Crystallographic identification of Ca^{2+} and Sr^{2+} coordination sites in synaptotagmin I C₂B domain

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Abstract

Synaptotagmin I has two tandem Ca^{2+} -binding C₂ domains, which are essential for fast synchronous synaptic transmission in the central nervous system. We have solved four crystal structures of the C₂B domain, one of them in the cation-free form at 1.50 Å resolution, two in the Ca^{2+} -bound form at 1.04 Å (two bound Ca^{2+} ions) and 1.65 Å (three bound Ca^{2+} ions) resolution and one in the Sr^{2+} -bound form at 1.18 Å (one bound Sr^{2+} ion) resolution. The side chains of four highly conserved aspartic acids (D303, D309, D363, and D365) and two main chain oxygens (M302:O and Y364:O), together with water molecules, are in direct contact with two bound Ca^{2+} ions (sites 1 and 2). At higher Ca^{2+} concentrations, the side chain of N333 rotates and cooperates with D309 to generate a third Ca^{2+} coordination site (site 3). Divalent cation binding sites 1 and 2 in the C₂B domain were previously identified from NMR NOE patterns and titration studies, supplemented by site-directed mutation analysis. One difference between the crystal and NMR studies involves D371, which is not involved in coordination with any of the identified Ca^{2+} sites in the crystal structures, while it is coordinated to Ca^{2+} in site 2 in the NMR structure. In the presence of Sr^{2+} , which is also capable of triggering exocytosis, but with lower efficiency, only one cation binding site (site 1) was occupied in the crystallographic structure.

Keywords: Synaptotagmin I; C₂B domain; X-ray crystallography; calcium binding; strontium binding

Synaptotagmins (Syt) are membrane trafficking proteins characterized by an N-terminal transmembrane domain and tandem cytoplasmic C₂ domains (C₂A and C₂B) that bind Ca^{2+} with different affinities (Sudhof 2002; Tucker and Chapman 2002; Koh and Bellen 2003; Yoshihara et al. 2003). The calcium binding properties of the C₂ domains are thought to determine whether kiss-and-run or full-fusion will occur at the plasma membrane (Wang et al. 2001, 2003). Furthermore, the involvement of distinct synaptotagmins with unique Ca^{2+} -binding signatures in diverse types of regulated exocytosis may explain how fusion of distinct secretory vesicles is differentially triggered at select cal-

cium concentrations. Synaptotagmin I (Syt I), located on the synaptic vesicle membrane, is most likely the Ca^{2+} -sensor controlling regulated exocytosis at the brain synapse (Gepert et al. 1994; Fernandez-Chacon et al. 2001). Biochemical and genetic studies suggest that the C₂B domain is essential for triggering Ca^{2+} -dependent fast exocytosis. In *Drosophila*, mutation of Ca^{2+} -binding aspartate residues within the C₂B domain abolish exocytosis (Mackler et al. 2002).

Sr^{2+} , an analog of Ca^{2+} in the alkaline earth series, is known to also trigger fast neurotransmitter release, but less efficiently (Goda and Stevens 1994; Shin et al. 2003). Although both the C₂A and the C₂B domain of Syt I bind Ca^{2+} in phospholipid complexes, only the C₂B domain forms Sr^{2+} /phospholipid complexes. Unlike Ca^{2+} , Sr^{2+} is unable to induce binding of Syt I to the SNARE complex (Shin et al. 2003). Thus, it has been proposed that Sr^{2+} binding to the Syt I C₂B domain is sufficient to trigger fast exocytosis (Shin et al. 2003).

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NMR spectral analysis has outlined the structure of the Ca^{2+} -bound C_2B domain of Syt I and proposed the coordination geometries of two adjacent Ca^{2+} -binding sites (Fernandez et al. 2001). Two Ca^{2+} ions were built into the final NMR structure based on NOE patterns and titration-based chemical shift changes, supplemented by the results of site-directed mutagenesis experiments. As a result, a two-site Ca^{2+} binding model was proposed, in which the side chains of five aspartic acid residues from two loops form a pair of adjacent Ca^{2+} binding sites. In addition, similar to what has been reported for the C_2A domain (Shao et al. 1998), Ca^{2+} binding does not introduce a conformational change in the C_2B domain (Fernandez et al. 2001), but rather most likely modifies the surface potential, thereby regulating lipid interactions.

