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Crystallization and preliminary X-ray crystallographic analysis of a helicase-like domain from a tomato mosaic virus replication protein

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Tomato mosaic virus belongs to the genus *Tobamovirus* in the alphavirus-like superfamily of positive-strand RNA viruses. The alphavirus-like superfamily includes many plant and animal viruses of agronomical and clinical importance. These viruses encode replication-associated proteins that contain a putative superfamily 1 helicase domain. No three-dimensional structures for this domain have been determined to date. Here, the crystallization and preliminary X-ray diffraction analysis of the 130K helicase domain are reported. Diffraction data were collected and processed to 2.05 and 1.75 Å resolution from native and selenomethionine-labelled crystals, respectively. The crystals belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 85.8, b = 128.3, c = 40.7 Å.

1. Introduction

Helicases are motor proteins that use the free energy of nucleoside triphosphate (NTP) hydrolysis to catalyze the unwinding of duplex nucleic acids. They are required for gene replication, transcription, translation, recombination and repair (Abdelhaleem, 2010). Many genes in organisms, including viruses, encode helicases and they have been classified into six superfamilies (SF1–6) according to their amino-acid sequence similarities and conserved helicase motifs (Gorbalenya & Koonin, 1993; Singleton *et al.*, 2007). For example, SF1 helicases are found in alphaviruses, SF2 helicases are found in potyviruses and flaviviruses (NS3-like proteins) and SF3 helicases are found in picornaviruses (2C-like proteins). Three-dimensional structures have been solved for the helicases from hepatitis C virus (Love *et al.*, 1996; Kim *et al.*, 1996, 1998) and dengue virus (Murthy *et al.*, 1999, 2000), both of which belong to the SF2 group. Crystal structures of SF3 viral helicases have also been determined (Hickman & Dyda, 2005). However, although the SF1 helicases from *Bacillus stearothermophilus* and *Escherichia coli* have been found to have almost the same catalytic core fold (two RecA-like folds) as the SF2 helicase from the hepatitis C virus (Yao *et al.*, 1997), a three-dimensional structure is not available for viral SF1 helicases.

Tomato mosaic virus (ToMV) and tobacco mosaic virus (TMV) belong to the *Tobamovirus* genus and are also classified as members of the alphavirus-like superfamily of positive-strand RNA viruses. Tobamoviruses have been the seminal model for the study of plant positive-strand RNA virus multiplication and for characterization of the interactions between these viruses and their host organisms (Buck, 1999). The genome of TMV encodes at least four proteins (Goelet *et al.*, 1982). After infection, the genomic RNAs are translated in the cytoplasm of the host cell to produce proteins with an approximate molecular mass of 130 kDa (130K replication proteins) and 180 kDa (180K replication proteins, a read-through product of the 130K replication proteins). These proteins are both involved in viral RNA replication and they contain an SF1 helicase-like domain, which self-associates (Goregaoker & Culver, 2003) and interacts with more than ten host proteins (Ishibashi *et al.*, 2010). These results suggest that as well as unwinding the RNA duplex the helicase domain also plays multiple roles in mediating virus multiplication, elicitation of host responses and counter-defence.

Table 1
Summary of data collection from 130K HEL.

Values in parentheses are for the outermost resolution shell.

	Native 130K HEL	SeMet-labelled 130K HEL
Resolution range (Å)	200–2.05 (2.09–2.05)	50–1.75 (1.81–1.75)
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters		
a (Å)	85.8	85.5
b (Å)	128.3	128.6
c (Å)	40.7	40.6
Wavelength (Å)	1.0080	0.97914
Temperature (K)	100	100
$R_{\rm merge}$† (%)	0.074 (0.218)	0.079 (0.470)
Completeness (>1σ) (%)	100 (100)	93.6 (67.4)
No. of observed reflections (>1σ)	418292	168676
No. of unique reflections (>1σ)	28957	43116
Redundancy (>1σ)	7.7 (7.7)	3.9 (2.0)
$\langle I/\sigma(I)\rangle$	36.7 (11.7)	26.5 (2.0)
Molecules per asymmetric unit	1	1
$V_{\rm M}$ (Å^3 Da^{-1})	2.2	2.2
Solvent content (%)	45.2	44.2

