Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray analysis of squid neuronal Sec1

Andreas Bracher,[a] Thomas Dresbach,[b] Heinrich Betz[c] and Winfried Weissenhorn[a]*

[a]European Molecular Biology Laboratory (EMBL), 6 Rue Jules Horowitz, 38000 Grenoble, France, [b]Leibniz Institute for Neurobiology, Brenneckestrasse 6, 39118 Magdeburg, Germany, and [c]Max-Planck-Institut for Brain Research, Deutschordenstrasse 46, 60528 Frankfurt am Main, Germany

Correspondence e-mail: weissen@embl-grenoble.fr

Received 22 December 1999
Accepted 3 February 2000

Sec1 protein family members are involved in the regulation of all intracellular SNARE-mediated (SNARE = soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) vesicle-fusion processes in a step preceding membrane fusion and have been shown to interact with t-SNAREs. To better understand the structural basis and the role of Sec1 in the regulation of the SNARE-complex formation, neuronal Sec1 from the squid *Loligo pealei* has been expressed and crystallized; this invertebrate protein shows a high sequence homology to the human neuronal Sec1, Munc18a. Here, the production of diffraction-quality native crystals, which belong to space group $P3_121$ and diffract to 3.3 Å resolution, is described. In addition, selenomethionyl n-Sec1 crystals in space groups $P3_121$ and $P2_1$ have been generated. Preliminary analysis of the monoclinic space group indicates that these crystals diffract to a resolution higher than 2.5 Å.

1. Introduction

The selective transport of cargo between subcellular organelles is a crucial process in the generation of membrane compartments in eukaryotic cells. This process depends critically on selective and efficient membrane fusion. A number of genetic and biochemical experiments has attributed a central role in intracellular fusion events to SNARE proteins (Söllner *et al.*, 1993; Lian & Ferro-Novick, 1993; Nichols *et al.*, 1997; Sato & Wickner, 1998; Katz *et al.*, 1998). SNARE molecules represent prototypes of protein families whose members are distributed throughout the secretory pathway (Hardwick & Pelham, 1992). These complementary membrane-anchored proteins are localized on opposing membranes (Gerst, 1999) and form a highly stable ternary complex. Electron-microscopy studies and the recently solved structure of a proteolytic SNARE core complex have suggested a model by which SNARE molecules may catalyse membrane fusion (Hanson *et al.*, 1997; Sutton *et al.*, 1998). Consistent with this view, the SNARE complex has been shown to constitute a minimal membrane-fusion machinery *in vitro* (Weber *et al.*, 1998; Parlati *et al.*, 1999). *In vivo*, assembly of a SNARE fusion complex is a highly regulated specific process. A number of proteins, including Sec1 family members, are thought to be involved in the regulation of the fusion machinery (Mayer, 1999).

Neuronal Sec1 (n-Sec1) from the squid *L. pealei* (Dresbach *et al.*, 1998) belongs to an evolutionary conserved family of proteins generically named after the yeast protein Sec1 (Halachmi & Lev, 1996). n-Sec1 is involved in regulating the fusion of synaptic vesicles with the plasma membrane, resulting in the release of neurotransmitter into the synaptic cleft. The squid protein is a close sequence homologue of the mammalian neuronal protein Munc18a, indicating a high conservation of function. Sec1 family proteins have been shown to form binary complexes with t-SNAREs, as well as to inhibit SNARE-complex formation by competing for the t-SNARE *in vitro* (Garcia *et al.*, 1994; Pevsner *et al.*, 1994; Dulubova *et al.*, 1999). Consequently, Sec1 molecules have been postulated to exert a regulatory function in a step preceding SNARE-complex formation (Schulze *et al.*, 1994). Micro-injection studies at the squid giant synapse indicate that addition of squid n-Sec1 inhibits neurotransmission without affecting action potentials. Co-injection with a cytosolic fragment of its corresponding t-SNARE, neuronal squid syntaxin, restored proper neuronal function (Dresbach *et al.*, 1998), thus verifying its regulatory role in neurotransmission.

