crystallization communications

Purification, crystallization and X-ray diffraction analysis of human synaptotagmin 1 C2A-C2B

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Synaptotagmin acts as the Ca^{2+} sensor for neuronal exocytosis. The cytosolic domain of human synaptotagmin 1 is composed of tandem C2 domains: C2A and C2B. These C2 domains modulate the interaction of synaptotagmin with the phospholipid bilayer of the presynaptic terminus and effector proteins such as the SNARE complex. Human synaptotagmin C2A-C2B has been expressed as a glutathione-*S*-transferase fusion protein in *Escherichia coli*. The purification, crystallization and preliminary X-ray analysis of this protein are reported here. The crystals diffract to 2.7 Å and belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 82.37$, $b = 86.31$, $c = 140.2$ Å. From self-rotation function analysis, there are two molecules in the asymmetric unit. The structure determination of the protein using this data is ongoing.

1. Introduction

Ca^{2+}-dependent release of neurotransmitters into the synaptic space is one of the most fundamental concepts in modern neuroscience. While this phenomenon has been thoroughly described at the cellular level, identification of the molecular agents responsible for this activity has been challenging. Over the years, several candidate proteins have been posited as the Ca^{2+} receptor; these include an annexin (Pollard *et al.*, 1980), protein kinase C (Hutton, 1986) and calmodulin (DeLorenzo, 1982; Hutton, 1986). However, the vesicle-localized protein synaptotagmin has recently gained acceptance as the Ca^{+2} receptor in neurons (Sudhof, 2004; Chapman, 2002).

In general, the synaptotagmin family mediates docking, recycling and fusion of vesicles with target membranes (Yoshihara & Montana, 2004). While all isoforms possess a single transmembrane span, the main function of this protein is mediated through its peripheral association with acidic components of target phospholipid membranes. Some isoforms of synaptotagmin have evolved a sensitivity to Ca^{2+} gradients (Bhalla *et al.*, 2005), while others have dispensed with this activity (von Poser *et al.*, 1997). To date, there have been 16 paralogs of synaptotagmin identified within the human genome (Craxton, 2004). The cellular distribution of these isoforms ranges from ubiquitous expression (in most cell types) to exclusive localization in the brain, as is the case for synaptotagmin 1.

The molecular weight of the complete human synaptotagmin 1 protein is approximately 65 kDa. The short amino-terminal extracellular portion of the molecule is glycosylated and serves as the receptor for *Botulinum* neurotoxins (subtypes B and G; Nishiki *et al.*, 1994; Rummel *et al.*, 2004; Dong *et al.*, 2003). The single transmembrane helix of synaptotagmin 1 is followed by a long tethering linker which is rich in arginine and lysine residues. This linker is followed by the tandem C2 domains that are localized to the carboxy-terminal end of the molecule. These are labeled C2A and C2B. Both share considerable structural similarity with the C2 domain of protein kinase C (Perin *et al.*, 1991) and impart Ca^{2+}-dependent phospholipid-binding activity to the protein (Chapman & Jahn, 1994; Davletov & Sudhof, 1993).

Human synaptotagmin 1 C2A-C2B possesses ∼280 amino acids. Each C2 domain is composed of an eight-stranded β-sandwich (Sutton *et al.*, 1995; Fernandez *et al.*, 2001; Ubach *et al.*, 2001) and the two domains are joined by a short approximately five-amino-acid linker. To bind phospholipids, multiple calcium ions must bind in a cleft at one end of the fold. The divalent-cation-binding loops co-localize with residues implicated in phospholipid association. While there are published reports of Ca^{2+}-dependent structural changes in synaptotagmin 1 (Davletov & Sudhof, 1994), there is little structural evidence to support large-scale reorganizations of the C2 domains.

A considerable amount of structural analysis, both X-ray and NMR (Fernandez *et al.*, 2001; Shao *et al.*, 1998; Sutton *et al.*, 1995; Cheng *et al.*, 2004), has been devoted to the isolated C2 domains of synaptotagmin, yet little has been accomplished on the structure of the functional C2A-C2B pair. However, some evidence from NMR studies and X-ray structural studies suggests that the two domains do not interact (Arac *et al.*, 2006; Sutton *et al.*, 1999). Here, we report the crystallization and preliminary X-ray diffraction results of this domain in order to gain insight into the possible intercommunication between C2 domains and to address the possible role of oligomerization in synaptotagmin biology.

