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Crystallization and preliminary crystallographic studies of human septin 1 with site-directed mutations

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Septin 1 is a member of an evolutionarily conserved family of GTP-binding and filament-forming proteins named septins, which function in diverse processes including cytokinasis, vesicle trafficking, apoptosis, remodelling of the cytoskeleton, infection, neurodegeneration and neoplasia. Human septin 1 has been expressed and purified, but suffers from severe aggregation. Studies have shown that septin 1 with site-directed mutations of five serine residues (Ser19, Ser206, Ser307, Ser312 and Ser315) has a much lower degree of aggregation and better structural homogeneity and that the mutations cause only slight perturbations in the secondary structure of septin 1. This septin 1 mutant was crystallized and diffraction data were collected to 2.5 Å resolution. The space group is $P422$, with unit-cell parameters $a = b = 106.028$, $c = 137.852$ Å.

1. Introduction

Septins are an evolutionarily conserved group of GTP-binding and filament-forming proteins that belong to the large superclass of P-loop GTPases. The septins were first recognized in *Saccharomyces cerevisiae* as a set of homologous proteins (the products of the *CDC3*, *CDC10*, *CDC11* and *CDC12* genes) associated with the 10 nm filaments found at the cytoplasmic face of the plasma membrane in the mother-bud neck (Byers & Goetsch, 1976; Byers, 1981; Adams, 1984; Hearer & Pringle, 1987; Kim *et al.*, 1991; Ford & Pringle, 1991). The name septin was chosen to indicate the role of these proteins, which act in separating mother and daughter cells. In the recent years, more and more members of the septin family have been found. Members of this family have been identified in insects (*Drosophila*), amphibians (*Xenopus*) and mammals (both mouse and human). Septins are broadly expressed throughout the animal kingdom, but seem to be absent in plants (Frazier *et al.*, 1998).

Because septin genes were first identified in a genetic screen for *Saccharomyces cerevisiae* mutants defective in cytokinesis (Hartwell, 1971), they have long been believed to play a role in cytokinesis. However, recent research has indicated that septins not only play a role in cytokinesis but also have diverse cellular roles including polarity determination (Drees *et al.*, 2001; Faty *et al.*, 2002; Irazoqui & Lew, 2004), cytoskeletal reorganization, membrane dynamics, vesicle trafficking and exocytosis (DeMarini *et al.*, 1997; Hsu *et al.*, 1998; Beites *et al.*, 1999; Surka *et al.*, 2002; Finger, 2002; Finger *et al.*, 2003; Nagata *et al.*, 2003). To date, little is known about how they function. It is well known that the primary structures of septins are conserved between different species and that all septins have a common GTP-binding domain. One of the noticeable characteristics of septins is that they tend to aggregate, forming homo- and hetero-oligomeric structures which can form high ordered filaments *in vitro* and *in vivo* (Fares *et al.*, 1995; Frazier *et al.*, 1998; Mendoza *et al.*, 2002; Casamayor & Snyder, 2003).

As a member of the septin family, septin 1 may play an important role in the cellular processes mentioned above, although little is known about its biological function. It should be very useful to determine its structure in order to understand the function of septin 1. The secondary-structure prediction of wild-type septin 1 has been carried out using the *Jpred* (Kabsch & Sander, 1983; Lupas *et al.*, 1991; Barton, 1993; Altschul *et al.*, 1997; Wolf *et al.*, 1997; Eddy, 1998)

Table 1
Primers for site-directed mutation.

Primer name	Primer sequence
Sept1-S19A-A	GCTGCACCGCAAGGCTGTCAAGAAG
Sept1-S19A-B	CTTCTTGACAGCCTTGCGGTGCAGC
Sept1-S206A-A	CCGAATGTGACGCTGATGAAGATG
Sept1-S206A-B	CATCTTCATCAGCGTCACATTCGG
Sept1-S307A-A	GCGATCGAGCCGGCCGCAGTAAG
Sept1-S307A-B	CTTACTGCGGCCGGCTCGATCGC
Sept1-S312A-A	GCAGTAAGCTTGCCCGCCAGAGC
Sept1-S312A-B	GCTCTGGCGGGCAAGCTTACTGC
Sept1-S315A-A	TTTCCCGCCAGGGCGCCACAGAG
Sept1-S315A-B	CTCTGTGGCGCCCTGGCGGGAAA