We have initiated a research effort aimed at understanding the structural basis of calcium-triggered membrane fusion at the neuronal synapse. The eventual goal is to determine the structure of the synaptotagmin C_2 domains in complex with SNAREs and other regulatory components. As a first step, we report the crystal structures of the C_2B domain of Syt I in divalent cation-free form, in the presence of two and three bound Ca^{2+} ions and in the presence of a single bound Sr^{2+} ion. Our high resolution crystal structures of the C_2B domain have identified the coordination geometries at the three divalent cation-binding sites, as well as the protein side chains and water molecules involved in the coordination. This allows a direct comparison to be made between the Ca^{2+} coordination sites determined from crystallography with those proposed by NMR, which were supplemented by site-directed mutagenesis experiments (Fernandez et al. 2001). It also allows a comparison of divalent cation coordination geometries between Ca^{2+} and Sr^{2+} -bound C_2B domains.

Results

Crystallization conditions

Our efforts at growing diffraction quality crystals of the C_2B domain have been undertaken in the absence and presence of divalent cations. We first crystallized C_2B from a 1.8 M ammonium sulfate solution. These crystals, designated $\text{C}_2\text{B-A}$, diffracted on our in-house X-ray diffractometer up to 1.5 Å resolution. Following structure refinement, no visible electron density was detectable for loop 1, whose residues are known to participate in the Ca^{2+} coordination site(s). We next turned to crystallization in the presence of Mg^{2+} ions, which are known to stabilize the local structure of Syt III $\text{C}_2\text{A-C}_2\text{B}$ (Sutton et al. 1999). However, loop 1 could not be traced in the Syt I C_2B crystals grown from 1.0 M magnesium sulfate and 0.4 M ammonium sulfate.

Previous studies have demonstrated that crystals of Syt I C_2A domain can be grown in Li_2SO_4 solution, and Ca^{2+}

could be subsequently introduced into these crystals following soaking with low concentrations (0.1 mM) of CaCl_2 solution for 24 h (Sutton et al. 1995). It is likely that Ca^{2+} stabilizes the local structure of the Ca^{2+} -binding loops of synaptotagmin (Shao et al. 1998). We succeeded in growing diffraction-quality crystals of the complex of C_2B domain and Ca^{2+} using a similar approach. To avoid the precipitation of CaSO_4 (caused by CaCl_2 addition to crystals in ammonium sulfate buffer), C_2B crystals were carefully washed before transfer to CaCl_2 -containing soaking solutions, which included cryoprotectant. Further, we found that C_2B crystals can be kept in cryoprotectant solution for 5 min with only minor changes of crystallographic parameters and mosaicity. We obtained a crystal, designated $\text{C}_2\text{B-B}$, following 1 min soaking in cryoprotectant solution containing 200 mM CaCl_2 , and another crystal, designated $\text{C}_2\text{B-C}$, following 1 min soaking in cryoprotectant solution containing 600 mM CaCl_2 . We also obtained a crystal, designated $\text{C}_2\text{B-D}$, following 1 min soaking in cryoprotectant solution containing 200 mM SrCl_2 .

