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Crystallization and preliminary crystallographic analysis of extracellular fragment X3 of YWK-II/APPH: a human sperm membrane protein related to the Alzheimer βA4-amyloid precursor protein

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Crystals of extracellular fragment X3 of a human sperm membrane protein YWK-II/APPH have been grown at 291 K using 8% PEG 4000 as precipitant by the vapour-diffusion method. The diffraction pattern of the crystal extends to 2.9 Å resolution at 100 K using Cu $K\alpha$ radiation in-house. The crystals belong to space group $P2_1$, with unit-cell parameters $a = 46.0$, $b = 43.7$, $c = 90.2$ Å, $\alpha = \gamma = 90.0$, $\beta = 106.6^\circ$. Furthermore, a selenomethionine (SeMet) derivative of the protein was overexpressed in the same expression system and was purified in a reducing environment. The derivative crystals were obtained under similar conditions. Subsequently, a single-wavelength data set was collected to 2.38 Å resolution from the derivative crystal at ESRF. The crystals belong to space group $P2_1$, with unit-cell parameters $a = 46.2$, $b = 44.0$, $c = 88.3$ Å, $\alpha = \gamma = 90.0$, $\beta = 103.6^\circ$. The presence of one molecule per asymmetric unit gives a crystal volume per protein mass (V_M) of 2.8 Å^3 Da^{-1} and a solvent content of 56.4% by volume.

1. Introduction

YWK-II protein is a member of the amyloid precursor protein (APP) superfamily and is involved in fertilization. The protein is a type I sperm transmembrane protein which was initially identified as the target antigen to a monoclonal antibody (mAb) raised against proteins extracted from human spermatozoa (Yan *et al.*, 1987). The YWK-II mAb possessed potent sperm-agglutinating activity. The coding cDNA was isolated and its sequence determined (RSD-2: GenBank accession No. M31322). The deduced polypeptide, designated the YWK-II protein, contained a segment with high homology (70.6%) to the transmembrane-cytoplasmic domain of the βA4-amyloid precursor protein (APP) found in brain plaques of Alzheimer's disease patients (Yan *et al.*, 1990). It has subsequently been referred to as the human placenta amyloid precursor protein homologue (APPH) and rat amyloid precursor-like protein 2 (APLP2), which are apparently species-specific forms of the same component. The alternative splicing patterns of YWK II protein gene isoforms and APP gene family members are also similar (Sandbrink *et al.*, 1994*a*,*b*; Vidal *et al.*, 1992; Hanes *et al.*, 1993; Slunt *et al.*, 1994; Sprecher *et al.*, 1993; Wasco *et al.*, 1993). Of the members of the APLP family, the βA4-amyloid precursor protein (APP) is the most extensively studied. The occurrence of deposits of APP in the neurofibrillary tangles of the brain is a constant feature of Alzheimer's disease that has attracted a great deal of attention. Several mutations in the sequence of APP have been implicated in the pathogenesis of Alzheimer's disease (Goate, 1998).

A series of previous experimental results indicate that YWK-II protein is involved in sperm–egg interaction. YWK-II mAb agglutinates human and rat sperm (Yan *et al.*, 1986, 1987; Haneji & Koide, 1987) and prevents heterogamete fertilization (Wang *et al.*, 1987). Passive immunization of female mice with YWK-II mAb causes a significant reduction in the number of zygotes formed compared with controls. Additionally, immunization with a polypeptide corresponding to segment 594–610/763 of rat YWK-II protein effectively reduced fertility in both male and female mice (Vanage *et al.*, 1992). Furthermore, in null-mutation mice, both APP$^{-/-}$ individuals and APLP2$^{-/-}$ mice are fertile, while APP and APLP2 double-mutation mice are completely infertile (Von Koch *et al.*, 1997). These findings show that YWK-II protein/APLP2 plays a critical role in the fertilization process.

In our previous studies, we cloned and expressed the fragment HSD-226 of YWK-II in *Escherichia coli*. Müllerian-inhibiting substance (MIS) was found to interact with the HSD-226 fragment, indicating that HSD-226 may function as a receptor for Müllerian-inhibiting substance (Tian *et al.*, 2001). We obtained crystals of this peptide and preliminary diffraction data sets were collected at

100 K in-house on a Rigaku RU-2000 rotating Cu $K\alpha$ anode X-ray generator at 48 kV and 98 mA (λ = 1.5418 Å) with a MAR 345 mm image-plate detector (Yang *et al.*, 2002). The aim of the present study was to clarify the molecular role of YWK-II protein in the process of fertilization and to determine its structure. There are seven conservative regions of YWK-II protein: the cysteine-rich growth-factor-like region (named X1), the negative region (X2), the α-helix-rich region (X3), the KPI region (X4), the coil-rich region (X5) and the transmembrane region and cytoplasmic region (X6). We cloned and expressed the six peptides X1–X6. The six recombinant polypeptides were isolated, purified and screened for crystallization. In the present study, the crystallization and preliminary crystallographic analysis of the X3 (α-helix-rich region) fragment, consisting of residues 388–574, is reported.

