Yuzuru Itoh,^{a,b,c} Shun-ichi
 Sekine^{a,b} and Shigeyuki
 Yokoyama^{a,b*}

^aDepartment of Biophysics and Biochemistry,
 Graduate School of Science, The University of
 Tokyo, 7-3-1 Hongo, Bunkyo-ku,
 Tokyo 113-0033, Japan, ^bRIKEN Systems and
 Structural Biology Center, 1-7-22 Suehiro,
 Tsurumi, Yokohama 230-0045, Japan, and
^cLaboratory of Membrane and Cytoskeleton
 Dynamics, Institute of Molecular and Cellular
 Biosciences, The University of Tokyo,
 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032,
 Japan

Correspondence e-mail:
 yokoyama@biochem.s.u-tokyo.ac.jp

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Crystallization and preliminary X-ray crystallographic analysis of *Aquifex aeolicus* Sela, a bacterial selenocysteine synthase

Selenocysteine (Sec), the 21st amino acid, is synthesized on its specific tRNA (tRNA^{Sec}) via a multi-step process. In bacteria, tRNA^{Sec} is ligated first with serine by seryl-tRNA synthetase, which is followed by Ser-to-Sec conversion by Sec synthase (Sela). To elucidate its structure and catalytic mechanism, *Aquifex aeolicus* Sela was crystallized. Although wild-type Sela crystals diffracted X-rays poorly (to up to 8 Å resolution), the resolution was improved by introducing a quadruple point mutation targeting the loop regions and by methylating the lysine residues, which yielded 3.9 Å resolution diffraction data from a full-length Sela crystal. Truncation of the N-terminal region (ΔN) also improved the resolution. A 3.3 Å resolution data set for phase determination was obtained from a crystal of selenomethionine-substituted Lys-methylated Sela-ΔN.

1. Introduction

Selenocysteine (Sec) is a selenium-containing amino acid known as the 21st amino acid and is translationally incorporated into proteins (Böck *et al.*, 1991). Sec is widely distributed in all three domains of life: Bacteria, Archaea and Eukarya. In contrast to the canonical amino acids, Sec is synthesized on Sec-specific tRNA (tRNA^{Sec}) via a multi-step process. tRNA^{Sec} is ligated first with serine by seryl-tRNA synthetase (SerRS) to form seryl-tRNA^{Sec} (Ser-tRNA^{Sec}; Leinfelder *et al.*, 1988). In Bacteria, the hydroxyl group of the seryl moiety is replaced with a selenol group by Sec synthase (Sela or SecS) to form selenocysteinyl-tRNA^{Sec} (Sec-tRNA^{Sec}; Forchhammer & Böck, 1991). In contrast, the eukaryotic/archaeal Sec synthesis characteristically employs an intermediate, *O*-phosphoseryl-tRNA^{Sec} (Sep-tRNA^{Sec}). The hydroxyl group of Ser-tRNA^{Sec} is phosphorylated by *O*-phosphoseryl-tRNA kinase (PSTK) to yield Sep-tRNA^{Sec} (Carlson *et al.*, 2004), which is then converted to Sec-tRNA^{Sec} by the homotetrameric enzyme Sep-tRNA:Sec-tRNA synthase (SepSecS; Yuan *et al.*, 2006; Xu *et al.*, 2007). Sela and SepSecS are both members of the fold-type-I superfamily of pyridoxal phosphate (PLP) dependent enzymes (Tormay *et al.*, 1998; Ganichkin *et al.*, 2008; Araiso *et al.*, 2008), but their sequence similarity is limited to the PLP-binding motif. The selenophosphate generated by selenophosphate synthetase is utilized as the reactive selenium donor for Sec synthesis in both Bacteria and Eukarya/Archaea (Ehrenreich *et al.*, 1992; Low *et al.*, 1995).

A previous study of Sela, including electron-microscopic analyses, revealed that Sela exists as an ~500 kDa homodecamer (Engelhardt *et al.*, 1992). However, because of the low resolution, the catalytic mechanism and substrate specificity of Sela, as well as the biological significance of the homodecameric quaternary structure, have not been clarified. In this communication, we report the crystallization of Sela and a strategy for improving the diffraction resolution.