Structure refinement

The crystal structure of $\text{C}_2\text{B-A}$ in the absence of divalent cations was solved by molecular replacement against the published NMR-based solution structure of the Ca^{2+} -bound C_2B (Fernandez et al. 2001). Five contiguous residues within a loop segment ($\text{D}_{303}\text{V}_{304}\text{G}_{305}\text{G}_{306}\text{L}_{307}$), together with the last three residues (VKK) are disordered in the crystal structure of $\text{C}_2\text{B-A}$. In turn, the divalent-cation bound $\text{C}_2\text{B-B}$, $\text{C}_2\text{B-C}$ and $\text{C}_2\text{B-D}$ crystal structures were solved by molecular replacement against the $\text{C}_2\text{B-A}$ crystal structure. The $\text{D}_{303}\text{V}_{304}\text{G}_{305}\text{G}_{306}\text{L}_{307}$ segment could be traced in all three structures stabilized by bound divalent cations. In addition, a residue tag (SGGGGGIL) from the fusion protein cleavage site, was also observed in the 1.04 Å $\text{C}_2\text{B-B}$ and 1.18 Å $\text{C}_2\text{B-D}$ structures. Further, double conformations were assigned to the side chains of E295, M302, I314, N336, S344, V376, S391, and V409 in these two-high resolution structures. The final structural statistics data for $\text{C}_2\text{B-A}$ (no divalent ions bound), $\text{C}_2\text{B-B}$ (two bound Ca^{2+} ions), $\text{C}_2\text{B-C}$ (three bound Ca^{2+} ions) and $\text{C}_2\text{B-D}$ (one bound Sr^{2+} ion) are listed in Table 1. Ramachandran analysis of the main-chain conformations showed that for each structure, ~90% of the residues lay in the most-favored regions, while none fell in disallowed regions (data not shown). The $\text{C}_2\text{B-B}$ structure contained one bound acetate molecule from the crystallization buffer, while all the crystal structures contained one bound glycerol molecule from the soaking buffers. None of the crystal structures contained bound sulfate.

Overall structure

The four C_2B crystal structures have the same overall topology as the NMR-based solution structure (Fernandez et

Table 1. Summary of crystallographic data collection and refinement

	C ₂ B-A	C ₂ B-B	C ₂ B-C	C ₂ B-D
Space group: P3 ₂ 21				
Cell dimensions				
a = b (Å)	54.5	54.7	54.6	54.6
c (Å)	104.2	103.8	103.6	103.3
Data collection				
Resolution (Å)	1.50 (1.53–1.50)	1.04 (1.06–1.04)	1.65 (1.69–1.65)	1.18 (1.20–1.18)
Observation	203,470	477,779	162,737	618,517
Unique reflections	28,428 (1,201)	85,544 (4,126)	22,306 (1,430)	56,844 (1,639)
Completeness	96.3% (61.6%)	99.0% (96.6%)	99.9% (98.7%)	95.5% (56.2%)
I/σ(I)	33.6 (3.0)	19.3 (1.6)	24.2 (4.1)	31.1 (3.4)
R _{merge} ^a	0.052 (0.302)	0.103 (0.416)	0.095 (0.386)	0.048 (0.190)
Redundancy	7.2	5.6	7.3	10.9
Refinement				
Resolution (Å)	20.0–1.50	20.0–1.04	20.0–1.65	20.0–1.18
Completeness	96.22%	99.04%	99.83%	95.47%
Reflections	26,931	81,211	21,129	53,935
R-factor ^b	0.188	0.165	0.163	0.155
R _{free}	0.214	0.183	0.197	0.173
Mean B-factor	20.1	12.3	15.4	13.4
RMS bonds (Å)	0.014	0.018	0.015	0.017
RMS angles (°)	1.5	1.7	1.4	1.7
PDB code	—	1TJX	1UOV	1TJM

$$^a R_{\text{merge}} = \sum |I - \langle I \rangle| / \sum I.$$

$$^b R\text{-factor} = \sum_{\text{hkl}} |F_o - F_c| / \sum_{\text{hkl}} |F_o|.$$

al. 2001). For example, the 1.04 Å C₂B-B structure shows that protein is composed of two layers of anti-parallel β sheets, along with two short α helices (Fig. 1A). The β-stranded core of the C₂B domain adopts the same topology as its related C₂A domain (Sutton et al. 1995) and presents a convex surface for further recognition. Two additional α-helices, one between strand β7–β8 and the other at the C terminus, make one face of the C₂B domain significantly different from that observed for the corresponding C₂A-domain. Three loops, designated 1, 2, and 3, encompass the calcium binding sites. The terminal α-helix, which is rich in acidic residues (E410, E411, E412, and D414), introduces a negatively charged patch, which is close to a region that is rich in basic residues (K313, R322, K325, K327, and K375; Fig. 1B).