Sec1 family molecules are cytosolic proteins which are usually associated with membranes *in vivo*, probably through their interaction with t-SNAREs (Halachmi *et al.*, 1995; Carr *et al.*, 1999). Their consensus sequence comprises no known structural building blocks. Here, we

© 2000 International Union of Crystallography
Printed in Denmark – all rights reserved

report the crystallization of a Sec1 family protein, n-Sec1 from squid, and present preliminary data on its X-ray analysis.

2. Materials and methods

2.1. Expression and purification

Squid n-Sec1 was expressed as a fusion protein with a 6-His-tag at the N-terminus as described previously (Dresbach *et al.*, 1998) in *Escherichia coli* cells Bl21/pUBS (Brinkmann *et al.*, 1989). Cells were grown in LB medium to an optical density (600 nm) of 0.6 and protein expression was induced with 1 m*M* isopropylthio-β-D-galactoside for 3 h. *E. coli* cultures were harvested by centrifugation and lysed in 50 ml lysis buffer (50 m*M* HEPES/KOH pH 7.4, 200 m*M* KCl, 10 m*M* mercaptoethanol) per 10 g of bacterial pellet by sonification. To prevent proteolysis, a cocktail of protease inhibitors was added prior to sonification (1 m*M* phenylmethylsulfonyl fluoride, 5 μ*M* leupeptin, 0.3 μ*M* pepstatin, 0.005 trypsin inhibitor units aprotinin, 0.05 m*M* tosyl-L-lysine chloromethyl ketone). The supernatant was cleared by centrifugation (20 000 rev min^{-1}, 1 h) and loaded onto a column containing Ni^{2+}-chelating Sepharose FF (Pharmacia Biotech) pre-equilibrated with lysis buffer. The n-Sec1 fusion protein was recovered by applying a linear gradient of 0–350 m*M* imidazole in lysis buffer. After dialysis against a buffer containing 20 m*M* HEPES/KOH pH 8.2 and 5 m*M* DTT, the preparation was applied onto a Q-Sepharose column (Pharmacia), which was eluted with a linear gradient of 0–150 m*M* KCl. A final purification step included gel-filtration chromatography on Superose-12 (Pharmacia) in a buffer containing 20 m*M* HEPES/KOH pH 7.4, 100 m*M* KCl and 10 m*M* DTT. For crystallization experiments, the protein was concentrated to 4–6 mg ml^{-1} as determined by the Biorad protein assay. Selenomethionine-derivatized n-Sec1 was expressed as described by Van Duyne *et al.* (1993) and the same purification protocol as described above was employed.

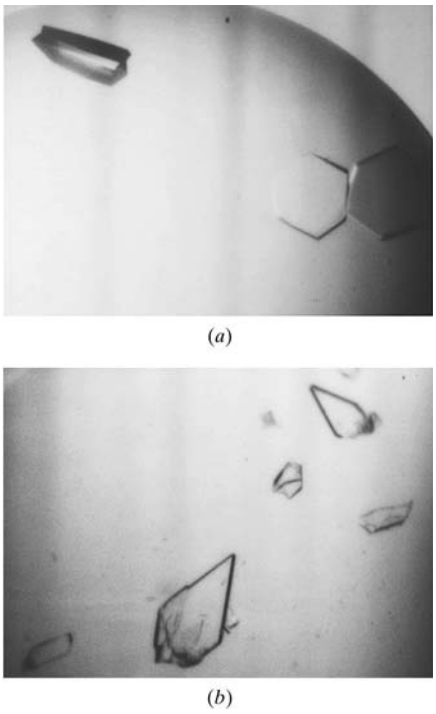

Figure 1
(*a*) Crystals of native n-Sec1. Hexagonal plates were grown from solutions containing 15%(*w*/*v*) PEG 1000, 0.4 *M* ammonium sulfate, 0.1 *M* sodium citrate pH 5.6 and 20 m*M* DTT. Crystals grew to maximum dimensions of 0.6 × 0.6 × 0.1 mm in 5 d. (*b*) Crystals of selenomethionine-derivatized n-Sec1. These crystals grew in the storage buffer at 277 K and have maximum dimensions of 0.2 × 0.2 × 0.1 mm.

