Xiaojie Yan^{a*} and Yuequan Shen^b
^aDepartment of BMB, Basic Medical College, Tianjin Medical University, Tianjin 300070, People's Republic of China, and ^bState Key Laboratory of Medicinal Chemical Biology, College of Life Sciences, Nankai University, Tianjin 300071, People's Republic of China

Correspondence e-mail: xjyan@tjmu.edu.cn

Received 29 January 2013

Accepted 4 March 2013

Preliminary crystallographic analysis of the kinase domain of SAD-1, a protein essential for presynaptic differentiation in *Caenorhabditis elegans*

SAD-1 is a serine/threonine kinase which plays an important role in the regulation of both neuronal polarity and synapse formation in *Caenorhabditis elegans*. The kinase domain of SAD-1 from *C. elegans* was overexpressed in *Escherichia coli* BL21 (DE3) cells and purified to homogeneity using nickel-nitrilotriacetic acid metal-affinity, ion-exchange and gel-filtration chromatography. Diffraction-quality crystals were grown using the sitting-drop vapour-diffusion technique from a condition consisting of 1 M CAPSO pH 9.6, 10% (w/v) polyethylene glycol 3350. The crystals belonged to the monoclinic space group C2, with unit-cell parameters $a = 205.4$, $b = 57.1$, $c = 71.7$ Å, $\beta = 106.1^\circ$. X-ray diffraction data were recorded to 3.0 Å resolution from a single crystal using synchrotron radiation.

1. Introduction

Nervous-system development is a series of events that includes the maturation of neurons as well as the establishment of synapses. Mature neurons are highly polarized cells which receive input signals via dendrites and transmit output signals along axons. These mature neurons undergo a series of morphological transformations to acquire their axonal and dendritic characteristics (Dotti *et al.*, 1988). This process can be divided into several stages. Initially, the nascent neuron extends multiple short neurites. The single neurite that extends the most rapidly then develops into an axon and the remaining neurites become dendrites. As the axon grows and contacts its targets, synapses are formed so as to establish communication in neuronal networks. According to recent studies, the process of axon/dendrite specification is driven by both extracellular cues, including morphogens and growth factors, and intracellular mechanisms such as signalling kinase cascades, their associated scaffolding proteins and factors affecting actin-cytoskeleton and microtubule dynamics (Arimura & Kaibuchi, 2005; Wiggin *et al.*, 2005; Barnes & Polleux, 2009; Conde & Cáceres, 2009; Polleux & Snider, 2010).

Only a few of these polarity regulators have been verified to be related to axon/dendrite specification *in vivo*. One of them is a family of Ser/Thr kinases called SAD (synapses of amphids defective). SAD-1, which has been implicated in synapse formation and neuronal polarity in *Caenorhabditis elegans*, was the first to be shown to be required for neuronal polarization *in vivo* (Kishi *et al.*, 2005; Hung *et al.*, 2007). Both aberrant synaptic organization and defects in neuronal polarity are observed in *sad-1* loss-of-function mutants (Crump *et al.*, 2001; Hung *et al.*, 2007), implying the importance of SAD-1 during the establishment of neuronal polarity and synaptic organization.

SAD-1 has been extensively studied genetically and biochemically *in vivo* and *in vitro* (Crump *et al.*, 2001; Hung *et al.*, 2007; Kim *et al.*, 2008). Many structures of kinases from the same family as SAD-1 from various organisms such as *Homo sapiens* (Fedorov *et al.*, 2007), *Bos taurus* (Bettayeb *et al.*, 2008), *Escherichia coli* (Bakali *et al.*, 2007), *Enterococcus faecalis* (Fong *et al.*, 2011) and *Mycobacterium tuberculosis* (Young *et al.*, 2003) have contributed to understanding of the kinase mechanism of SAD-1 at the molecular level; however, its

© 2013 International Union of Crystallography
All rights reserved

own structural characterization is required for a complete understanding of the molecular basis of SAD-1 function. With this aim, structural studies of uncharacterized SAD-1s have been initiated by our group. Since we failed to express full-length *C. elegans* SAD-1 in *E. coli*, and we found that residues 42–298 corresponded to its kinase domain (Crump *et al.*, 2001), we constructed a truncated form (amino acids 39–300) according to secondary-structure prediction. The function of residues 1–38 of SAD-1 is as yet unknown. Here, we expressed and crystallized the kinase domain of SAD-1 (amino acids 39–300) and we also report the preliminary X-ray diffraction studies of SAD-1.