2. Materials and methods

2.1. Overexpression and purification of SelA

The *Aquifex aeolicus* SelA gene (NCBI Gene ID 1193774; locus tag aq_1031) was cloned into the *NdeI* and *SalI* restriction sites of the vector pET25b (Novagen). The mutant SelA gene corresponding to SelA- Δ N (amino-acid residues 62–452) was cloned into the *NdeI* and *XhoI* restriction sites of the vector pET22b (Novagen). The wild-type SelA encoded in the vector does not have any artificial sequences at its N- or C-termini, while SelA- Δ N only has an artificially introduced initiating Met residue followed by residues 62–452. Since the cloned genes retained their natural stop codons, the C-terminal His tags that were encoded in the vectors were not expressed.

The point mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). *Escherichia coli* strain Rosetta 2 (DE3) (Stratagene) was transformed with the expression plasmids and the proteins were overexpressed in LB medium at 310 K. Selenomethionine-substituted SelA- Δ N was overexpressed in *E. coli* Rosetta 2 (DE3) transformed by the same vector as that encoding the native protein. The cells were grown in M9 minimal medium at 310 K to an OD₆₀₀ of 0.4, and 60 mg l⁻¹ L-selenomethionine, 100 mg l⁻¹ each of L-threonine, L-lysine and L-phenylalanine and 50 mg l⁻¹ each of L-leucine, L-isoleucine and L-valine were then added to the medium. After a 15 min pre-incubation, gene expression was induced by adding 0.5 mM isopropyl β -D-1-thiogalactopyranoside. The cells were further cultured at 310 K overnight prior to harvesting.

The harvested cells expressing wild-type SelA were resuspended in 20 mM Tris-HCl buffer pH 8.1 containing 500 mM NaCl, 500 mM MgCl₂ and 10 mM β -mercaptoethanol (β -ME) and disrupted using an ultrasonic homogenizer. The supernatant of the lysate was incubated at 358 K for 30 min to denature and remove most of the *E. coli* proteins. After centrifugation, ammonium sulfate was added to the supernatant (to a final concentration of 2 M), which was then applied onto a Toyopearl Phenyl-650 column (Tosoh). The column was washed with Tris-HCl buffer pH 7.5 containing 2.0 M ammonium sulfate and 10 mM β -ME and the protein was eluted with 20 mM Tris-HCl buffer pH 7.5 containing 10 mM β -ME. The protein was subsequently loaded onto an SP-Sepharose Fast Flow column (GE Healthcare) and eluted with a linear gradient of 0–1.2 M NaCl. It was dialyzed against 20 mM Tris-HCl buffer pH 7.5 containing 200 mM NaCl and 10 mM β -ME and concentrated to 5.2 mg ml⁻¹. The mutant SelAs were purified in a similar manner to the wild type.

2.2. Gel-filtration analysis

The total molecular mass of SelA was analyzed by gel-filtration analysis. Wild-type SelA (2 mg) was loaded onto a HiLoad 26/60 Superdex 200 gel-filtration column (GE Healthcare) and chromatographed using Tris-HCl buffer pH 7.5 containing 300 mM NaCl and 10 mM β -ME. An elution peak was detected by monitoring the absorbance at 280 nm (protein) and 412 nm (protein-bound PLP). The column was calibrated using a standard protein marker kit (Sigma) and the molecular mass of SelA was calculated from its elution volume.

2.3. Lysine methylation

Lysine-residue methylation of the wild-type and mutant SelAs was performed using formaldehyde and dimethylamine-borane complex as described by Walter *et al.* (2006). Prior to methylation, the proteins were diluted with 20 mM HEPES-NaOH buffer pH 7.5 containing 0.4–0.8 M NaCl and 10 mM β -ME. The methylation reagents were

removed by repeated dialyses against 20 mM Tris-HCl buffer pH 7.5 containing 0.2–0.3 M NaCl and 10 mM β -ME.

2.4. Crystallization and diffraction data collection

Crystallization was performed at 293 K by the sitting-drop vapour-diffusion method by mixing 0.75 μ l protein solution with 0.75 μ l reservoir solution. Seven screening kits, Crystal Screen, Crystal Screen 2, Natrix, SaltRx, Index (Hampton Research) and Wizard I and II (Emerald BioSystems), were used for initial screening of the crystallization conditions. These conditions were refined by optimizing the reservoir-solution composition, including the precipitant concentration, the buffer pH and the use of additives.