2.2. Crystallization and X-ray analysis

Crystallization trials were set up at room temperature and initial screenings were performed with commercial Crystal Screen kits (Hampton Research) in hanging-drop vapour-diffusion experiments. Subsequent optimization of crystallization buffers resulted in hexagonal shaped plates which grew on mixing 2 µl of protein solution with 1 µl of reservoir buffer containing 15%(*w*/*v*) PEG 1000, 0.4 *M* ammonium sulfate, 0.1 *M* sodium citrate pH 5.6, 20 m*M* DTT and 10 m*M* $CaCl_2$. Native crystals were soaked in reservoir buffer containing 0.2 m*M* $K_2Pt(CN)_4$ for 12 h for heavy-metal derivatization.

The selenomethionyl-derivatized protein crystallized under the same conditions as native protein. In addition, a different crystal form could be obtained from the storage-buffer solution at 277 K (20 m*M* HEPES/KOH pH 7.4, 100 m*M* KCl, 20 m*M* DTT).

Prior to cryo-mounting using 20 µm cryoloops (Hampton Research), crystals were crushed with a glass needle in order to separate the halves resulting from macroscopic twinning. Small crystals were frozen in a 100 K nitrogen stream after rapid transfer into a reservoir buffer containing 20% glycerol. Larger crystals were transferred to 10% PEG 1000, 0.27 *M* ammonium sulfate, 0.133 *M* KCl, 0.067 *M* sodium citrate, 0.027 *M* HEPES/KOH pH 6.1 and 20 m*M* DTT and then transferred to buffers containing 5, 10, 15 and 20% glycerol in 1 h steps.

Native data were collected at the ID13 Microfocus beamline of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) at a wavelength of 0.784 Å. The data set contains two segments of 30° (1° oscillation frames) which were recorded on a MAR CCD detector.

Table 1
Summary of data-collection statistics.

Numbers in parentheses refer to the final resolution shell.

	Native	PtCN†
ESRF beamline	ID13	ID14 EH3
Wavelength (Å)	λ = 0.784	λ = 0.931
Maximum resolution (Å)	3.3	3.5
R_{merge}‡	6.2 (29.7)	6.5 (28.1)
Completeness (%)	95.8 (96.3)	97.9 (93.5)
No. of reflections	79526	96949
No. of unique reflections	22649	20022
Average multiplicity	3.5 (3.2)	4.8 (4.4)
$\langle I/\sigma(I)\rangle$	15.5 (4.3)	19.3 (3.7)
Unit-cell dimensions (Å)	$a = b$ = 118.5, c = 192.6	$a = b$ = 118.1, c = 195.0

† Potassium tetracyanoplatinate(II) soak. ‡ $R_{merge} = \sum_h \sum_i |I_i(h) - \langle I(h)\rangle| / \sum_h \sum_i I_i(h)$, where $I_i(h)$ is the ith measurement and $\langle I(h)\rangle$ is the weighted mean of all measurements of $I(h)$.

The data were processed and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The solvent content was calculated with the program *MATTHEWS_COEFF* (Collaborative Computational Project, Number 4, 1994) and the self-rotation functions were calculated using *GLRF* (Tong & Rossmann, 1990).

3. Results and discussion

Squid n-Sec1 was expressed in *E. coli* and purified as a 6-His-tag fusion protein with a final yield of 0.5 mg per litre of culture. The purified protein was eluted as a sharp band from a Superose-12 gel-filtration column, indicating the homogeneity of the protein preparation. Crystals were obtained from solutions containing 15%(*w*/*v*) PEG 1000, 0.4 *M* ammonium sulfate, 0.1 *M* sodium citrate pH 5.6 and 20 mM DTT by the vapour-diffusion method using hanging drops. The His tag did not interfere with the crystallization; hexagonal-shaped crystals grew to 0.6 mm in the longest dimension (Fig. 1*a*). The size of the crystals was improved by lowering the nucleation rate *via* addition of 10 m*M* $CaCl_2$ to the crystallization drop. Hexagonal-shaped crystals exhibited systematic macroscopic twinning, usually two stacked plates which could be easily separated from each other mechanically.