Not surprisingly, the backbone folds of all four crystal structures of C₂B are very similar (Fig. 2), except for the disordered peptide segment observed in C₂B-A. The root-mean-square deviation (RMSD) of the Cα atoms for the four C₂B crystal structures is less than 0.34 Å. This result is also consistent with NMR studies, where it was shown that cation-binding does not impact on the global structure of C₂B, but rather stabilized the conformation of loop 1 (Fernandez et al. 2001). The greatest variability among the three divalent cation-coordinated crystal structures is found in the loop connections that encircle the Ca²⁺ binding sites. The X-ray (C₂B-B) and NMR (C₂B-NMR; Fernandez et al. 2001) structures of C₂B coordinated by two bound Ca²⁺ ions exhibit an RMSD for all Cα atoms of 0.91 Å.

Cation-binding sites

Loop 1 was disordered in the C₂B-A crystal structure in the absence of Ca²⁺ ions, but could be traced in the C₂B-B and C₂B-C crystal structures in the presence of Ca²⁺ ions, consistent with the conformational stabilization of loop 1 by bound Ca²⁺ (Shao et al. 1998). Two Ca²⁺ binding sites, designated 1 and 2, are common to the C₂B-B (Fig. 3B) and C₂B-C (Fig. 3C) crystal structures and the C₂B-NMR (Fig. 3A) structure. In the NMR structure, where Ca²⁺ coordination is deduced from NOE patterns and divalent cation-induced chemical shift perturbations, supplemented by mutagenesis studies, site 1 is coordinated by the side-chain oxygens of D303, D309, D363, and D365, and main chain oxygen of Y364, while site 2 is coordinated with side-chain oxygens of D303, D363, D365, and D371, and the main chain oxygen of M302 (Fig. 3A). The coordination geometries of sites 1 and 2 in the C₂B-B (Fig. 3B) and C₂B-C (Fig. 3C) crystal structures exhibit pentagonal-bipyramidal coordination (Fig. 4B), with site 1 coordinated to five protein residues (side-chain oxygens of D303, D309, D363, and D365 and main-chain oxygen of Y364) and one water molecule, while site 2 is coordinated to four protein residues (side-chain oxygens of D303, D363, and D365 and main chain oxygen of Y302) and three water molecules (Table 2). A third pentagonal-bipyramidal Ca²⁺ coordination site, designated site 3, was identified in the C₂B-C crystal structure (Fig. 3C) soaked with high CaCl₂ concentration, where the side chain of N333 appears to rotate and cooperate with

Figure 1. Structure of C₂B-B. (A) Front and side view of ribbon diagrams of the structure of C₂B-B with 2 Ca²⁺ ions. β -Strands are labeled from 1 to 8, while helices are labeled H1 and H2. Ca²⁺ ions are labeled Ca1 and Ca2. (B) Front and back view of electrostatic potential surface of C₂B-B. The acidic and basic residues are colored green and orange, respectively.

D309 to bind the third Ca²⁺ ion. Site 3 is coordinated to three protein residues (side-chain oxygens of D309 and N333) and four water molecules (Figs. 3C, 4B). The most noteworthy difference between the C₂B-B and C₂B-C crystal structures and the C₂B-NMR structure is that D371, which is involved in coordination to Ca2 in the NMR structure (Fig. 3A), is not involved in Ca²⁺-binding in the crystal structures (Fig. 3B,C).

In the presence of Sr²⁺ ions, C₂B shows a different pattern of divalent cation binding. The Sr²⁺ ion is hexa-coordinated, with Sr²⁺ occupying site 1, where it is coordinated to four protein residues (side-chain oxygens of D303, D309, and D363 and main-chain oxygen of Y364), and one water molecule, thereby adopting an approximate tetragonal bipyramidal geometry (Fig. 4C).

Discussion

We have solved four crystal structures of the C₂B domain, three of which are in the presence of divalent cations. Two

of these structures were solved at high resolution, namely 1.04 Å for C₂B-B containing two bound Ca²⁺ ions (Fig. 3B) and 1.18 Å for C₂B-D containing one bound Sr²⁺ ion (Fig. 3D). These structures therefore provide details of coordination within three separate divalent cation-binding sites (Table 2), the order of occupancy by bound divalent cations, and insights into the minimum number of divalent cations necessary for triggering exocytosis.

