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Crystallization and crystallographic analysis of yeast Sec2p, a guanine nucleotide-exchange factor for the yeast Rab GTPase Sec4p

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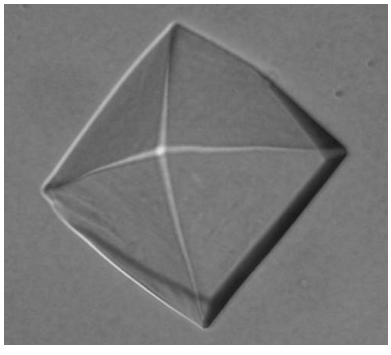
Sec2p is a guanine nucleotide-exchange factor (GEF) for the yeast Rab GTPase Sec4p. Sec2p accelerates GDP release from Sec4p and promotes GDP–GTP exchange for Sec4p activation. In order to elucidate this nucleotide-exchange mechanism using X-ray crystallography, three constructs of native Sec2p ($Sec2_{1–160}$p, $Sec2_{18–160}$p and $Sec2_{31–160}$p) and three constructs of selenomethionine-labelled Sec2p [$Sec2_{31–160}$p, $Sec2_{31–160}$p (M115L) and $Sec2_{31–160}$p (M115L, K121M, T142M)] were crystallized. These six crystals diffracted to 8.8, 4.8, 2.6, 4.0, 3.3 and 3.0 Å resolution, respectively. The data set from the SeMet-labelled $Sec2_{31–160}$p (M115L, K121M, T142M) crystal was processed for SAD phasing; the crystal belonged to space group $P2_12_12_1$, with unit-cell parameters a = 101.9, b = 176.6, c = 181.5 Å.

1. Introduction

In eukaryotic cells, various biological molecules are secreted or transported to defined areas of the plasma membrane by exocytosis (Lee *et al.*, 2004). In yeast, Sec4p, a Rab GTPase, regulates the exocytic process in a GTP-dependent fashion (Salminen & Novick, 1987). GTP-bound Sec4p interacts with a multiprotein complex, Exocyst, to tether the secretory vesicles to the proper sites of the plasma membrane (TerBush *et al.*, 1996; Guo *et al.*, 1999). Recycling of the secretory vesicle is coupled to the nucleotide-bound state of Sec4p (Zajac *et al.*, 2005), which is controlled by two types of regulators: a GTPase-activating protein (GAP) and a guanine nucleotide-exchange factor (GEF) (Quilliam *et al.*, 1995). The GAP stimulates GTP hydrolysis by Sec4p to produce the inactive GDP-bound form of Sec4p. The GEF triggers GDP release and thus promotes GDP–GTP exchange and activation of Sec4p. In yeast, Sec2p has been identified as the GEF for Sec4p (Walch-Solimena *et al.*, 1997). Sec2p is essential for the polarized cell growth of yeast. The N-terminal region encompassing residues 1–160 interacts with Sec4p and exhibits full GEF activity (Ortiz *et al.*, 2002), but shares no homologous sequence with any other GEFs of known structure. Here, we report the crystallization and X-ray crystallographic analysis of the five constructs of the Sec2p GEF domain [$Sec2_{1–160}$p, $Sec2_{18–160}$p, $Sec2_{31–160}$p, $Sec2_{31–160}$p (M115L) and $Sec2_{31–160}$p (M115L, K121M, T142M)] with the aim of elucidating its GDP–GTP exchange mechanism.



2. Materials and methods

2.1. Protein preparation

The genes encoding $Sec2_{1–160}$p, $Sec2_{18–160}$p and $Sec2_{31–160}$p were PCR-amplified from *Saccharomyces cerevisiae* genomic DNA. Site-directed mutations were generated by PCR. The amplified PCR products were cloned into the pGEX-6P-1 expression vector (GE Healthcare) with *Bam*HI and *Xho*I sites to produce the N-terminal GST-fusion protein. *Escherichia coli* strain Rosetta (DE3) (Invitrogen) was transformed with each expression vector and cultured in LB medium containing 100 mg l^{-1} ampicillin. When the optical density at 600 nm of the culture reached ∼0.5, expression was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 m*M* and the *E. coli* cells were incubated