2. Materials and methods

2.1. DNA construction, protein expression and purification

A gene fragment containing the kinase domain (residues 39–300) of *C. elegans* SAD-1 (hereafter referred to as ceSKD) was amplified from the cDNA library of *C. elegans* using the polymerase chain reaction. The DNA fragment was introduced into the *Bam*HI and *Xho*I sites of an in-house-modified version of the pET32a vector (Novagen, San Diego, California, USA) in which the S-tag and the thrombin-recognition site were replaced by a PreScission protease-cleavable segment (LEVLFQGP). The amplified sequence was confirmed by DNA sequencing (Invitrogen, People's Republic of China). The resulting protein contained two additional amino acids (GS) between the PreScission protease-cleavage site and ceSKD as a cloning artifact.

The expression vector was transformed into *E. coli* BL21 (DE3) cells. The cells were grown to an OD₆₀₀ of 0.6 in LB broth medium containing 50 µg ml⁻¹ ampicillin at 310 K; they were then induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cultured for a further 4–6 h at 298 K. The cells were harvested by centrifugation at 6000g for 30 min and stored at 193 K until further use. Cell lysis was performed by sonication on ice in nickel-nitrilotriacetic acid (NTA) resin binding buffer (20 mM Tris, 300 mM NaCl

pH 7.5). All of the following purification steps were carried out at 277 K. The lysate was clarified by centrifugation at 18 000g for 40 min. The supernatant was loaded onto an Ni-NTA resin column (Qiagen) and nonspecifically bound contaminants were removed by washing the column with washing buffer (binding buffer plus 10 mM imidazole). Finally, Trx-6×His-ceSKD (where Trx indicates a thio-redoxin tag) was eluted in elution buffer (binding buffer plus 500 mM imidazole). After concentration using an Amicon Ultra filter (Millipore), the fusion protein was dissolved in low-salt buffer (20 mM Tris, 20 mM NaCl pH 7.5). The Trx-6×His-ceSKD protein was then digested using PreScission protease (Freuler *et al.*, 2008) at 277 K for 12 h and the cleaved protein, which had four additional residues (GPGS) at its N-terminus, was purified on a HiTrap SP HP column (GE Healthcare) using a linear NaCl gradient from 20 to 800 mM. The protein peak corresponding to ceSKD was pooled, concentrated, loaded onto a HiLoad 16/60 Superdex 200 pg column (GE Healthcare Life Sciences, USA) and eluted with gel-filtration buffer (20 mM Tris, 20 mM NaCl pH 7.5, 1 mM DTT). A sharp peak corresponding to the ceSKD was pooled and concentrated to 9 mg ml⁻¹. The purity of the protein was assessed by 13% Coomassie-stained SDS-PAGE (Fig. 1). The yield of pure protein was about 5 mg per litre of culture.

2.2. Crystallization and data collection

Sitting-drop crystallization trials of ceSKD were performed at 293 K by mixing 1 µl protein solution (9 mg ml⁻¹ in gel-filtration buffer) with 1 µl precipitant solution in 48-well sitting-drop plates. Initial screening using Index, Crystal Screen, Crystal Screen 2, PEGRx 1, PEGRx 2, SaltRx 1 and SaltRx 2 (Hampton Research) produced two promising crystallization conditions. Rod-like single crystals appeared in both Crystal Screen condition No. 36 [0.1 M Tris pH 8.5, 8% (w/v) PEG 8000] and PEGRx 1 condition No. 30 [0.1 M sodium HEPES pH 7.5, 20% (w/v) PEG 3350]. Initial crystallization conditions were optimized by changing the concentrations of the precipitants, by changing the pH and by the use of additives. Finally, diffraction-quality crystals were obtained after one week in a condition consisting of 1 M CAPSO pH 9.6, 10% (w/v) PEG 3350 using the 9 mg ml⁻¹ ceSKD stock (Fig. 2).