Our crystallographic studies establish that loop 1 is disordered in the divalent cation-free state (C₂B-A structure) or in the presence of Mg²⁺, but becomes ordered in the presence of divalent cations Ca²⁺ (C₂B-B and C₂B-C structures) and Sr²⁺ (C₂B-D structure). Divalent cation binding site 1 is occupied in both Ca²⁺-bound structures (Fig. 3B,C) and the Sr²⁺-bound structure (Fig. 3D). Divalent-cation site 2 is occupied in both Ca²⁺-bound structures (Fig. 3B,C), but not in the Sr²⁺-bound structure (Fig. 3D). It is therefore likely that site 1 is the highest-affinity cation-binding site, followed closely by site 2. NMR titration studies have estimated dissociation constants of 300–400 μ M and 500–600 μ M, for Ca²⁺-binding sites 1 and 2 on C₂B, respectively (Fernandez et al. 2001). It should be noted that despite both Ca²⁺ sites exhibiting pentahedral-bipyramidal coordination, site 1 is coordinated to six oxygens from protein residues, while site 2 is coordinated to four oxygens from protein residues. Four aspartic acid residues (D303, D309, D363, and D365) participate in divalent cation coordination of conserved sites 1 and 2. These four acidic residues are invariant among Syt I–VII (Fig. 4A). It remains unclear at this time whether site 3, which is the weakest of the three Ca²⁺-binding sites, plays a functional role in Ca²⁺-mediated synaptic transmission.

Figure 2. Superimposition of crystal structures C₂B-A, C₂B-B, C₂B-C and C₂B-D and 20 C₂B-NMR structures. The α traces of C₂B-A, C₂B-B, C₂B-C and C₂B-D are colored yellow, red, blue, and green, respectively. The C₂B-NMR structures are colored gray. The orientations are the same as in Figure 1A.

Figure 3. Cation-binding sites in (A) superpositioned C₂B-NMR structures and (B) C₂B-B, (C) C₂B-C, and (D) C₂B-D crystal structures. The Ca²⁺ and Sr²⁺ ions are colored orange. The oxygen atoms that coordinate the cations are colored red. Water molecules are represented as small red spheres. Dashed lines indicate pentagonal bipyramidal coordination to Ca²⁺ sites (B,C) and tetragonal bipyramidal coordination to Sr²⁺ site (D) in the crystal structures.

The identification of a single bound Sr²⁺ in divalent cation-binding site 1 in the C₂B-D crystal structure (Fig. 3D) following soaking of the C₂B-A crystal in 200 mM SrCl₂, contrasts with the identification of Ca²⁺ ions bound in cation-binding site 1 and 2 in the C₂B-B crystal structure (Fig. 3B) following soaking of the C₂B-A crystal in 200 mM CaCl₂. The side chain of D365 is not involved in coordinating the Sr²⁺ in site 1 in the C₂B-D crystal structure (Fig.

3D), as it is in coordinating the Ca²⁺ in site 1 in the C₂B-B crystal structure (Fig. 3B). Nevertheless, Sr²⁺ can trigger fast exocytosis (Goda and Stevens 1994; Shin et al. 2003), which implies that a single bound divalent cation in site 1 is sufficient for maintaining the C₂B fold, and therefore is competent for triggering membrane fusion. However, since transmitter release in hippocampal neurons has been shown to have an Sr²⁺ cooperativity of 4, other factors such as the

Figure 4. (A) Partial amino acid sequences alignment of Syt homology group. This includes Syt I: synaptotagmin I (GenBank accession no. P21707); Syt II: synaptotagmin II (P29101); Syt III: synaptotagmin III (P40748); Syt IV: synaptotagmin IV (P50232); Syt V: synaptotagmin V (P47861); Syt VI: synaptotagmin VI (AAA87724); Syt VII: synaptotagmin VII (AAA87725), from *Rattus norvegicus*. The secondary structural elements of Syt I are also indicated. Schematic of Ca^{2+} -binding sites 1, 2 and 3 (B) and Sr^{2+} binding site 1 (C). The cation binding sites are indicated by larger pink balls, while their coordination to amino acids and waters are indicated by smaller pink and yellow balls, respectively. The three Ca^{2+} coordination sites in B are of the pentagonal bipyramidal type, while the single Sr^{2+} coordination site in C is of the tetragonal bipyramidal type. These coordinations (distances of ≈ 2.5 Å) are indicated by blue lines. One additional, slightly longer coordination (distances of ≈ 2.7 Å) was observed for Ca^{2+} sites 1 and 2 (dashed green lines), D363:OD2 to Ca1 and D365:OD1 to Ca2, respectively (Table 2). These two interactions are from the bidentate aspartate, which is in coordination with the Ca^{2+} -binding site (D363:OD1 to Ca1 and D365:OD2 to Ca2), and therefore excluded from the coordination system. Two hydrogen bonds are indicated by dashed blue lines.