2. Materials and methods

2.1. Protein expression and purification

The recombinant X3 protein was synthesized in *E. coli* by overexpression of the X3 gene cloned from the HSD-2 gene. The construct, consisting of residues 388–574 from the *YWK-II/APPH* gene, was amplified by the PCR method using the two primers 5′-GCG GAT CCC GCT TCC AGA AGG-3′ and 5′-GAC AAT TGG TCC ATA TCC GCT CGC TG-3′. Two tags of the vector were added at the ends of these fragments. The PCR product was restricted with *Bam*HI and *Mfe*I, purified and ligated into *Bam*HI- and *Mfe*I-restricted sites of the pET30a(+) vector (Novagen Inc.) with T_4 DNA ligase. A further transformation into *E. coli* DH5α competent cells was performed and the positive clones with an insert of the right size were identified by double digestion with *Bam*HI and *Mfe*I. The sequence of the insert was verified by sequencing. The plasmid was transformed into *E. coli* strain BL21 (DE3) and the transformants were selected on LB agar plates containing 25 µg ml^{-1} kanamycin. The cells were cultured at 310 K in LB medium containing 50 µg ml^{-1} kanamycin. When the culture density reached 0.6–0.8 (A_{600}), the culture was induced with 0.25 m*M* IPTG at 296 K and grown for an additional 12 h before the cells were harvested.

The bacterial cell pellet was resuspended in lysis buffer (20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 10 m*M* imidazole, 1 m*M* PMSF, 1 m*M* β-mercaptoethanol) and lysed using a French cell. The lysate was centrifugated at 20 000*g* for 30 min to remove the cell debris. The supernatant was filtrated through 0.45 µm filters and then purified using a HiTrap Chelating HP column (1 ml) on an ÄKTA Prime system (Amersham Pharmacia). The contaminating protein was washed with a buffer similar to the lysis buffer but with the addition of 20 m*M* imidazole. The target protein was eluted with elution buffer (20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 200 m*M* imidazole, 1 m*M* PMSF, 1 m*M* β-mercaptoethanol). The elution buffer was changed to buffer *A* (25 m*M* MOPS pH 7.2, 50 m*M* NaCl, 1 m*M* β-mercaptoethanol) by passage through a G-25 column and purified using a Resouce-S (5/5) ion-exchange column (buffer *A*, 25 m*M* MOPS pH 7.2, 50 m*M* NaCl, 1 m*M* β-mercaptoethanol; buffer *B*, 25 m*M* MOPS pH 7.2, 0.5 *M* NaCl, 1 m*M* β-mercaptoethanol). The protein was concentrated and then chromatographed using a Superdex-75 column (buffer *A*). The final purification was using a Mono-S ion-exchange column (buffers *A* and *B*). All pre-packed columns were purchased from Amersham Pharmacia and all steps were performed on an ÄKTA Explor system. The purified protein was analyzed using SDS–PAGE.

2.2. Crystallization

For crystallization, the purified protein was dialyzed into crystallization buffer (10 m*M* NaCl, 10 m*M* MOPS pH 7.2, 5 m*M* DTT) and was concentrated to 2.5–5 mg ml^{-1} using an Ultrafree 10 000 NMWL filter unit. Protein concentrations were estimated spectroscopically by absorbance at 280 nm, assuming an A_{280} of 0.430 for a 1.0 mg ml^{-1} solution. Crystallization trials were set up in 16-well tissue-culture plates using the hanging-drop vapour-diffusion method, using standard protocols. Preliminary crystallization conditions were established using Hampton Research Crystal Screen kits I and II at 291 K, followed by refinement of the conditions through variation of the precipitant. The purified selenomethionine derivative was concentrated to 1.2 mg ml^{-1}. Crystallization trials were set up based on the optimum conditions used for native protein.

2.3. Data collection and preliminary X-ray crystallographic analysis

Preliminary diffraction data sets were collected at 100 K in-house on a Rigaku RU-2000 rotating Cu $K\alpha$ anode X-ray generator operating at 48 kV and 98 mA (λ = 1.5418 Å) with a MAR 345 mm image-plate detector. Single-wavelength anomalous dispersion (SAD) data were collected on beamline ID14-4 under cryoconditions at the synchrotron-radiation source at ESRF (Grenoble, France). Crystals were frozen in the crystallization buffer with 25% ethylene glycol as cryoprotectant prior to data collection. All intensity data were indexed, integrated and scaled using the *HKL* programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results

The final purified protein was confirmed to be homogenous by SDS–PAGE analysis and was fit for use in crystallization trials. Small needle-shaped and clustered crystals appeared after 2 d from several different conditions using Crystal Screen kits I and II (Hampton Research) containing PEG 2000, PEG 4000 and PEG 8000 as precipitants (reagents 15, 17, 22, 36, 41 from kit I and reagents 22, 26, 38, 48 from kit II). The conditions were further optimized by variation of the precipitants, buffer pH and protein concentration.