and *NNPREDICT* (McClelland & Rumelhart, 1988; Kneller *et al.*, 1990) online prediction servers, which predict a 45% α-helix content, with β-sheets and coils making a comparatively small contribution to the overall secondary structure (Fig. 1). Our studies have shown that the recombinant septin 1 aggregates severely in solution, making both X-ray diffraction and NMR studies impossible. This may be the main reason why there is no detailed tertiary structure information about septin 1 available to date. Considering the effect of site-directed mutation on protein crystallization (Pande *et al.*, 2001), we mutated five serine residues in septin 1 and obtained a protein sample with much improved homogeneity. Useful crystals have been grown using this septin 1 mutant, named 5mSept1.

2. Materials and methods

2.1. Strains and plasmid

Escherichia coli DH5α and BL21(DE3) were used for plasmid propagation and protein purification, respectively. Plasmid PGEX-5X-1 was used for the cloning of human septin 1.

2.2. Plasmid construction of human septin 1 and mutation introduction

The gene encoding human septin 1 (NCBI ID NM_052838) was cloned into PGEX-5X-1 vector between *Eco*RI and *Xho*I sites after PCR amplification.

Mutations were introduced using the Quikchange Site-Directed Mutagenesis Kit (Stratagene), followed by sequencing with an ABI 3730 DNA Sequencer to ensure that there were no mistakes.

The mutant with three mutated residues S307A, S312A, S315A is named 3mSept1 and the mutant with five mutated residues S19A, S206A, S307A, S312A, S315A is named 5mSept1. The primer pairs used for mutations are shown in Table 1.

2.3. Expression and purification

For expression of GST-tagged wild-type septin 1 and the mutants 3mSept1 and 5mSept1, the corresponding recombinant plasmids were transformed into *E. coli* BL21(DE3) cells. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 0.4 m*M* when the A_{550} reached 0.7 and the culture was cultivated for 4 h at 298 K. Cells were collected by centrifugation, after which the cell pellet was resuspended in lysis buffer with 10 m*M* Na_2HPO_4/NaH_2PO_4 pH 7.4 and 0.5 *M* NaCl and lysed by sonication. The cell debris was removed by centrifugation and the supernatant was subjected to affinity chromatography using a glutathione Sepharose 4 fast-flow column (Amersham Biosciences); the eluted recombinant proteins were further purified with a Superose 12 column (Tricorn, Amersham Biosciences) and a fusion protein was finally obtained. There was a segment of a tether sequence (IEGRGIP) between the GST tag and the septin 1 protein and the GST tag was not removed.

2.4. Dynamic light scattering

Dynamic light-scattering (DLS) experiments were carried out on a DynaPro DLS instrument with a 781.8 nm wavelength laser (Protein Solutions). The protein solution was pre-treated by centrifugation at 277 K and 10 000*g* for 10 min. Dynamic light-scattering experiments were run at 293 K and at least 15 measurements were taken for each sample. Regularization histogram analyses of samples were carried out using the software *DYNAMICS* v.5.25.44.

2.5. Circular dichroism

Samples were prepared by dissolving 0.2 mg protein in 1 ml 10 m*M* PBS. The CD spectra were recorded over the range 250–190 nm on a Jasco J-715 circular-dichroism system (Jasco Inc.) at room temperature. The optical path length was 0.1 mm and 10 m*M* PBS was used as a reference. The software *J-700* v.1.10.00 was used to estimate the proportions of secondary-structure elements (α-helix, β-sheet, turn, random or unordered forms).

2.6. Crystallization and data collection

Crystallization of the mutant 5mSept1 was carried out by the hanging-drop vapour-diffusion method. A 1.5 µl sample of reservoir solution [0.2 *M* ammonium sulfate, 30%(*w*/*v*) polyethylene glycol 8000] was mixed with 1.5 µl protein solution (16 mg ml^{-1} fusion protein, 20 m*M* Tris pH 8.0) and cultivated at 277 K against 0.5 ml reservoir. Larger crystals of octahedral shape grew in about five months. Diffraction data were collected from mutant 5mSept1 crystals using a wavelength of 0.9801 Å at 100 K on a MAR CCD 165 at beamline 3W1A of the Beijing Synchrotron Radiation Facility (BSRF). A total of 125 images were collected with an oscillation range of 1° per frame and a crystal-to-detector distance of 200 mm. Data were processed with the *HKL*2000 program package (Otwinowski & Minor, 1997).