presence of lipids, the tandem C_2A domain, or synaptotagmin oligomerization may provide additional Sr^{2+} -binding sites (Goda and Stevens 1994).

There is in general good overall agreement for coordination of Ca^{2+} at sites 1 and 2 on C_2B from NMR (Fernandez et al. 2001) and crystallographic (this study) approaches. The only difference relates to the D371, which coordinates to Ca2 in the NMR structure (Fig. 3A), but not in any of the crystal structures (Fig. 3B,D). High resolution crystal structures unambiguously identify the divalent cation binding sites and the amino acid and water molecules involved in

coordination. Nevertheless, there is always a concern that a key contact in crystal structures could be perturbed by packing interactions. In this regard, there exists a potential weak hydrogen bond ($\text{N}\cdots\text{O}$ distance ≈ 3.12 Å) between one of the carboxylate oxygens of D371 and the side chain NH_3^+ of K324 from an adjacent molecule in the crystal lattice (Fig. 4B). In our view, C_2B would have to undergo a main-chain conformational change that D371 could directly coordinate to Ca2, since this would not be achievable solely through side-chain rotation of D371. The NMR approach does not directly monitor Ca^{2+} ions or the amino acid ligands coor-

Table 2. Summary of the oxygen-ion distances (Å)

	C ₂ B-NMR ^a		C ₂ B-B		C ₂ B-C			C ₂ B-D Sr
	Ca1	Ca2	Ca1	Ca2	Ca1	Ca2	Ca3	
D303:OD1	2.80~2.83		2.57		2.54			
D303:OD2	2.80~2.84	2.49~2.80	2.51	2.46	2.43	2.37		2.50
D309:OD1	2.18~2.64		2.50		2.35		2.60	2.44
D309:OD2							2.37	
D363:OD1			2.52		2.50			2.41
D363:OD2	2.80~2.81	2.80~2.83	2.67 ^b	2.44	2.70 ^b	2.35		2.68
D365:OD1	2.60~2.81	2.46~2.80	2.48	2.75 ^b	2.42	2.74 ^b		
D365:OD2		2.25~2.81		2.62		2.52		
N333:OD1							2.30	
M302:O		2.81~2.83		2.48		2.37		
Y364:O	2.80~2.83		2.41		2.35			2.49
D371:OD1		2.81~2.83						
H ₂ O			2.41	2.35, 2.43, 2.62	2.45	2.33, 2.35, 2.52	2.25, 2.32, 2.33, 2.35	2.28

^a Distances were measured for each of the NMR models individually. These distances are given to two decimal places, with the caveat that they represent estimates only, given the indirect nature of the measurements for these NMR structures.

^b Distances between noncoordination pairs are indicated as italic.

minated to them. Rather, it uses a combination of NOE and chemical shift titration patterns to deduce this information, supplemented where available by relevant mutational data. In this regard, the D371N mutation appears to have a less severe effect on Ca²⁺ binding to the C₂B domain than does the D309N mutation (Fernandez et al. 2001). Thus, there appears to be no obvious explanation for the difference between the crystallographic and NMR approaches regarding the role of D371 in Ca2 coordination at this time. Position 371 is occupied by either aspartic acid or glutamine among C₂B homologs (Fig. 4A). It is therefore conceivable that the longer glutamine side chain at position 371 could reach and coordinate Ca²⁺ in site 2, in different Syt isoforms.

In summary, our high-resolution crystal structures have identified the protein groups and water molecules that coordinate the Ca²⁺ and Sr²⁺ cations in each of the three sites, as well as the local conformational changes within the C₂B domain on proceeding from the free to the divalent cation-bound forms. The distinct cation-binding patterns observed for the C₂B domain could provide insights into the mechanism of Ca²⁺-dependent synaptotagmin/SNARE complex interaction and Sr²⁺-triggered membrane fusion.