2. Experimental procedures and results

2.1. Cloning, expression and purification

Human synaptotagmin 1 (Syt1) C2A-C2B was obtained *via* PCR from a human hippocampal Quick-Clone cDNA library (Clontech). An 858-nucleotide fragment comprising Syt1 C2A-C2B residues 140–422 was directly cloned into PCR2.1 (Invitrogen), excised using *Bam*HI and *Xho*I and subsequently subcloned into pGEX4T-1 (GE-Healthcare). DNA-sequence analysis confirmed insertion in the correct reading frame with respect to the GST fusion partner. BL21 cells containing the Rosetta plasmid were transformed with the pGEX4T-1-SytI plasmid and the resultant colonies were grown in 10 l ECPM1 medium (Bernard & Payton, 1995) in a BioFlo 3000 fermentor at 310 K with 100 µg ml⁻¹ ampicillin as the selection agent. Heterologous expression of the synaptotagmin fusion protein was initiated with 400 µ*M* IPTG and expression took place for 4 h at 298 K. The culture was immediately centrifuged at 277 K and the pellets were frozen in liquid nitrogen and stored at 193 K.

Frozen cells were thawed in cold lysis buffer (50 m*M* sodium phosphate buffer, 50 m*M* Tris, 300 m*M* NaCl, 5 m*M* DTT, 10 m*M* EDTA, 0.5 m*M* PMSF, 1% Triton X-100 pH 7.4). A hand blender was used to homogenize the cells. They were disrupted using a Microfluidizer M110-EH (Microfluidics). The lysate was then centrifuged at 40 000 rev min⁻¹ for 60 min at 277 K. All subsequent chromatography columns and chromatography media were obtained from GE-Healthcare. Buffer exchanges were performed using G-25 Sephadex medium packed in an XK 26/20 column. The lysate supernatant was filtered using 0.2 µm syringe filters and loaded at a flow rate of 300 cm h⁻¹ onto Glutathione Sepharose 4 Fast Flow packed in an XK 26/20 column equilibrated with lysis buffer. The column was then washed with the following buffers at a flow rate of 600 cm h⁻¹ until $\Delta A_{280} < 5$ mAU: lysis buffer, wash buffer (50 m*M* sodium phosphate buffer, 50 m*M* Tris, 300 m*M* NaCl, 5 m*M* DTT pH 7.4), nuclease buffer (50 m*M* sodium phosphate buffer, 200 µg ml⁻¹ DNase, 20 µg ml⁻¹ RNase, 2 m*M* MgCl₂ pH 7.4), wash buffer, high-salt buffer (wash buffer + 1 *M* NaCl pH 7.4) and finally wash buffer. The fusion protein was eluted with elution buffer (wash buffer + 15 m*M* reduced glutathione pH 8.0). The eluant was buffer-exchanged into 50 m*M* sodium phosphate, 150 m*M* NaCl pH 7.4. Human α-thrombin

(Haematologic Technologies Inc) was added to the eluant at 50 NIH units per milligram of fusion protein. The protease cleavage reaction was allowed to proceed for 36 h at 297 K with occasional gentle mixing. The cleavage product was spun, filtered using a 0.2 µm syringe filter, buffer-exchanged into wash buffer and loaded onto the Glutathione Sepharose 4 Fast Flow column at 300 cm h⁻¹. The flowthrough was collected and buffer-exchanged into IEX$_A$ buffer (50 m*M* HEPES, 150 m*M* NaCl pH 7.2) and loaded onto an equilibrated SP Sepharose HP XK16/20 column at 480 cm h⁻¹, washed until the A_{280} was stable and developed using a gradient from IEX$_A$ buffer to IEX$_B$ buffer (IEX$_A$ + 1 *M* NaCl pH 7.2) over 20 column volumes. The desired fractions were combined and concentrated using 10 kDa molecular-weight cutoff Ultra Centrifugal Filter Devices (Millipore). Gel filtration was performed using Superdex 75 Prep Grade in an XK 16/60 column equilibrated with 25 m*M* sodium phosphate, 50 m*M* Tris, 100 m*M* NaCl pH 7.2. The pure protein fractions were mixed and concentrated to 17 mg ml⁻¹ using YM-10 centrifugal filters (Millipore) for immediate crystallization setup.