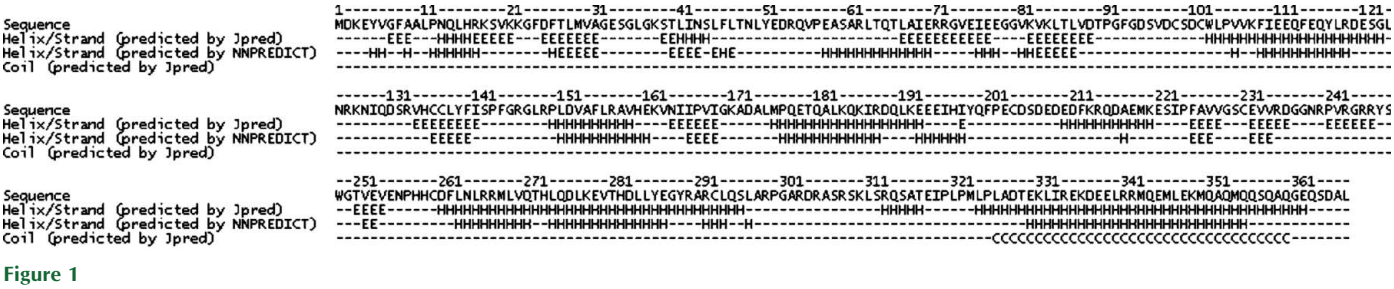

Figure 1
Predicted secondary structure of wild-type septin 1 according to the predictions of the *Jpred* and *NNPREDICT* online servers. H, helix; E, strand; C, coil; -, no prediction.

3. Results and discussion

3.1. Behaviour of recombinant human septin 1

Since it is known that purified *Drosophila*, rat and yeast septin proteins can form filaments *in vitro* and are thought to act as cytoskeletal polymers (Field & Kellogg, 1999), we investigated the aggregation behaviour of the wild-type human septin 1 that makes structural study of septin 1 very difficult.

Wild-type septin 1 was expressed and purified to about 90% purity using affinity chromatography and gel-filtration chromatography (Fig. 2). During purification using gel-filtration chromatography, we found that wild-type septin 1 has at least two forms of aggregation in solution (Fig. 3). Subsequent DLS examination shows that there are two aggregates in solution: one has a hydrodynamic radius (R_h) of 13.10 nm and a calculated molecular weight of 1384 kDa, accounting for 76.2% of the total protein mass, and the other has a hydrodynamic radius of 43.98 nm and a calculated molecular weight of 23 560 kDa, accounting for 23.7% of the total protein mass (Fig. 4). The theoretical molecular weight of this recombinant protein is 69 kDa; therefore, it could be concluded that the recombinant wild-type septin 1 aggregates severely in solution.

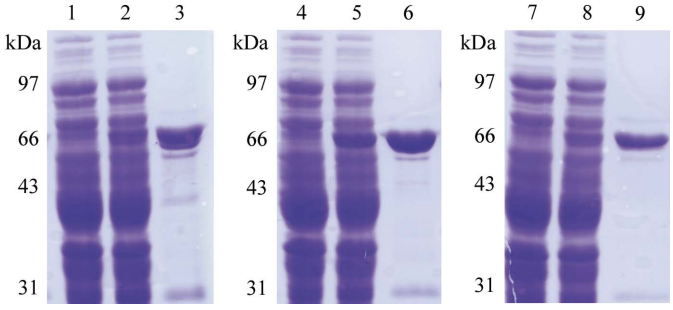

Figure 2
SDS–PAGE analysis of human septin 1 expressed in *E. coli* BL21(DE3). (*a*) Lane 1, extracts of wild-type septin 1 expression strain whole cells of BL21(DE3) transformed without induction; lane 2, extracts of wild-type septin 1 expression strain whole cells of BL21(DE3) transformed after 3 h of IPTG induction; lane 3, purified wild-type septin 1. (*b*) Lane 4, extracts of 3mSept1 expression strain whole cells of BL21(DE3) transformed without induction; lane 5, extracts of 3mSept1 expression strain whole cells of BL21(DE3) transformed after 3 h of IPTG induction; lane 6, purified 3mSept1. (*c*) Lane 7, extracts of 5mSept1 expression strain whole cells of BL21(DE3) transformed without induction; lane 8, extracts of 5mSept1 expression strain whole cells of BL21(DE3) transformed after 3 h of IPTG induction; lane 9, purified 5mSept1.