Prior to the X-ray diffraction experiment, crystals were transferred into a cryoprotective solution and flash-cooled in a cryocooled N₂ stream (90 K). Diffraction data were collected on BL41XU at SPring-8, Hyogo, Japan and on BL5A, BL17A and NW12A at the Photon Factory, Tsukuba, Japan. The data were processed with *HKL-2000* (Otwinowski & Minor, 1997) and the solvent content was estimated using *MATTHEWS_COEF* (Winn *et al.*, 2011).

2.5. Soaking of the heavy-atom reagent

The crystals obtained were washed with reservoir solution and transferred into a solution of the heavy-atom reagent in reservoir solution. After a 10–15 h incubation, the crystals were transferred into a cryoprotective solution and flash-cooled. The X-ray absorption fine structure (XAFS) was measured to monitor the heavy-atom labelling and to estimate the peak and edge wavelengths of the anomalous scattering.

3. Results

3.1. Crystallization and diffraction analysis of wild-type SelA

We performed a gel-filtration analysis to determine whether the recombinant *A. aeolicus* SelA is indeed a homodecameric protein. The elution peak monitored by the absorbance of aromatic protein residues (280 nm) and protein-bound PLP (412 nm) indicated a molecular mass as large as 517 kDa (Fig. 1), which was in agreement with the theoretical value (10 $\times$ 50.8 kDa).

Initial crystallization screening of wild-type SelA performed by mixing 5.2 mg ml⁻¹ protein solution and the reservoir solutions identified many crystallization conditions. Seven screening kits, Crystal Screen, Crystal Screen 2, Natrix, SaltRX, Index and Wizard I and II, were used and crystals were obtained under 72 conditions

Figure 1
Gel-filtration analysis of wild-type SelA. The elution volume was measured by monitoring the absorbance at 280 nm (protein) and 412 nm (protein-bound PLP). The calculated molecular mass was 517 kDa, which is close to the theoretical value (508 kDa) for the SelA decamer.

within two months. The main precipitants in each condition were polyethylene glycol (PEG), 2-methyl-2-propanol, ($\pm$)-2-methyl-2,4-pentandiol (MPD), Jeffamine M-600, ammonium sulfate, NaNO₃, NaCl, trisodium citrate, sodium formate, Na₂HPO₄/KH₂PO₄, sodium malonate, KSCN, (NH₄)₂HPO₄, diammonium tartrate and potassium sodium tartrate. However, most of them yielded needle-shaped or thin plate-shaped crystals. Three-dimensional crystals were only obtained using crystallization reagents that contained MPD as the

precipitant, such as Wizard II condition No. 2 (100 mM MES–NaOH buffer pH 6.0 containing 35% MPD and 200 mM Li₂SO₄). The optimized reservoir solution (100 mM Na MES–HCl buffer pH 6.5 containing 35% MPD and 200 mM Li₂SO₄) generated hexagonal column crystals (Fig. 2*a*). The crystals only diffracted to 8 Å resolution using snapshot X-ray diffraction experiments. The unit-cell parameters were $a = 150$, $b = 165$, $c = 295$ Å (primitive orthorhombic).

Table 1
Data-collection and phasing statistics.

Values in parentheses are for the highest resolution shell.

	SelA-ΔN, Lys methylation	SelA-ΔN, SeMet, Lys methylation		SelA-ΔN, SeMet, Lys methylation, K ₂ PtCl ₄ derivative	SelA ^{4KA} , Lys methylation
		Peak	Edge		
Data collection					
Beamline	BL5A, Photon Factory	BL41XU, SPring-8		BL17A, Photon Factory	BL41XU, SPring-8
Wavelength (Å)	1.00000	0.97917	0.97947	1.07117	1.00000
Space group	<i>P</i> 1	<i>P</i> 2 ₁		<i>P</i> 2 ₁	<i>P</i> 4 ₁ 2 ₁ 2 or <i>P</i> 4 ₃ 2 ₁ 2
Unit-cell parameters (Å, °)	$a = 92.4$, $b = 116.4$, $c = 124.8$, $\alpha = 102.1$, $\beta = 93.4$, $\gamma = 106.1$	$a = 92.9$, $b = 280.9$, $c = 98.1$, $\beta = 113.5$		$a = 92.8$, $b = 280.9$, $c = 97.6$, $\beta = 113.4$	$a = b = 167.0$, $c = 211.1$
Resolution (Å)	50.0–3.25 (3.37–3.25)	50.0–3.30 (3.42–3.30)	50.0–3.40 (3.52–3.40)	50.0–3.20 (3.31–3.20)	50.0–3.90 (4.04–3.90)
Unique reflections	75475 (7499)	69327 (6903)	63781 (6362)	75145 (7212)	26396 (2358)
Completeness (%)	99.3 (98.6)	99.8 (99.9)	99.9 (99.8)	99.5 (96.0)	94.1 (86.8)
Multiplicity	3.9 (3.8)	7.5 (7.0)	7.5 (7.0)	7.4 (6.2)	9.1 (8.1)
$R_{\text{merge}}^{\dagger}$	0.129 (0.538)	0.114 (0.859)	0.107 (0.891)	0.090 (0.674)	0.103 (0.736)
$\langle I/\sigma(I) \rangle$	12.7 (2.05)	18.1 (2.47)	19.2 (2.17)	22.4 (2.50)	15.3 (2.02)
Phasing					
No. of identified Se sites		60			
Phasing power					
Isomorphous (acentric/centric)		—	0.254/0.213		
Anomalous		0.729	0.364		
FOM (before DM/after DM) ‡		0.250/0.598			