overnight at 293 K. The cells were harvested by centrifugation at 8000*g* for 15 min and were disrupted by sonication in phosphate-buffered saline (PBS) containing 1 m*M* dithiothreitol (DTT) and 1 m*M* phenylmethylsulfonyl fluoride (PMSF). The lysate was centrifuged at 12 000*g* for 40 min and the supernatant was loaded onto a Glutathione Sepharose FF column (GE Healthcare) pre-equilibrated with PBS containing 1 m*M* DTT. The GST-fused Sec2p was eluted with 50 m*M* Tris–HCl buffer pH 8.0 containing 50 m*M* NaCl, 1 m*M* DTT and 15 m*M* reduced glutathione. The GST tag was cleaved using PreScission protease (GE Healthcare) and reduced glutathione was removed by dialysis against 50 m*M* Tris–HCl buffer pH 8.0 containing 50 m*M* NaCl and 1 m*M* DTT. The cleaved GST tag was then removed by chromatography on a Glutathione Sepharose FF column (GE Healthcare). The eluate was loaded onto a MonoQ anion-exchange column (GE Healthcare) pre-equilibrated with 50 m*M* Tris–HCl buffer pH 8.0 containing 1 m*M* DTT. The protein was eluted with a linear gradient of 0–1.0 *M* NaCl. The fractions rich in Sec2p were loaded onto a Superdex 200 16/60 (prep-grade) column (GE Healthcare) pre-equilibrated with 10 m*M* Tris–HCl buffer pH 7.2 containing 50 m*M* NaCl and 5 m*M* β-mercaptoethanol. The purified Sec2p GEF domain was directly concentrated to around 10 mg ml^{-1} by ultrafiltration using an Amicon Ultra 15 filter (Millipore) for use in crystallization.

SeMet-labelled Sec2p GEF domain was overproduced in *E. coli* strain B834 (DE3) (Invitrogen) and cultured in Core medium (Wako) with 30 mg l^{-1} selenomethionine and was purified in the same manner as the native Sec2p GEF domain.

2.2. Crystallization

Crystallization experiments were performed using the hanging-drop vapour-diffusion method at 293 K. Drops were prepared by mixing 1 µl reservoir with 1 µl protein solution and were equilibrated against 500 µl reservoir solution. Identical parameters (*i.e.* temperature, reservoir volume and drop volume) were used for all of the crystallization experiments. Reagents for the initial screening and optimization were purchased from Hampton Research.

2.3. X-ray data collection and processing

Ethylene glycol, glycerol or 2-methyl-2,4-pentanediol (MPD) was used as a cryoprotectant for data collection under cryogenic conditions. The crystals were soaked in cryoprotectant-containing mother liquor and flash-cooled in a nitrogen stream at 100 K. Diffraction data were collected with 1° oscillation per image at SPring-8 BL41XU (Harima, Japan), PF-AR NW12 (Tsukuba, Japan) or PF BL5A (Tsukuba, Japan). The diffraction data sets were processed with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Sec2$_{1–160}$p

The initial crystals of Sec2$_{1–160}$p appeared in 50 m*M* cacodylate buffer pH 6.5 containing 10%(*w*/*v*) PEG 4000, 0.2 *M* KCl and 10 m*M* $MgCl_2$ (Natrix No. 23 from Hampton Research). The optimized condition was 30 m*M* cacodylate buffer pH 6.5 containing 2.4%(*w*/*v*) PEG 20 000, 2.4%(*w*/*v*) PEG 4000, 80 m*M* KCl and 4.5 m*M* $MgCl_2$. The crystals grew to maximum dimensions of 150 × 150 × 150 µm in a week (Fig. 1*a*). The Sec2$_{1–160}$p crystals were cryoprotected using 30%(*v*/*v*) ethylene glycol. Diffraction data were collected at SPring-8 BL41XU using an ADSC Quantum 315 CCD detector at a wavelength of 1.0000 Å. The crystals diffracted poorly to 8.8 Å resolution (Fig. 2*a*), probably owing to partial disorder of the Sec2$_{1–160}$p structure.

In order to obtain a higher resolution data set, we generated seven truncated mutants of Sec2p (Sec2$_{18–160}$p, Sec2$_{1–142}$p, Sec2$_{1–292}$p, Sec2$_{1–502}$p, Sec2$_{31–160}$p, Sec2$_{18–195}$p and Sec2$_{18–221}$p) and obtained crystals of Sec2$_{18–160}$p and Sec2$_{31–160}$p (see below).