Materials and methods

Gene cloning, protein expression, and purification

The cDNA encoding the rat Syt I C₂B domain (amino acids 271–421) was amplified by PCR and inserted into pGEX-KG plasmid between EcoRI and XhoI cleavage sites. A single colony of BL21 (ΔDE3) bacteria harboring the expression vector was cultured in 80 mL Luria-Bertani broth overnight, and was then used to inoculate 4 L media containing 50 μg/mL ampicillin. Cells were grown

at 37°C until an OD₆₀₀ of 0.8 was reached, and protein expression was induced overnight with 0.3 mM IPTG at room temperature. The bacteria were collected and resuspended in 250 mL buffer A (25 mM HEPES at pH 7.5, 300 mM NaCl). After disrupting the cells with a cell disruptor, bacteria were centrifuged at 235,000g for 1 h. The recovered clean lysate was loaded onto a 25 mL GSTrap column (Amersham) that was pre-equilibrated by buffer A. The column was washed with buffer A and the fusion protein was eluted with 25 mM Tris buffer (pH 8.0) in the presence of 10 mM glutathione. Digestion of the fusion protein was performed with 500 U of thrombin at 4°C, for 3 h. Syt I C₂B domain was further purified by a heparin column and the main fraction was eluted with 720 mM NaCl and then applied to a Superdex-75 column. Pooled fractions containing the C₂B domain were finally concentrated to ≈22 mg/mL.

Crystallization

Crystals were grown using the hanging-drop vapor diffusion method at 4°C. The crystallization drop was made by mixing 1 μL protein sample and 1 μL well solution (1.8 M ammonium sulfate, 100 mM sodium acetate at pH 5.0, and 5 mM DTT). The crystal was cryoprotected by soaking with 40% glycerol and 60% well solution. This crystal form, named C₂B-A, belongs to P3₂21 space group with one molecule in the asymmetric unit (18.3 kDa; 2.439 Å³/Da; 48% solvent volume). The remaining crystals were obtained by soaking in divalent cation solutions. C₂B-A crystals were washed four times with cryoprotectant solution (40% [v/v] glycerol, 100 mM sodium acetate at pH 5.0), and then transferred to cryoprotectant solution containing defined concentrations of calcium or strontium cations for 1 min, prior to freezing them in liquid nitrogen. The C₂B-A crystals soaked in 200 and 600 mM CaCl₂ were designated C₂B-B and C₂B-C, respectively, whereas the one soaked in 200 mM SrCl₂ was designated C₂B-D.

X-ray data collection

All X-ray data sets were collected at 100 K. The data set for C₂B-A and C₂B-C were collected on our in-house Rigaku R-Axis HTC

with 10 min exposure per 0.5° frame. The data set for C₂B-B was collected at Advanced Photon Source, 14BM-C, Argonne National Laboratory, using 10 sec exposure per 0.2° frame for high-resolution data, followed by 0.65 sec exposure per 0.5° frame for low-resolution data. The data set for C₂B-D was collected at APS 19BM, Argonne National Laboratory using 4 sec exposure per 0.25° frame. The statistics for each data set are given in Table 1.

Structure determination

The crystal structures were determined by a molecular replacement procedure using the program Molrep from the CCP4 suite (Collaborative Computational Project 1994). The coordinates of the NMR-based C₂B solution structure (1K5W; Fernandez et al. 2001) was used as the search model. Crystallographic refinement was done with CNS (Brunger et al. 1998) and Refmac5 (Murshudov et al. 1997). We used 5% of the data for the R_{free} calculation. Iterative cycles of interactive manual refitting of the model were carried out using the program Turbo, which made use of maps created with CNS or Refmac5 to complete and correct the model. Restrained refinement was carried out using a maximum likelihood target function and anisotropic temperature factors for individual atoms. Additional alternate conformations and water molecules were added. During the later stages of refinement, difference ($F_o - F_c$) maps were used to place the divalent atoms.

Coordinates deposition

The crystal structures of C₂B-B, C₂B-C and C₂B-D have been deposited in PDB as 1TJX, 1UOV, and 1TJM, respectively.

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