The best data set thus far was collected on the ESRF ID13 micro-focusing beamline from a fragment of a hexagonal crystal plate, with data to 3.3 Å resolution and an overall completeness of 96% (Table 1). The auto-

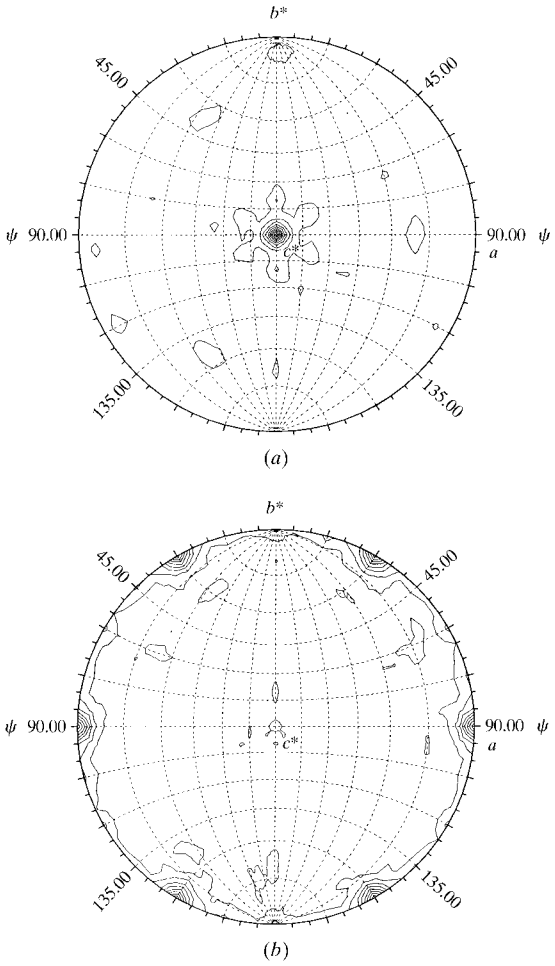

Figure 2
Self-rotation function maps of n-Sec1 for (*a*) the $\kappa = 120^\circ$ section and (*b*) the $\kappa = 180^\circ$ section (using data in the resolution range 10–4 Å).

indexing procedure using *DENZO* indicated that the crystals belong to the trigonal crystal system with unit-cell parameters $a = b = 118.52$, $c = 192.62$ Å. Systematic absences along (0, 0, *l*) indicated a threefold screw axis and the lattice was assigned to space group $P3_121$ (or $P3_221$) in accordance with the self-rotation function results (Fig. 2).

Considering a molecular weight of 68 136 Da for the squid n-Sec1, the Matthews coefficient was determined to be 2.87 Å^3 Da^{-1}, assuming two molecules per asymmetric unit with a corresponding solvent content of 56.74%. However, no significant non-crystallographic peaks were observed in the self-rotation function from 0–360°; the solvent content for one molecule per asymmetric unit would be 78.37%.

Initial screening of heavy-metal derivatized native crystals indicated a high degree of non-isomorphism among data sets. The statistics for a data set from a platinum-derivatized crystal are included in Table 1. Therefore, the n-Sec1 protein derivatized with selenomethionine was also produced, which crystallized as hexagonal plates belonging to space group $P3_121$, in a similar manner to the native crystals. Initial testing on a rotating-anode generator equipped with a MAR image-plate detector showed that these crystals diffract to approximately 4 Å, which is comparable with the native crystals on the rotating-anode X-ray source. Surprisingly, large crystals of the selenomethionine-derivatized n-Sec1 also grew from the storage buffer at 277 K (Fig. 1*b*). These crystals grow to a maximum size of $0.2 \times 0.2 \times 0.05$ mm and initial screening on the ESRF ID14 EH4 beamline showed diffraction to better than 2.5 Å at a relatively low X-ray intensity (single-bunch mode). Initial indexing indicates that these crystals belong to space group $P2_1$, with unit-cell parameters $a = 49.2$, $b = 122.9$, $c = 63.7$ Å, $\beta = 110.9^\circ$. This second crystal form will now be employed in order to solve the structure by the MAD technique. The n-Sec1 protein contains 21 methionine residues, which should allow successful phase determination by collecting data at the selenium *K* absorption edge.