3.2. Sec2$_{18–160}$p

The initial crystals of Sec2$_{18–160}$p appeared in 0.1 *M* Tris–HCl buffer pH 8.5 containing 25%(*v*/*v*) *t*-butanol (Crystal Screen No. 40 from Hampton Research). The optimized crystallization condition was 0.1 *M* Tris–HCl buffer pH 8.5 containing 14%(*v*/*v*) *t*-butanol, 19%(*v*/*v*) MPD and 0.1 *M* DTT. The crystals grew to maximum dimensions of 200 × 400 × 10 µm in a week (Fig. 1*b*). The Sec2$_{18–160}$p crystals were cryoprotected using 20%(*v*/*v*) MPD. Diffraction data were collected at PF-AR NW12 using an ADSC Quantum 210 CCD detector at a wavelength of 1.0000 Å. The crystal belongs to space group $P2_12_12$, with unit-cell parameters $a = 230.3$, $b = 359.8$, $c = 51.7$ Å. While diffraction spots corresponding to 4.8 Å resolution were observed (Fig. 2*b*), diffraction was seriously anisotropic, thus limiting the effective resolution to only 9.0 Å.

3.3. Sec2$_{31–160}$p

The initial crystals of Sec2$_{31–160}$p appeared in 0.1 *M* Bicine-Na buffer pH 9.0 containing 2%(*v*/*v*) dioxane and 10%(*w*/*v*) PEG 20 000 (Crystal Screen 2 No. 48 from Hampton Research). The optimized crystallization condition was 0.1 *M* Bicine-Na buffer pH 8.5 containing 0.8%(*w*/*v*) PEG 20 000, 0.8%(*w*/*v*) PEG 8000 and 15 m*M* $CaCl_2$. The crystals grew to maximum dimensions of 200 × 200 × 150 µm in a week (Fig. 1*c*). The Sec2$_{31–160}$p crystals were cryoprotected using 30%(*v*/*v*) ethylene glycol. Diffraction data were collected at PF-AR NW12 using an ADSC Quantum 210 CCD detector at a wavelength of 1.0000 Å. The crystal belongs to space group $P2_12_12_1$, with unit-cell parameters a = 101.6, b = 179.4, c = 182.4 Å. The data set was collected and processed to 2.6 Å resolution (Fig. 2*c*).

For phasing by anomalous dispersion using the selenium edge, we also produced SeMet-labelled Sec2$_{31–160}$p crystals. The optimized

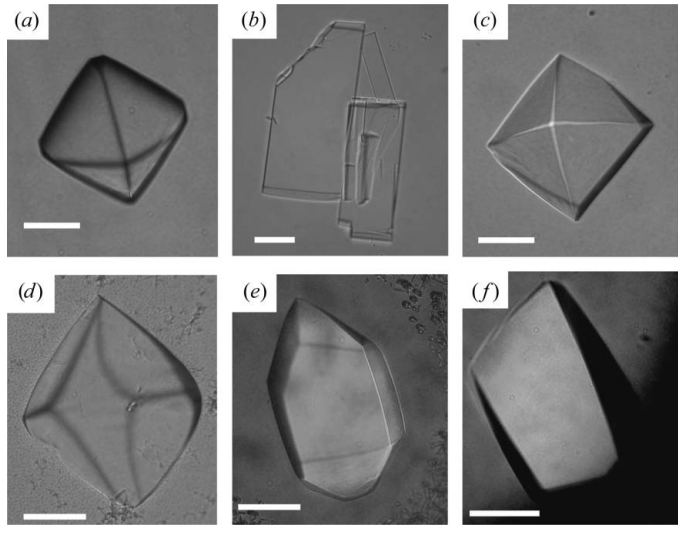


Figure 1
Crystal forms of (*a*) Sec2$_{1–160}$p, (*b*) Sec2$_{18–160}$p, (*c*) Sec2$_{31–160}$p, (*d*) SeMet-labelled Sec2$_{31–160}$p, (*e*) SeMet-labelled Sec2$_{31–160}$p (M115L) and (*f*) SeMet-labelled Sec2$_{31–160}$p (M115L, K121M, T142M) proteins. The scale bar is 100 µm.