The protein quantitation and overall purity at each step was assessed using the Bradford method and SDS–PAGE (Fig. 1). MALDI–TOF was performed on purified samples in the University of Texas Medical Branch Mass Spectrometry Laboratory. The average calculated molecular weight for the fragment 140–422 of human synaptotagmin C2A-C2B after thrombin cleavage is 32 487.47 Da; MALDI analysis yielded a weight of 32 635.1 Da. The identity of the resultant protein as synaptotagmin 1 C2A-C2B was also confirmed by its mostly β-sheet circular-dichroism signature (data not shown) and Western blot analysis (data not shown) using commercially available polyclonal Ab produced against the C2A specific peptide from synaptotagmin 1 (Synaptic Systems, GmbH).

2.2. Crystallization and data collection

Initial crystallization conditions were obtained by screening with crystallization kits from Hampton Research (Laguna Niguel, CA, USA) and Molecular Dimensions Inc. (Apopka, FL, USA). All experiments were performed using the hanging-drop and sitting-drop vapor-diffusion method at 293 K. 2 µl of 17 mg ml⁻¹ protein solution was mixed with 2 µl reservoir solution over wells containing 500 µl precipitant solution. After one week, small crystals appeared in Hampton SaltRx Screen formulation 12 (3.2 *M* sodium chloride, 100 m*M* sodium acetate pH 4.6). Crystallization conditions were optimized to 3 *M* sodium chloride, 75 m*M* sodium acetate pH 4.5. Microseeding techniques yielded crystals of dimensions 0.1 × 0.1 × 0.05 mm that were suitable for diffraction studies (Fig. 2). While the precipitant alone was rather acidic, the resultant 2 µl protein solution + 2 µl precipitant had a pH of ∼5.8.
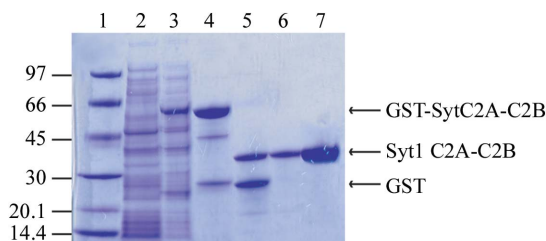
Figure 1
20% PAGE gel summarizing human synaptotagmin C2A-C2B purification. Lane 1, molecular-weight markers (kDa); lane 2, pre-induction cell lysate; lane 3, post-IPTG induction cell lysate; lane 4, glutathione-Sepharose purified fusion protein; lane 5, GST-Syt1-C2A-C2B cut with thrombin; lane 6, thrombin cut protein re-purified over glutathione-Sepharose; lane 7, overloaded sample of Mono S/gel-filtered final protein.

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Synchrotron radiation	Beamline 11-1, SSRL, $\lambda = 0.979$ Å
Detector	ADSC Quantum 4 CCD, $D_{fx} = 400$ mm
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 82.37$, $b = 86.31$, $c = 147.2$
Resolution range (Å)	50.0–2.70
Total reflections	116148
Unique reflections	29346
Completeness (%)	99.5 (99.9)
R_{merge} (%)	8.6 (44)
$I/\sigma(I)$	13 (2.8)
Unit-cell volume (Å^3)	1046000
Solvent content† (%)	70
Multiplicity	4.0 (4.0)

† Assuming two molecules in the asymmetric unit.

2.3. X-ray diffraction

The crystals for data collection were harvested from sitting drops when 0.1 mm in size. These crystals were picked up in 0.1–0.2 mm nylon loops, soaked in 5 *M* sodium chloride, 75 m*M* sodium acetate pH 4.5 for 10 min and then flash-frozen in liquid nitrogen. Initial X-ray diffraction experiments were performed at 100 K with an oscillation range of 1° per frame over 30 min using a MacScience MO6XHF X-ray generator and a MacScience DIP2030H-VLM dual 30 cm diameter imaging-plate detector at the University of Texas Medical Branch X-ray Crystallography Core facility. Final X-ray data sets were collected at beamline 11-1 at the Stanford Synchrotron Radiation Laboratory (SSRL; Stanford, CA, USA) at 100 K. All data were processed with *MOSFLM* (Leslie, 1992) and *CCP*4 (Collaborative Computational Project, Number 4, 1994). The optimal data-collection strategy was computed using the 'Strategy' option in *MOSFLM*. Structure factors were calculated with *TRUNCATE* (French & Wilson, 1978).