Because the X3 protein contains 20 glutamines and seven asparagines, which

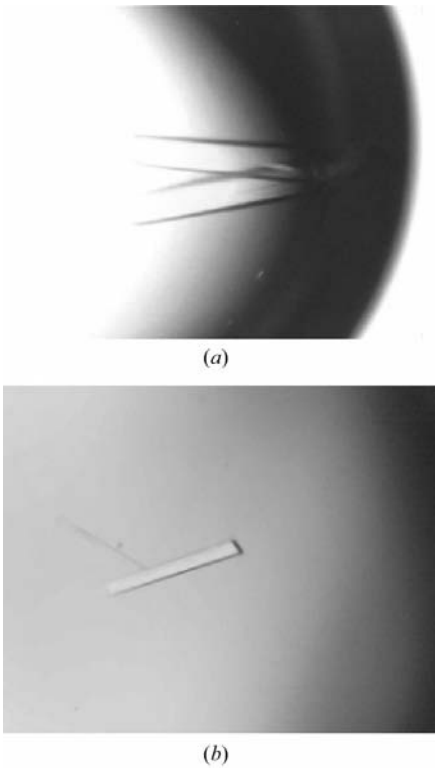

Figure 1
Crystals of X3 protein. (*a*) Sheet-like native crystals (the size of a single crystal is approximately 2 × 0.3 × 0.05 mm). (*b*) A rod-shaped selenomethionine-derivative crystal (the size of a single crystal is approximately 0.1 × 0.1 × 0.8 mm).

Table 1
Data collection and processing statistics of SAD at ESRF from a single X3 selenomethionine-derivative crystal.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 46.2, b = 44.0, c = 88.2, α = 90, β = 103.6, γ = 90.0
Matthews coefficient (Å^3 Da^{-1})	2.8
Resolution (Å)	50–2.38
Total observations	86534
Unique reflections	13836
Anomalous diffraction ratios for F_{obs} (%)	5.2
Redundancy	6.25
Average $I/\sigma(I)$	6.55 (6.12)
R_{merge}† (%)	8.5 (35.5)
Data compleness (%)	97.6 (79.7)

† $R_{merge} = 100\sum|I_i - \langle I\rangle|/\sum I_i$, where I_i is the intensity of the ith observation.

would undergo a deamidation reaction in lower or higher pH solution, we screened the PEG conditions in the pH range 6.0–7.0. We found that the crystals grew more quickly and to an optimum size in 0.1 *M* MES pH 6.2–6.5, 5–13% PEG 4000. By further refinement, varying the concentrations of protein and precipitant, larger prism-like crystals which are reproducible and are suitable for X-ray diffraction were obtained. The largest sheet-shaped crystals (Fig. 1) grew to 2 × 0.3 × 0.05 mm in size from a reservoir solution comprising 0.1 *M* MES pH 6.5, 8% PEG 4000. The crystals obtained using the optimum reservoir solution condition are compact and stable, as demonstrated by their diffraction to 2.9 Å following storage for about 10 d at 291 K.

Diffraction data to 2.9 Å resolution were collected in-house from a native crystal. The crystal belongs to the space group $P2_1$, with unit-cell parameters a = 46.0, b = 43.7, c = 90.2 Å, $\alpha = \gamma$ = 90.0, β = 106.6°. There is one molecule in the asymmetric unit. R_{merge} is 6.3% and the completeness is 98.1%.

Since there are ten methionine residues in the X3 protein, the SAD method was considered. Selenomethionine-derivative crystals were obtained using similar conditions to those for the native protein, except for a lower pH (0.1 *M* MES pH 6.3) and protein concentration (1.2 mg ml^{-1}). SAD data were collected from a single selenomethionine-derivative crystal to 2.38 Å resolution (λ = 0.9807 Å). Statistics for data collection from the selenomethionine-derivative crystal are shown in Table 1. With the addition of two tags of the vector to each terminus of the protein, the total number of residues is 263. The presence of one molecule per asymmetric unit gives a Matthews coefficient (V_M; Matthews, 1968) of 2.8 Å^3 Da^{-1} and a solvent content of 56.4% by volume.

Structure determination of the X3 fragment is currently under way.

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