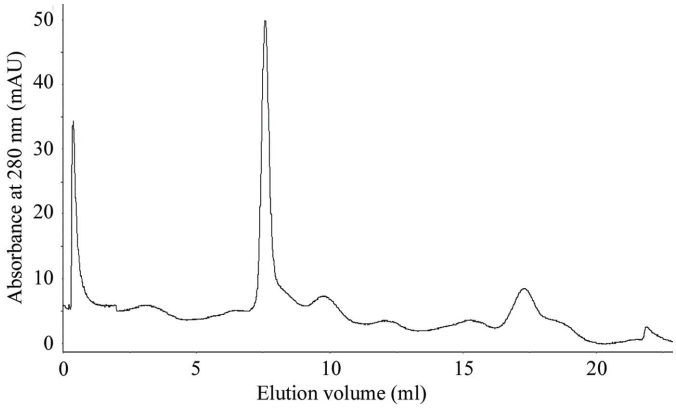

Figure 3
Purification profile of the recombinant septin 1 using molecular-filtration chromatography.

3.2. Improvement of septin 1 homogeneity by site-directed mutagenesis

To obtain septin 1 samples with higher homogeneity, we first tried using several types of detergents, to no effect. Considering the effect of site-directed mutations on protein crystallization and enlightened by the crystallization study of wild-type and R36S mutant human γD crystallin (Pande *et al.*, 2001), we ultimately chose the mutagenesis method to improve the sample homogeneity.

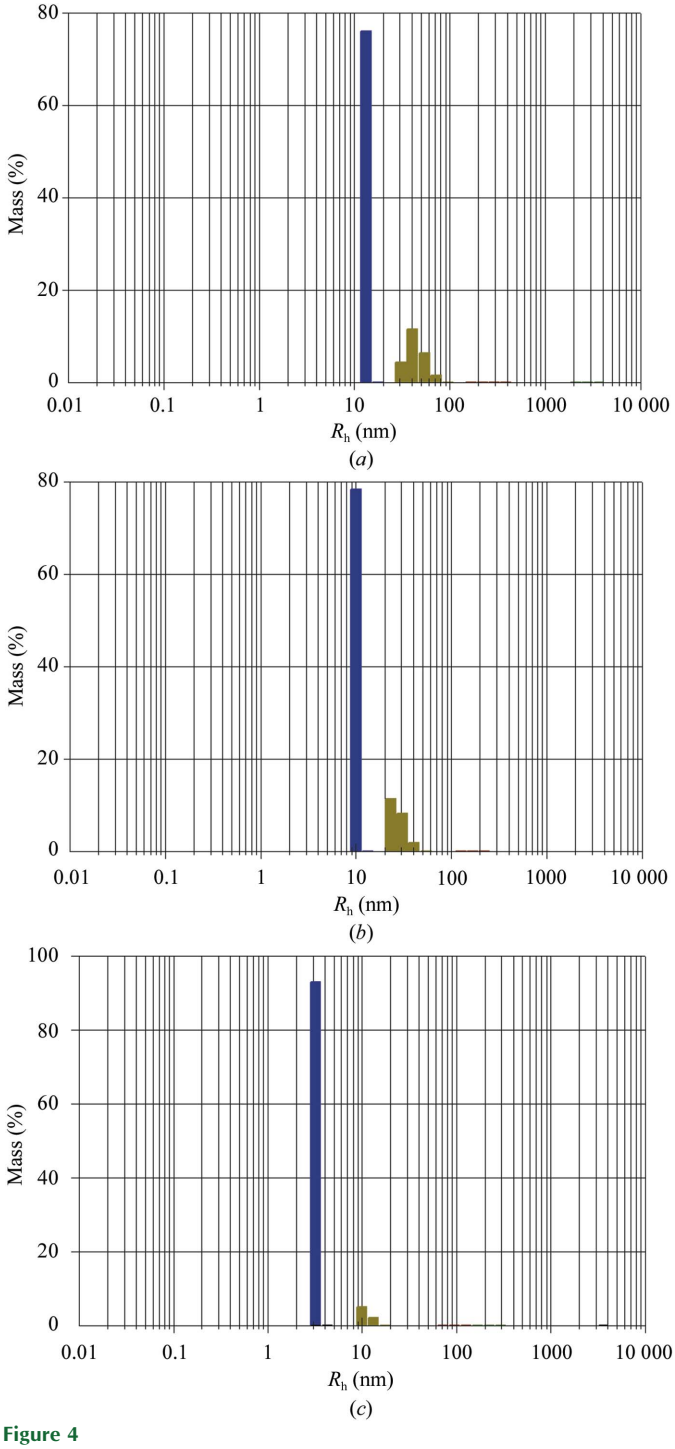

Figure 4
DLS regularization histograms. (*a*) Wild type; (*b*) 3mSept1; (*c*) 5mSept1.