We thank Dr A. Perrakis (EMBL, Grenoble) and Dr G. Leonard (ESRF, Grenoble) for help with data collection.

References

Brinkmann, U., Mattes, R. E. & Buckel, P. (1989). *Gene*, **85**, 109–114.
Carr, C. M., Grote, E., Munson, M., Hughson, F. M. & Novick, P. J. (1999). *J. Cell. Biol.* **146**, 333–344.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Dresbach, T., Burns, M. E., O'Connor, V., DeBello, W. M., Betz, H. & Augustine, G. J. (1998). *J. Neurosci.* **18**, 2923–2932.
Dulubova, I., Sugita, S., Hill, S., Hosaka, M., Fernandez, I., Südhof, T. C. & Rizo, J. (1999). *EMBO J.* **18**, 4372–4382.
Garcia, E. P., Gatti, E., Butler, M., Burton, J. & De Camilli, P. (1994). *Proc. Natl Acad. Sci. USA*, **91**, 2003–2007.
Gerst, J. E. (1999). *Cell. Mol. Life Sci.* **55**, 707–734.
Halachmi, N., Feldman, M., Kimchie, Z. & Lev, Z. (1995). *Eur. J. Cell. Biol.* **67**, 275–283.
Halachmi, N. & Lev, Z. (1996). *J. Neurochem.* **66**, 889–897.
Hanson, P. I., Roth, R., Morisaki, H., Jahn, R. & Heuser, J. E. (1997). *Cell*, **90**, 523–535.
Hardwick, K. G. & Pelham, H. R. (1992). *J. Cell. Biol.* **119**, 513–521.
Katz, L., Hanson, P. I., Heuser, J. E. & Brennwald, P. (1998). *EMBO J.* **17**, 6200–6209.
Lian, J. P. & Ferro-Novick, S. (1993). *Cell*, **73**, 735–745.
Mayer, A. (1999). *Curr. Opin. Cell Biol.* **11**, 447–452.
Nichols, B. J., Ungermann, C., Pelham, H. R., Wickner, W. T. & Haas, A. (1997). *Nature (London)*, **387**, 199–202.
Otwinowski, Z & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Parlati, F., Weber, T., McNew, J. A., Westermann, B., Söllner, T. H. & Rothman, J. E. (1999). *Proc. Natl Acad. Sci. USA*, **96**, 12571–12576.
Pevsner, J., Hsu, S. C., Braun, J. E., Calakos, N., Ting, A. E., Bennett, M. K. & Scheller, R. H. (1994). *Neuron*, **13**, 353–361.
Sato, K. & Wickner, W. (1998). *Science*, **281**, 700–702.
Schulze, K. L., Littleton, J. T., Salzberg, A., Halachmi, N., Stern, M., Lev, Z. & Bellen, H. J. (1994). *Neuron*, **13**, 1099–1108.
Söllner, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H. & Rothman, J. E. (1993). *Cell*, **75**, 409–418.
Sutton, R. B., Fasshauer, D., Jahn, R. & Brunger, A. T. (1998). *Nature (London)*, **395**, 347–353.
Tong, L. & Rossmann, M. G. (1990). *Acta Cryst.* A**46**, 783–792.
Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L. & Clardy, J. (1993). *J. Mol. Biol.* **299**, 105–124.
Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Söllner, T. H. & Rothman, J. E. (1998). *Cell*, **92**, 759–772.