3. Results and discussion

Crystals grown in sodium chloride diffracted to 2.7 Å at SSRL. The refined mosaicity was low (0.6°). We tried to soak these crystals in solutions containing divalent cations, such as ∼100 µ*M* Ca^{2+} or Pb^{2+}; however, the diffraction limit decreased and the mosaicity increased markedly. Attempts to cocrystallize this protein with calcium ion yielded crystals that diffracted to ∼25 Å resolution (personal
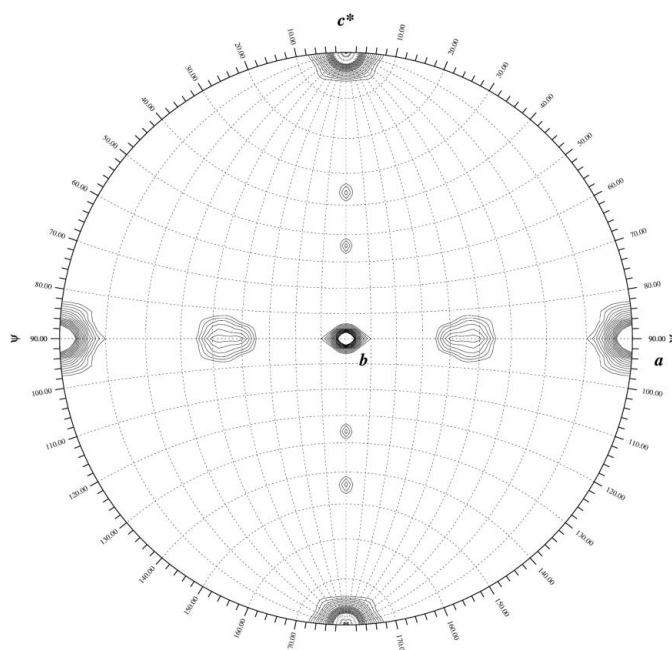

Figure 2
Crystals of human synaptotagmin 1 C2A-C2B. The largest crystals are approximately 0.1 mm in size.

communication from Dr Byron DeLaBarre, Stanford University). These results could be indicative of a substantial conformational change within these crystals upon divalent cation binding.

The crystal belong to space group $P2_12_12_1$, with unit-cell parameters $a = 82.37$, $b = 86.31$, $c = 147.2$ Å (Table 1). In this case, the Matthews coefficient is ambiguous. For two molecules in the asymmetric unit, the Matthews coefficient is calculated as 4.09 Å^3 Da^{-1}. This implies ∼70% solvent content in the crystal. However, three molecules per asymmetric unit yields a Matthews coefficient of 2.73 Å^3 Da^{-1} and 55% solvent content. Two molecules per asymmetric unit is consistent with the rather high solvent content found in the related synaptotagmin III structure (Sutton *et al.*, 1999); however, the value corresponding to three molecules per asymmetric unit is more characteristic of well diffracting protein crystals. To resolve this ambiguity, a self-rotation function was calculated using the available X-ray data (Fig. 3). At $\kappa = 180°$, there were two major peaks that could not be attributed to crystallographic symmetry. Both these peaks were found ∼45° about the crystallographic *c* axis. There were no major peaks observed at $\kappa = 120°$. Therefore, we conclude that this crystal form possesses a non-crystallographic dimer of synaptotagmin molecules. The self-rotation function was calculated using *GLRF* (Tong & Rossmann, 1997). The Patterson integration radius was 40 Å and data were used in the resolution range 15.0–4.0 Å.

While crystallographic and NMR structures of C2A (Sutton *et al.*, 1995; Shao *et al.*, 1997) and C2B (Fernandez *et al.*, 2001; Cheng *et al.*, 2004) exist, the functional synaptotagmin protein comprises the two domains in tandem. Therefore, the structural information obtained from this crystal structure at atomic resolution will certainly augment our understanding of Ca^{2+}-dependent exocytosis and synaptotagmin biology in general. Also, since this crystal represents the first instance of synaptotagmin as a homodimer, it is hoped that we will gain insight into the structural mechanism of synaptotagmin oligomerization and perhaps the interdomain interactions between C2A and C2B.

Figure 3
Self-rotation function calculated using the human synaptotagmin 1 crystals. A self-rotation search with $\kappa = 180°$ was used to identify the non-crystallographic twofold axis of symmetry. Contours start at 2 standard deviations, with intervals of 0.5. Calculations and plots were performed and produced using *GLRF* (Tong & Rossmann, 1997).

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