crystallization condition was 0.1 *M* Bicine-Na buffer pH 8.5 containing 1.25%(*w*/*v*) PEG 20 000, 1.25%(*w*/*v*) PEG 8000 and 60 m*M* ammonium dihydrogen phosphate. The crystals grew to maximum dimensions of 300 × 250 × 150 µm in a week (Fig. 1*d*). The SeMet-labelled Sec2$_{31–160}$p crystals were cryoprotected with 35%(*v*/*v*) ethylene glycol. The SAD data set was collected at SPring-8 BL41XU using an ADSC Quantum 315 CCD detector at a wavelength of 0.97884 Å. The crystal belongs to space group $P2_12_12_1$, with unit-cell parameters a = 99.7, b = 178.4, c = 180.9 Å. However, the crystals only diffracted to 4.0 Å resolution (Fig. 2*d*), probably owing to disturbance of the dimerization interactions and/or the crystal-packing interactions by incorporation of the Se atoms (see below). We could not identify any selenium sites with this data set, even when using the powerful direct method with the program *SnB* (Weeks & Miller, 1999).

3.4. Sec2$_{31–160}$p (M115L)

In order to obtain better crystals, we generated two Sec2$_{31–160}$p mutants by replacing the intrinsic Met115 and Met158 by leucine residues. SeMet-labelled Sec2$_{31–160}$p (M158L) precipitated after purification, whereas SeMet-labelled Sec2$_{31–160}$p (M115L) could be purified and crystallized as the wild-type Sec2$_{31–160}$p. The optimized crystallization condition was 0.1 *M* Bicine-Na buffer pH 8.5 containing 1.2%(*w*/*v*) PEG 20 000, 1.2%(*w*/*v*) PEG 8000 and 3.5% ethanol. The crystals grew to maximum dimensions of 300 × 200 × 200 µm in a week (Fig. 1*e*). The SeMet-labelled Sec2$_{31–160}$p (M115L) crystals were cryoprotected using 35%(*v*/*v*) ethylene glycol. The SAD data set was collected at SPring-8 BL41XU using an ADSC Quantum 315 CCD detector at a wavelength of 0.97924 Å. The crystal belongs to space group $P2_12_12_1$, with unit-cell parameters a = 103.5, b = 176.3, c = 182.0 Å. A data set was collected to 3.3 Å resolution (Fig. 2*e*). However, anomalous dispersion was not observed from this data set, probably owing to the high temperature factor of the remaining SeMet residue (SeMet158).

3.5. Sec2$_{31–160}$p (M115L, K121M, T142M)

In order to detect effective anomalous dispersion, we increased the number of SeMet residues as follows. Firstly, we performed a primary