Table 2
Composition of protein solutions.

	Radius (nm)	MW (kDa)	Mass (%)
Wild type	13.10	1384	76.2
	43.98	23560	23.7
3mSept1	9.885	716.5	78.5
	27.33	7739	21.4
5mSept1	3.57	66.12	92.9
	10.80	880.8	7.0

We first mutated the three serine residues Ser307, Ser312 and Ser315 near the C-terminus of septin 1, which may be involved in molecular interactions (Martinez *et al.*, 2004). In addition, the predicted secondary structure of septin 1 has shown that residues Ser307, Ser312 and Ser315 are not involved in the secondary-structure elements that are important for the overall three-dimensional structure of septin 1 (Fig. 1). Therefore, mutation of those three serine residues could improve the sample homogeneity while having no significant impact on the overall three-dimensional structure. This mutant (3mSept1) was expressed and purified in the same way as for the wild type (Fig. 2*b*) and examined by DLS. As shown in Fig. 4(*b*), the protein sample of 3mSept1 in solution is made up of two aggregates: one has a hydrodynamic radius of 9.885 nm, accounting for 78.5% of the total protein mass, while the other has a hydrodynamic radius of 27.33 nm, accounting for 21.4% of the total protein mass. Compared with the wild-type septin 1, we can conclude that the aggregation has been partly alleviated and the mutation is effective in overcoming protein aggregation. Based on the knowledge that heterotypic septin assembly is dependent on the conserved central core domain and that homotypic interactions require the N- and C-termini of each protein (Martinez *et al.*, 2004), we have chosen two more mutation sites, Ser19 and Ser206. Ser206 is not involved in the elements of secondary structure according to the secondary-structure prediction, while Ser19 seems to be located within a short strip of helix in the N-terminus of the protein (Fig. 1) and mutation of Ser19 does not visibly alter the overall structure. The mutant 5mSept1 was constructed on the basis of 3mSept1. The mutant 5mSept1 was expressed and purified in the same way as for the wild type and was also examined by DLS. As shown in Table 2, the experimental results indicate that 92% of the 5mSept1 molecules are monomers in solution, while the other 8% still form aggregates.

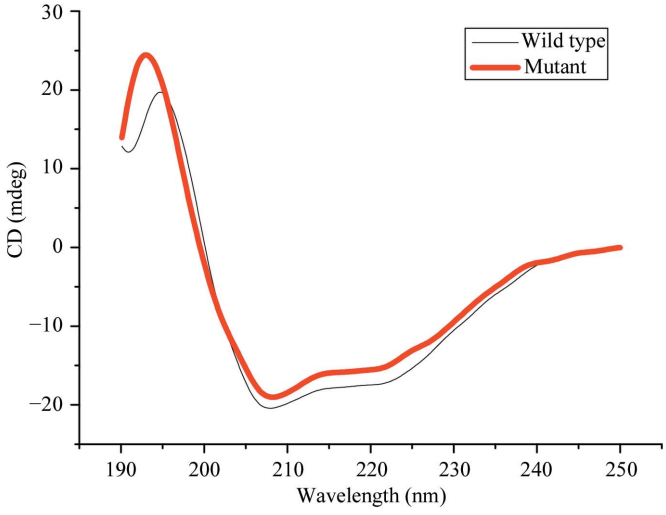

Figure 5
The circular-dichroism spectrum of wild-type septin 1 and the mutant 5mSept1.

Table 3
The predicted secondary structures of wild-type septin 1 and 5mSept1.

