5.0 using 20 m*M* citric acid. The precipitate from the 80% saturated ammonium sulfate cut was dissolved in 20 m*M* Na HEPES, 1 *M* NaCl, 5 m*M* imidazole pH 7.0. The protein was directly applied onto a column (AK26×20, GE Healthcare) packed with 80 ml chelating Sepharose Fast Flow agarose (GE Healthcare) charged with zinc ions. The bound protein was then eluted with 20 m*M* Na HEPES, 1 *M* NaCl, 0.5 *M* imidazole pH 7.0. The eluate was collected and concentrated to less than 1.5 ml using Centriprep YM-3 concentrators (Millipore) before being loaded onto a HiLoad 16/60 Superdex 200pg column (GE Healthcare). The sample was eluted with 20 m*M* HEPES, 100 m*M* NaCl pH 7.0. Fractions containing EA4 protein were identified using SDS–PAGE and concentrated. Finally, the sample was dialyzed against 10 m*M* Na HEPES buffer pH 7.0 containing 100 m*M* NaCl. The purified EA4 protein was used for crystallization at a concentration of 1.7 mg ml^{-1}. 100 pupae, weighing a total of 180–200 g, generally yielded 2–3 mg pure protein.

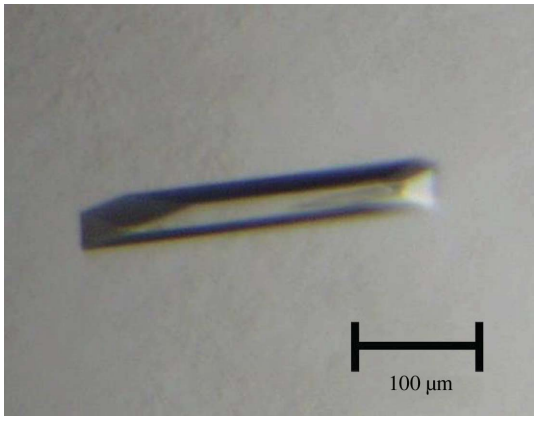

Figure 1
A crystal of native EA4. The dimensions of the crystal are approximately 50 × 50 × 400 µm.

Table 1
Data-collection statistics for EA4.

Values in parentheses are for the highest resolution shell (2.18–2.1 Å).

Resolution range (Å)	50.0–2.1
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 47.10, b = 73.89, c = 47.45, β = 104.1
Redundancy	4.1
Wilson B factor (Å^2)	24
No. of measured reflections	70486
No. of unique reflections	17500
Completeness (%)	95.5 (85.8)
$R_{\rm merge}$† (%)	5.6 (11.9)
Redundancy	4.1
Mean $\langle I/\sigma(I)\rangle$	23.8

† $R_{\rm merge} = \sum|I_i - \langle I\rangle|/\sum I_i$, where I_i is the intensity of an observation and $\langle I\rangle$ is the mean value of its unique reflection; the summations are over all reflections.

2.3. Mass-spectrometric analysis

To confirm that the protein was correctly modified with the expected sugar chain, purified EA4 was analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI–TOF) mass spectrometry before and after deglycosylation. Peptide-*N*-glycosidase F (PNGaseF) was purchased from New England BioLabs. Complete deglycosylation was achieved by incubation of 5 µg recombinant EA4 protein with 5 units of PNGaseF in 20 µl reactions at 310 K for 1 d. Prior to deglycosylation, the recombinant EA4 protein was denatured in a buffer containing 0.5% SDS and 1% β-mercaptoethanol at 373 K for 5 min. Samples were cooled to room temperature and, in order to avoid inactivation of the enzyme by SDS, 1/10 volume each of 10× incubation buffer (0.5 *M* sodium phosphate pH 7.5) and 10% NP-40 were added to the reaction mixture prior to the addition of PNGaseF.

2.4. Crystallization and data collection of EA4

Crystallization of glycosylated EA4 protein was performed using the hanging-drop vapour-diffusion method at 293 K. Crystal Screens 1 and 2 and PEG/Ion Screen (Hampton Research) were used for screening. Optimal conditions for obtaining crystals were found to be 27%(*w*/*v*) PEG 3350, 500 m*M* $MgCl_2$, 20 m*M* NaF. 1 µl protein solution and 1 µl precipitant solution were mixed and equilibrated against 1 ml precipitant solution. Crystals grew to maximum dimensions (50 × 400 µm in length) within one week (Fig. 1). A crystal was flash-cooled to 100 K in a nitrogen-gas stream after washing with mother liquor containing 20%(*v*/*v*) glycerol as cryoprotectant and X-ray diffraction data were collected using synchrotron radiation (wavelength 1.0 Å) and an ADSC Quantum 210 mm CCD detector at AR-NW12A at the Photon Factory, KEK, Japan. The data were processed using *HKL*-2000 (Otwinowski & Minor, 1997). A total of 17 500 unique reflections were obtained with an $R_{\rm merge}$ of 5.6%. The data-collection statistics are summarized in Table 1. Molecular replacement was carried out using the atomic coordinates of bovine copper–zinc superoxide dismutase (PDB code 1e9p) as a search model. The program *MOLREP* (Collaborative Computational

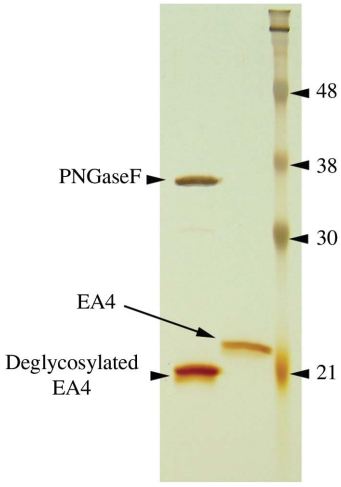

Figure 2
SDS–PAGE of purified EA4. The right-hand lane contains molecular-weight markers; the weights of the markers are indicated in kDa. The left-hand lane contains PNGaseF-treated denatured EA4 (deglycosylated form), which has the expected molecular weight for the 156-residue monomer. The middle lane contains EA4 before deglycosylation.

Project, Number 4, 1994) readily built a preliminary model of EA4 with two protein molecules in the asymmetric unit.

3. Results and discussion

As the sugar chain attached to native EA4 is apparently essential for function, we have expressed recombinant EA4 in silkworm pupae and purified it. The resulting protein can be completely deglycosylated enzymatically (Fig. 2), confirming the presence of the sugar chain. MALDI–TOF mass spectrometry shows that the purified EA4 had the expected molecular weight and an attached sugar chain of four or five sugar units. The space group of the crystals was found to be $P2_1$, with unit-cell parameters a = 47.1, b = 73.9, c = 47.4 Å, β = 104.1°. Two molecules of EA4 in the asymmetric unit give a crystal volume per unit protein weight of 2.3 Å^3 Da^{-1}, corresponding to a solvent content of approximately 47%. EA4 has 53% amino-acid sequence identity with bovine copper–zinc superoxide dismutase, which was used as a search model for molecular replacement. *MOLREP* (Collaborative Computational Project, Number 4, 1994) found two rotation–translation solutions which were rigid-body refined using *CNS* (Brünger, 1998). The current model includes only polypeptide backbone atoms and has an R factor of 26.5% and a free R factor of 32.6%. Refinement of the complete EA4 structure, including the sugar chain and copper and zinc ions, is in progress.

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