$^{\dagger} R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th measurement of hkl and $\langle I(hkl) \rangle$ is the average value of $I_i(hkl)$ for all i th measurements. ‡ FOM, figure of merit; DM, density modification (solvent flattening and NCS averaging).

Figure 2
Crystals of wild-type SelA and mutant SelAs. Typical crystals of wild-type SelA (*a*), Lys-methylated wild-type SelA (*b*), SelA-ΔN (*c*), SeMet-substituted SelA-ΔN with Lys methylation (*d*) and Lys-methylated SelA^{4KA} (*e*) are shown. The yellow colour of the crystals arises from the PLP cofactor. The scale bars indicate 200 μm.

3.2. Effect of Lys methylation

In order to improve the diffraction resolution, methylation of the lysine residues of SelA was examined. Lys-methylated wild-type SelA (14 mg ml^{-1}) required initial screening of crystallization conditions. Four screening kits, Crystal Screen 2, Natrix and Wizard I and II, were used and crystals were obtained under 12 conditions within two weeks. The main precipitant reagent in each condition was PEG 2000–8000. The initially obtained crystals were all needle-shaped or thin rod-shaped and the best reservoir solution was Wizard II condition No. 43 [100 mM Tris–HCl buffer pH 7.0 containing 10% (w/v) PEG 8000 and 200 mM MgCl_2]. During reservoir-solution optimization, we noticed that octahedral crystals were obtained at a high NiCl_2 concentration. Finally, the following reservoir solution was employed: 100 mM Tris–HCl buffer pH 7.5 containing 10–12% (w/v) PEG 4000, 200 mM MgCl_2 , 60–120 mM NiCl_2 , 50 mM $\text{Na}_2\text{S}_2\text{O}_3$ and

50 mM L-serine (Fig. 2*b*). The crystals diffracted to $6 \text{ \AA}$ resolution using an optimized cryoprotective solution (50 mM Tris–HCl buffer pH 7.5 containing 25% ethylene glycol, 12% PEG 1000, 12% PEG 8000 and 20 mM MgCl_2). The unit-cell parameters were $a = b = 172$, $c = 250 \text{ \AA}$ (primitive tetragonal). Diffraction improvement (from 8 to $6 \text{ \AA}$ resolution) and alteration of the crystal system were observed upon Lys methylation. However, the diffraction resolution was not sufficient to determine the crystal structure.

3.3. Improvement by the truncation of the N-terminal region

In addition to the core and C-terminal regions that are conserved among PLP-dependent enzymes, SelA possesses a unique N-terminal region. To improve the diffraction resolution, the truncated mutant SelA- ΔN lacking the N-terminal region (residues 1–61) was exam-

Figure 3
X-ray diffraction images. Typical X-ray diffraction images from crystals of SeMet-substituted SelA- ΔN with Lys methylation (*a*) and Lys-methylated SelA^{4KA} (*b*) are shown.