	Wild type	5mSept1
α-Helix (%)	20.7	21.2
β-Strand (%)	40.1	44.7
Turn (%)	10.6	4.0
Random (%)	28.5	30.1
Total (%)	100.0	100.0

Studies have revealed that the mutation of specific residues obviously alters the free energy of binding ($\Delta G_{\rm bind}$; Desrosiers & Peng, 2005; Almlöf *et al.*, 2006) and this is the case in our mutations involving the five serine residues. One possible explanation for this is that the serine residues Ser19, Ser206, Ser307, Ser312 and Ser315 may be dispersed on the molecular surface and participate in intermolecular interactions: according to the study of Martinez *et al.* (2004), all five of these residues are located in sites that are involved in intermolecular contact.

As shown in Table 3 and Fig. 5, the CD spectra show that some conformational changes may occur in the secondary structure of septin 1 after mutation. Compared with the wild type, the ratio of turn in total secondary structure has evidently lowered (from 10.6 to 4.0%) in the mutant 5mSept1 and the ratio of β-sheet has slightly increased (from 40.1 to 44.7%). However, these small conformational differences predicted by CD measurements may not affect the main features of the septin 1 structure that are crucial for its function. The three-dimensional structure of 5mSept1 may provide important structural information for further functional study of septin 1.

3.3. Crystals of septin 1 mutant

A crystal of 5mSept1 mutant grew to dimensions of 57 × 63 × 55 µm after five months of cultivation and diffraction data were collected to 2.5 Å resolution. A diffraction pattern is shown in Fig. 6. The space group of the crystals was determined to be *P*422, as there is

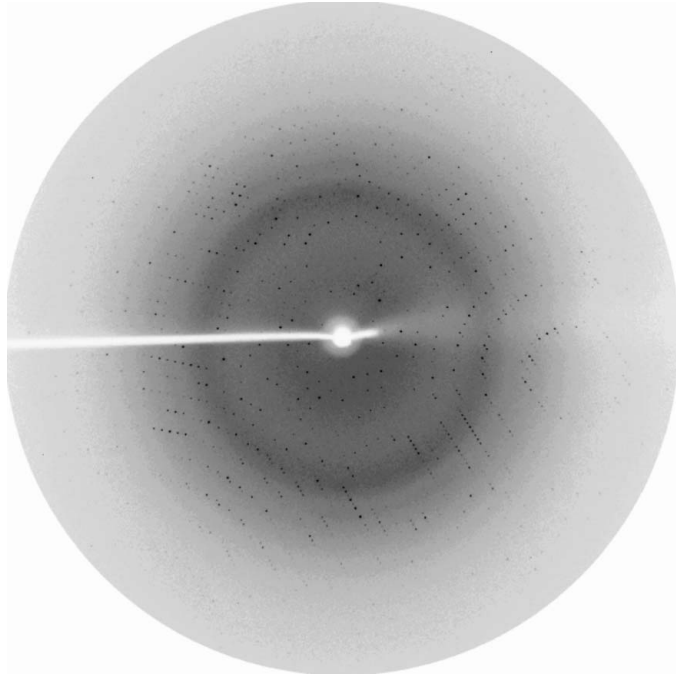

Figure 6
Diffraction pattern of the 5mSept1 crystals.

Table 4
Data-collection statistics of the human 5mSept1 mutant crystal.

Values in parentheses are for the highest resolution shell.

Space group	*P*422
Unit-cell parameters (Å)	$a = b = 106.028$, $c = 137.852$
Observed reflections	191895
Unique reflections	25990
Resolution (Å)	20.0–2.5 (2.59–2.50)
Completeness (%)	95.4 (96.9)
Redundancy	7.4 (6.6)
V_M (Å^3 Da^{-1})	2.8
Solvent content (%)	55.8
No. of molecules in ASU	1
$I/\sigma(I)$	17.9 (5.8)
R_{merge}† (%)	10.6 (26.6)

† $R_{merge} = \sum |I_j - \langle I \rangle| / \sum \langle I \rangle$, where I_j is the intensity of reflection j and $\langle I_j \rangle$ is the average intensity of reflection j.

no systematic extinction of reflections in the intensity data. Crystal and data-collection statistics are listed in Table 4. The statistics show that there is no significant deficiency in molecular packing. Further work should consist of an attempt to obtain usable crystals of selenomethionine-labelled protein and to obtain preliminary phases by the SAD or MAD method.

In summary, this work has provided a method to determine the human septin 1 structure and to further facilitate its functional studies. In addition, this research has verified that the site-directed mutation of several serine residues may be a feasible way of improving the homogeneity of protein samples.

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