Data collection from native crystals was performed at 100 K using a wavelength of 0.9792 Å at the Shanghai Synchrotron Radiation Facility (SSRF), Shanghai, People's Republic of China. For data collection under cryogenic conditions, crystals were soaked for a few

Figure 1
SDS-PAGE profile of the purified ceSKD. The gel was stained with Coomassie Brilliant Blue R-250. Lane 1, molecular-mass marker (labelled in kDa); lane 2, purified ceSKD. 30 µg of protein was loaded onto the gel.

Figure 2
Crystals of ceSKD. The approximate dimensions of the ceSKD crystals were 55 × 10 × 5 µm.

seconds in mother liquor containing 25%(v/v) glycerol. Crystals were then mounted on the beamline in a nylon loop and flash-cooled in a nitrogen-gas stream at 100 K. Data sets were collected by rotation through 180° with an increment of 0.5° per frame at a crystal-to-detector distance of 200 mm. Data sets were processed using the *HKL-2000* package (Otwinowski & Minor, 1997). Data-collection and processing statistics are summarized in Table 1.

3. Results and discussion

SAD-1 is a serine/threonine kinase expressed in the nervous system that localizes to synapse-rich regions of the axons and is crucial for

Table 1

Data-collection statistics for the SAD-1 kinase domain.

Values in parentheses are for the highest resolution shell.

Data collection	
Beamline	BL-17U1
Wavelength (Å)	0.9792
Detector	MAR 245 CCD
Crystal parameters	
Space group	<i>C2</i>
Unit-cell parameters (Å, °)	<i>a</i> = 205.4, <i>b</i> = 57.1, <i>c</i> = 71.7, β = 106.1
Resolution range (Å)	50–3.0
Molecules per asymmetric unit	2 or 3
Solvent content (%)	63.49 or 45.24†
Matthews coefficient (Å ³ Da ^{−1})	3.37 or 2.24†
Data processing	
No. of observed reflections	59048
No. of unique reflections	16119
Multiplicity	3.7 (3.7)
Completeness (%)	99.3 (99.7)
<i>R</i> _{merge} ‡ (%)	9.9 (68.1)
<i>I</i> / <i>σ</i> (<i>I</i>)	13.4 (3.7)

† The values given are for two and three molecules in the crystal asymmetric unit, respectively. ‡ $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

Figure 3

A representative diffraction image from the ceSKD crystal. The resolution at the edge of the detector is 1.5 Å; a complete data set was collected to 3.0 Å resolution.

presynaptic differentiation in *C. elegans* (Crump *et al.*, 2001). ceSKD (cloned in an in-house-modified version of the pET32a vector) was overexpressed in *E. coli* BL21 (DE3) cells and was purified to homogeneity using nickel–nitrilotriacetic acid metal-affinity, ion-exchange and gel-filtration chromatography. The purified protein had a purity of greater than 95% (Fig. 1).

Figure 4

Stereographic projection of the self-rotation function, with the crystallographic *a* axis along $\varphi = 0^\circ$, $\psi = 90^\circ$ and the *c** axis perpendicular to the plane of the figure. The φ angle varies from 0 to $\pm 180^\circ$ around the plot, while the ψ angle varies from 0° at the centre to 90° at the edge of the ring. (a) Section with $\kappa = 180^\circ$; there are two obvious peaks (30, -180 , 180° and 60, 0, 180°) in this section. (b) Section with $\kappa = 120^\circ$. The resolution of the data used was 15.0–3.0 Å and the radius of integration was 30 Å.

Diffraction-quality crystals (Fig. 2) of ceSKD were obtained by the sitting-drop vapour-diffusion technique. The crystals diffracted to 3.0 Å resolution (Fig. 3) and a complete native data set was collected. The data were processed and scaled using the *HKL-2000* (Otwinowski & Minor, 1997) and *CCP4* (Winn *et al.*, 2011) program suites. The crystals of ceSKD belonged to space group *C2*, with unit-cell parameters $a = 205.4$, $b = 51.7$, $c = 101.5$ Å, $\beta = 106.1^\circ$, and may contain two or three molecules per asymmetric unit based on solvent-content calculations (Matthews, 1968). The data-processing statistics are given in Table 1. The self-rotation function was calculated using *MOLREP* (Vagin & Teplyakov, 2010) from the *CCP4* suite (Winn *et al.*, 2011) with a radius of integration of 30 Å using diffraction data within the 15.0–3.0 Å resolution range. There are two obvious peaks potentially generated by a single noncrystallographic twofold axis perpendicular to the space-group twofold axis (Fig. 4*a*) and these peaks remained present when the radius of integration was varied. No obvious peak corresponding to a threefold axis (Fig. 4*b*) was observed. Molecular replacement using the coordinates of the kinase domain of human 5'-AMP-activated protein kinase catalytic subunit alpha-2 (PDB entry 2h6d; Littler *et al.*, 2010) as a template was attempted with the *Phaser* molecular-replacement program (McCoy, 2007) as implemented in the *CCP4* suite (Winn *et al.*, 2011); no valid solution was obtained. This result may be a consequence of conformational differences between the two kinase domains.