ined. Initial crystallization screening of SclA- Δ N (2.4 mg ml⁻¹) was also performed. Six screening kits, Crystal Screen, Crystal Screen 2, Natrix, SaltRX and Wizard I and II, were used and crystals were obtained under 47 conditions within two months. The main precipitants in the conditions were PEG, ethanol, 2-propanol, 2-methyl-2-propanol, 1,6-hexanediol, 1,4-butanediol, MPD, Jeffamine M-600, ammonium sulfate, Li₂SO₄, NaCl, trisodium citrate, sodium formate, sodium acetate, ammonium acetate, Na₂HPO₄/KH₂PO₄, (NH₄)₂HPO₄ and potassium sodium tartrate. Like the wild-type SclA crystals, most of the mutant protein crystals were needle-shaped or thin plate-shaped. The best reservoir solution was Crystal Screen 2 condition No. 36 (Na HEPES-HCl buffer pH 7.5 containing 4.3 M NaCl) and rod-shaped crystals were obtained by using a further concentrated protein solution (20 mg ml⁻¹) and the optimized reservoir solution 100 mM Na MES-HCl buffer pH 6.5 containing 2.5 M NaCl (Fig. 2c). The crystals diffracted to 4.5 Å resolution using

the cryoprotective solution 100 mM Na MES-HCl buffer pH 6.5 containing 4.0 M LiCl, 1.0 M NaCl, 100 mM L-serine and 200 mM Na₂S₂O₃. The crystals belonged to space group *P*2₁, with unit-cell parameters $a = 93$, $b = 347$, $c = 141$ Å, $\beta = 108^\circ$.

Lys methylation of SclA- Δ N was also performed. Two screening kits, Crystal Screen 2 and Natrix, were used for initial crystallization screening of Lys-methylated SclA- Δ N (6.9 mg ml⁻¹) and crystals were obtained under eight conditions within two weeks. The main precipitant in each condition was PEG 4000–20 000 or ammonium sulfate. Most conditions yielded three-dimensional crystals, although they were smaller than 30 µm in size. The best reservoir solution was Natrix condition No. 44 [50 mM Tris-HCl buffer pH 7.5 containing 10% (w/v) PEG 4000, 200 mM KCl and 50 mM MgCl₂], which was optimized to the following reservoir: 100 mM Tris-HCl buffer pH 7.5 containing 12% PEG 4000, 800 mM NaNO₃, 160 mM KCl, 50 mM L-serine and 50 mM Na₂S₂O₃. The crystals diffracted to 3.25 Å resolution using a cryoprotective solution (100 mM Tris-HCl buffer pH 7.5 containing 35% PEG 4000, 800 mM NaBr and 160 mM KCl; Table 1). The unit-cell parameters were $a = 92$, $b = 116$, $c = 125$ Å, $\alpha = 102$, $\beta = 93$, $\gamma = 106^\circ$ (*P*1). However, this cryoprotective solution was not sufficiently optimized, as most of the crystals only diffracted to ~4.0 Å resolution.

To obtain phase information, SeMet-substituted SclA- Δ N with Lys methylation (6.0 mg ml⁻¹) was first crystallized in the same reservoir solution as used for the native protein. Further optimization yielded the following reservoir solution: 100 mM Tris-HCl buffer pH 7.5 containing 11% PEG 4000, 400 mM CaCl₂, 160 mM KCl, 5.0 mM NiCl₂, 50 mM Na₂S₂O₃ and 50 mM L-serine (Fig. 2d). Optimization of the cryoprotective solution was also required. Finally, the following cryoprotective solution was employed: 50 mM Tris-HCl buffer pH 7.5 containing 38% PEG 4000, 100 mM NaNO₃ and 20 mM MgCl₂, which also contributed to the improvement of the resolution. A multi-wavelength anomalous dispersion (MAD) data set for phasing was collected to a resolution of 3.3 Å (Fig. 3a and Table 1). On the other hand, the highest resolution data (3.20 Å) were obtained from a platinum-labelled crystal of SeMet-substituted Lys-methylated SclA- Δ N prepared by soaking in 0.2 mM K₂PtCl₄ (Table 1). The unit-cell parameters were $a = 93$, $b = 281$, $c = 98$ Å, $\beta = 113^\circ$ (*P*2₁). One SclA decamer is expected to be present in the asymmetric unit, with a solvent content of ~55%.

Figure 4

Electron-density maps of SeMet-substituted SclA- Δ N with Lys methylation. (a) The overall F_0 map (1.0 σ level) calculated from the phase after density modification (solvent flattening and NCS averaging) is shown as a blue mesh. The 60 identified Se sites, represented as spheres, revealed the NCS rotation axes (one fivefold and five twofold axes). The anomalous difference Fourier map (3.5 σ level), indicating the ordered SeMet Se sites, is shown as a red mesh. (b–d) Typical close-up F_0 maps calculated from the phases without density modification (b), after density modification with solvent flattening (c) and after density modification with solvent flattening and NCS averaging (d). The putative C α traces are shown.