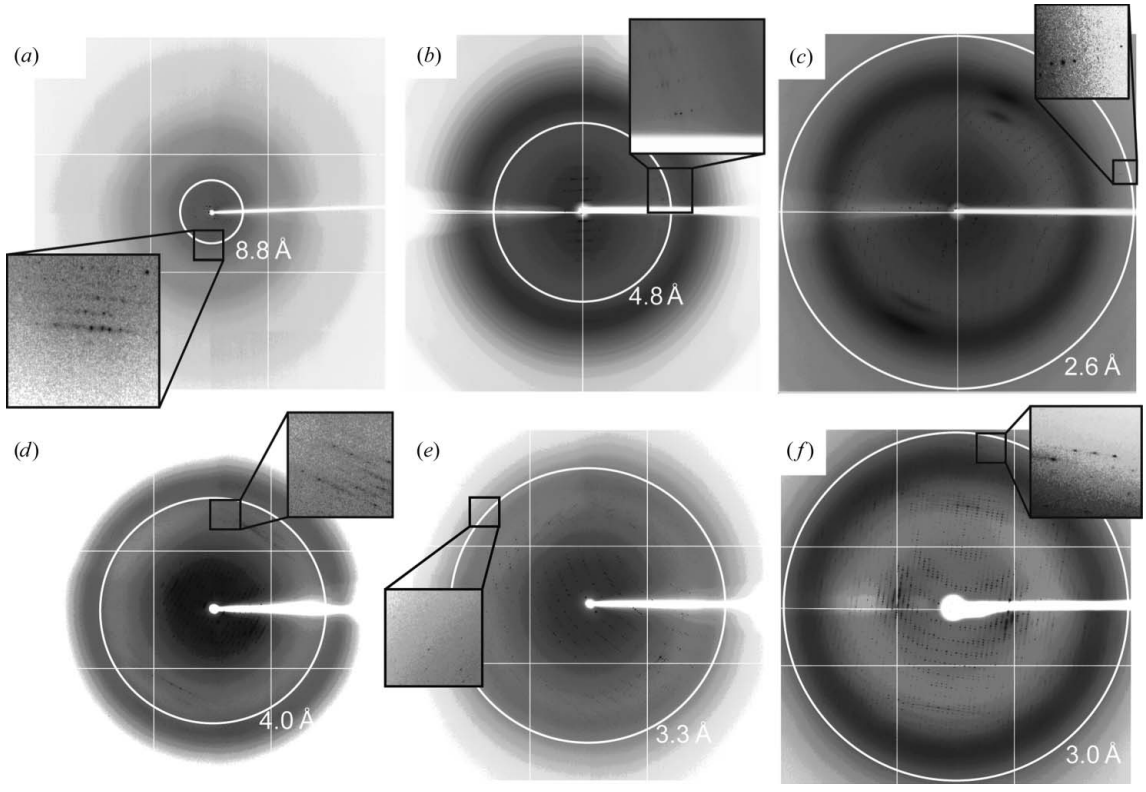


Figure 2
X-ray diffraction patterns of crystals of (*a*) Sec2$_{1–160}$p, (*b*) Sec2$_{18–160}$p, (*c*) Sec2$_{31–160}$p, (*d*) SeMet-labelled Sec2$_{31–160}$p, (*e*) SeMet-labelled Sec2$_{31–160}$p (M115L) and (*f*) SeMet-labelled Sec2$_{31–160}$p (M115L, K121M, T142M) proteins.

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                  31        40        50        60        70        80        90
Saccharomyces cerevisiae Sec2p  LEEQLNKSLKTIASQKAAIENYNQLKEDYNTLKRELSDRDD..EVKRLREDIAKENE..LRTKAEEEADK
Ashbya gossypii ARF670Wp        LEEQLQHAKKALAAQKDVVKQCESARGEAQQLRSQLADRDT....ELERQGEELEEARKARLAAELAVER
Rattus norvegicus GRAB          .WEEAGGEEHPAAAP.........LDVSRLRS.SSMEIREKGSEFLKEELYKAQKELKLKDEECERLCKV

                 100       110       120       130       140       150       160
Saccharomyces cerevisiae Sec2p  ...LNKEVEDLTASLFDEANNMVADARKEK...YAIEILNKRLTEQLREKDTLLDTLTLQLKNLKKVMHS
Ashbya gossypii ARF670Wp        ...LNKEIEDLTTSLFDEANRMVSKARKEK...HVVEQHNARLQEQLREKDMLLDTLTIQLKNLKDVLYK
Rattus norvegicus GRAB          RAQLEQELEELTASLFEEAHKMVREANMKQAAS.........EKQLKEAWGKIDMLQAEVTALKTLVIT
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Figure 3
Sequence alignment of the GEF domain of Sec2p. The residue numbers of yeast Sec2p are shown above the alignment. Identical residues are shown on a red background. Asterisks indicate residues replaced by Met for phase determination. This figure was drawn using the program *ESPript* (http://espript.ibcp.fr/ESPript/ESPript/index.php).

Table 1
Data-collection statistics.

Values in parentheses are for the last shell.

	$Sec2_{1–160}$p	$Sec2_{18–160}$p	$Sec2_{31–160}$p	SeMet $Sec2_{31–160}$p	SeMet $Sec2_{31–160}$p (M115L)	SeMet $Sec2_{31–160}$p (M115L, K121M, T142M)
X-ray source	SPring-8 BL41XU	PF-AR NW12	PF-AR NW12	SPring-8 BL41XU	SPring-8 BL41XU	PF BL5A
Wavelength (Å)	1.00000	1.00000	1.00000	0.97884	0.97924	0.97934
No. of frames	1	180	180	350	350	360
Space group	—	$P2_12_12$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	—	a = 230.3, b = 359.8, c = 51.7	a = 101.6, b = 179.4, c = 182.4	a = 99.7, b = 178.4, c = 180.9	a = 103.5, b = 176.3, c = 182.0	a = 101.9, b = 176.6, c = 181.5
Resolution (Å)	—	50–9.00 (9.15–9.00)	50–2.60 (2.69–2.60)	50–4.00 (4.07–4.00)	50–3.30 (3.36–3.30)	50–3.00 (3.11–3.00)
Unique reflections	—	3685	101988	28291	50870	66164
Total reflections	—	15730	565468	158787	377615	655703
Completeness (%)	—	89.9 (86.3)	98.8 (96.1)	96.8 (94.8)	97.8 (96.0)	99.3 (98.2)
$I/\sigma(I)$	—	15.7 (8.9)	13.1 (3.7)	8.4 (2.0)	8.9 (2.5)	10.3 (2.2)
$R_{\rm merge}$†	—	0.063 (0.117)	0.080 (0.281)	0.083 (0.380)	0.072 (0.306)	0.080 (0.422)