The crystallization and preliminary X-ray crystallographic studies of ceSKD provide the possibility of solving its structure. Attempts to use a selenomethionine derivative for MAD/SAD phasing are in progress; there are five methionine residues that are suitable for replacement with selenomethionine in ceSKD. Further structure determination of ceSKD will provide a detailed description and structural insights into the kinase domain and will contribute to the understanding of the functional mechanism of *C. elegans* SAD-1.

We are grateful to the staff of the SSRF, Shanghai, People's Republic of China for support during data collection.

References

- Arimura, N. & Kaibuchi, K. (2005). *Neuron*, **48**, 881–884.
- Bakali, H. M., Herman, M. D., Johnson, K. A., Kelly, A. A., Wieslander, A., Hallberg, B. M. & Nordlund, P. (2007). *J. Biol. Chem.* **282**, 19644–19652.
- Barnes, A. P. & Polleux, F. (2009). *Annu. Rev. Neurosci.* **32**, 347–381.
- Bettayeb, K., Oumata, N., Echaliier, A., Ferandin, Y., Endicott, J. A., Galons, H. & Meijer, L. (2008). *Oncogene*, **27**, 5797–5807.
- Conde, C. & Cáceres, A. (2009). *Nature Rev. Neurosci.* **10**, 319–332.
- Crump, J. G., Zhen, M., Jin, Y. & Bargmann, C. I. (2001). *Neuron*, **29**, 115–129.
- Dotti, C. G., Sullivan, C. A. & Banker, G. A. (1988). *J. Neurosci.* **8**, 1454–1468.
- Fedorov, O., Marsden, B., Pogacic, V., Rellos, P., Müller, S., Bullock, A. N., Schwaller, J., Sundström, M. & Knapp, S. (2007). *Proc. Natl Acad. Sci. USA*, **104**, 20523–20528.
- Fong, D. H., Xiong, B., Hwang, J. & Berghuis, A. M. (2011). *PLoS One*, **6**, e19589.
- Freuler, F., Stettler, T., Meyerhofer, M., Leder, L. & Mayr, L. M. (2008). *Protein Expr. Purif.* **59**, 232–241.
- Hung, W., Hwang, C., Po, M. D. & Zhen, M. (2007). *Development*, **134**, 237–249.
- Kim, J. S. M., Lilley, B. N., Zhang, C., Shokat, K. M., Sanes, J. R. & Zhen, M. (2008). *Neural Dev.* **3**, 23.
- Kishi, M., Pan, Y. A., Crump, J. G. & Sanes, J. R. (2005). *Science*, **307**, 929–932.
- Littler, D. R., Walker, J. R., Davis, T., Wybenga-Groot, L. E., Finerty, P. J., Newman, E., Mackenzie, F. & Dhe-Paganon, S. (2010). *Acta Cryst.* **F66**, 143–151.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- McCoy, A. J. (2007). *Acta Cryst.* **D63**, 32–41.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Polleux, F. & Snider, W. (2010). *Cold Spring Harb. Perspect. Biol.* **2**, a001925.
- Vagin, A. & Teplyakov, A. (2010). *Acta Cryst.* **D66**, 22–25.
- Wiggin, G. R., Fawcett, J. P. & Pawson, T. (2005). *Dev. Cell*, **8**, 803–816.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.
- Young, T. A., Delagoutte, B., Endrizzi, J. A., Falick, A. M. & Alber, T. (2003). *Nature Struct. Biol.* **10**, 168–174.