3.4. Improvement by point mutations

To improve the diffraction resolution, point mutations targeted to the basic residues in the loop regions were introduced into full-length SelA. There are two sets of three consecutive Lys/Arg residues: Lys19-Lys20-Lys21 and Lys46-Arg47-Lys48. Both of them are found in the N-terminal region and thus the quadruple mutation K19A-K21A-K46A-K48A (4KA) was introduced. The structure of SelA^{4KA} is expected to be identical to that of the wild type, since these mutated residues are not conserved among SelAs. A 3.9 Å resolution diffraction data set was obtained from Lys-methylated SelA^{4KA} using 11.3 mg ml⁻¹ protein solution and the same reservoir and cryoprotective solutions as used for Lys-methylated wild-type SelA (Figs. 2e and 3b). The crystal belonged to space group *P*₄₁2₁2 or *P*₄₃2₁2, with unit-cell parameters *a* = *b* = 167, *c* = 211 Å (Table 1). One half of the SelA decamer (five subunits of SelA) is expected to be present in the asymmetric unit, with a solvent content of ~58%. The point mutations drastically improved the diffraction resolution (from 6 to 3.9 Å).

3.5. Phase determination

The two-wavelength data set for SeMet-substituted SelA-ΔN with Lys methylation was analyzed with *SHELXC* and *SHELXD* (Sheldrick, 2008) and 26 putative Se sites were detected. Subsequently, three of the 26 Se sites were deleted and 39 new sites were picked during several automated cycles of Se-site-based phasing using *autoSHARP* (Vonnrhein *et al.*, 2007). Finally, a total of 62 sites were obtained.

Each SelA-ΔN subunit contains eight Met residues; two of them are located in the N-terminal region and are presumably disordered. Therefore, we postulated that the 62 Se sites corresponded to the ordered 6 × 10 SeMet residues in decameric SelA-ΔN. The rearrangement of the 62 Se sites by the crystallographic symmetry operations revealed one fivefold and five twofold rotation axes of noncrystallographic symmetry (NCS). Five Se sites which did not agree with the NCS were deleted manually and three new sites were picked based on the NCS and the anomalous difference Fourier map (Fig. 4a). The resulting 60 Se sites were superposed on each other and nine NCS operators (72, 144, -72 and -144° rotations around the fivefold axis and 180° rotations around the five twofold axes) were calculated using *LSQKAB* (Winn *et al.*, 2011).

The phase was recalculated based on the 60 updated Se sites using *SHARP* (Vonnrhein *et al.*, 2007). Solvent flattening and NCS averaging using the calculated operators were performed with *DM* (Winn *et al.*, 2011). The phase was drastically improved by NCS averaging (Figs. 4b, 4c and 4d). The phasing statistics are shown in Table 1. The overall *F*_o map clearly indicated that the asymmetric unit contains a pentamer of dimers (Fig. 4a).

4. Discussion

The wild-type *A. aeolicus* SelA contains 49 lysine residues (10.84%), while SelA-ΔN contains 38 lysine residues (9.69%). The theoretical isoelectric points of wild-type SelA and SelA-ΔN are 9.4 and 9.2, respectively. In both full-length SelA and SelA-ΔN, Lys methylation changed the crystallization conditions and the crystal forms. The high lysine content is probably one of the reasons for these changes. Lys methylation also facilitated crystal formation and growth. Furthermore, a significant improvement in the diffraction resolution was observed.

Truncation of the N-terminal region caused drastic improvements (from 6 to 3.25 Å resolution with Lys methylation and from 8 to 4.5 Å resolution without Lys methylation). Accordingly, SelA-ΔN crystallized under different conditions and in different space groups from the full-length SelAs (wild type and the 4KA mutant). These results suggest that the N-terminal domain has some flexibility that negatively affects the crystallographic protein-protein contacts.

The point mutations introduced into the loop regions had no effect on either the crystallization conditions or the appearance of the crystals. However, a drastic improvement in the diffraction resolution was observed (from 6 to 3.9 Å). The unit-cell volume was reduced from 7.40 × 10⁶ to 5.88 × 10⁶ Å³ (20.4% shrinkage). Therefore, the improvement in resolution may have resulted from a slippage that reinforces the intermolecular interactions at the crystal-packing interface.

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