† $R_{\rm merge} = \sum|I_i - \langle I_i\rangle|/\sum\langle I_i\rangle$, where I_i is the observed intensity and $\langle I_i\rangle$ is the average intensity over symmetry-equivalent measurements.

sequence alignment of yeast Sec2p and its homologues. Secondly, we selected three nonconserved residues that are replaced by Met in one or more Sec2p homologues. Fig. 3 shows the sequence alignment of the Sec2p GEF domains from yeast and two other species, based on which we selected the residues to be replaced with Met. Finally, we introduced 1–2 additional Met residue(s) by PCR-directed mutagenesis. In this study, we selected residues 66, 121 and 142 and prepared three single methionine-substitution mutants and three double methionine-substitution mutants. Of these, initial crystals of SeMet-labelled $Sec2_{31–160}$p (M115L, K121M, T142M) appeared in 50 m*M* Tris–HCl buffer pH 7.5 containing 10 m*M* $MgCl_2$ and 5% 2-propanol (Natrix No. 42 from Hampton Research). The optimized crystallization condition was 50 m*M* Tris–HCl buffer pH 7.5 containing 10 m*M* $MgCl_2$ and 3% 2-propanol. The crystal grew to maximum dimensions of 350 × 200 × 200 µm in a week (Fig. 1*f*). The SeMet-labelled $Sec2_{31–160}$p (M115L, K121M, T142M) crystals were cryoprotected using 30%(*v*/*v*) glycerol. A SAD data set was collected at PF BL5A using an ADSC Quantum 315 CCD detector at a wavelength of 0.97934 Å. The crystal belongs to space group $P2_12_12_1$, with unit-cell parameters a = 101.9, b = 176.6, c = 181.5 Å. A data set was collected to 3.0 Å resolution (Fig. 2*f*). Using this data set, we could identify ten selenium sites, which were used for effective phasing. Details of the structure determination and description have been published elsewhere (Sato *et al.*, 2007).

4. Concluding remarks

Generally, the handling of some eukaryotic proteins, especially multi-domain proteins, is quite difficult. The present Sec2p is a case in point. Firstly, the quality (or diffraction) of the crystals depended strikingly on the construction of the GEF domain. Secondly, crystallization and the crystal quality were much affected by selenomethionylation of the protein; the replacement of one Met residue (Met115) successfully produced SeMet-labelled crystals, but the anomalous signal was not sufficient for phasing. An increase in the number of Met residues (Met121 and Met142) finally produced SeMet-labelled crystals that diffracted with sufficient anomalous signal, but only after many trials. In the previous section, we described the rational basis for the selection of the amino-acid residues that were replaced by Met for effective phasing. We now know the reason why the SeMet-labelled native crystals diffracted X-rays poorly. As the native and SeMet-labelled $Sec2_{31–160}$p crystals have similar unit-cell parameters and the same space group (Table 1), the crystal-packing interactions are expected to be almost identical in these crystals. In our recently reported structure of SeMet-labelled $Sec2_{31–160}$p (M115L, K121M, T142M), the C^{δ} atoms of Leu115 are not only involved in the dimerization interaction but also in the crystal-packing interaction (Sato *et al.*, 2007). Therefore, the S^{δ} atom of Met115 is also likely to be involved in these interactions and replacing Met115 with SeMet115 may disturb them. Control of the selenium sites by mutagenesis can be applied to the improvement of any poorly diffracting SeMet-labelled crystals and may lead to successful phasing.

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