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

To fully characterize the viral SF1 helicase domain, structural studies must be performed. We recently reported the determination of the stable helicase domain (Ser666–Gln1116) of ToMV 130K replication protein (130K HEL; molecular weight 50.4 kDa), which contains the putative helicase domain and an extra 155 residues to its N-terminus, and large amounts of the recombinant protein could be purified for structural analysis (Xiang *et al.*, 2012). In order to determine the SF1 helicase structure, which has not yet been determined for a virus, we have performed crystallization and preliminary crystallographic studies of 130K HEL.

2. Materials and methods

2.1. Expression and purification

E. coli Rosetta (DE3) cells were transformed with the expression vector pDEST-trx-130K HEL and grown in LB medium containing 50 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol to an A_{600} of ∼0.6. Expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 20 µ*M*. Cells were grown for an additional 16 h at 291 K and then centrifuged at 5000*g* for 15 min. The pellet was suspended in 10 ml 50 m*M* sodium phosphate pH 7.5, 0.5 *M* NaCl, 2.0 m*M* β-mercaptoethanol (β-ME), disrupted by sonication and centrifuged at 27 000*g* for 30 min at 277 K. The fusion protein was loaded directly onto a 5 ml Ni-chelating HP column (GE Healthcare Biosciences) and the bound proteins were eluted by increasing the imidazole concentration to 0.5 *M*. The protein that was eluted by 0.5 *M* imidazole was then applied onto a HiLoad 26/60 Superdex 200 pg column (GE Healthcare Biosciences) in 20 m*M* sodium phosphate pH 7.5, 150 m*M* NaCl, 2.0 m*M* β-ME. The Trx-His$_6$ tag was removed with 1.7 U thrombin (Invitrogen) per milligram of protein for 16 h at 288 K. The protein was applied onto a 5 ml HiTrap Chelating HP column (GE Healthcare Biosciences) and the flowthrough fraction was collected and applied onto a HiLoad 26/60 Superdex 75 pg column (GE Healthcare Biosciences) followed by 5 ml SP and Q Sepharose High Performance columns (GE Healthcare Biosciences). The expression and purification procedure is described in more detail elsewhere (Xiang *et al.*, 2012). For selenomethionine (SeMet) labelling of 130K HEL, *E. coli* B834 (DE3) pRARE cells were transformed with the expression vector pDEST-trx-130K HEL. B834 (DE3) pRARE is a derivative of the B834 line (Novagen) transformed with the pRARE plasmid from Rosetta (DE3) (Novagen). We prepared the competent cells ourselves. The overproduction and purification of SeMet-labelled 130K HEL in *E. coli* B834 (DE3) pRARE was as described above except for the use of LeMaster medium (LeMaster & Richards, 1985) instead of LB medium. 2 m*M* $MgCl_2$ and 0.1 m*M* ATPγS were added to the purified 130K HEL. The mixture was then concentrated to 2.0 mg ml^{-1} using a Centricon (Amicon) and buffer-exchanged into 20 m*M* sodium phosphate pH 7.5 containing 150 m*M* NaCl, 2 m*M* β-ME, 0.4 m*M* ATPγS and 0.4 m*M* $MgCl_2$.

2.2. Crystallization and data collection

Native and SeMet-labelled 130K HEL were both crystallized using the hanging-drop vapour-diffusion method at 285 K under the same conditions as follows. The crystals were grown on a siliconized cover slip by equilibrating a mixture consisting of 1.5 µl protein solution (2.0 mg ml^{-1} protein in 20 m*M* sodium phosphate pH 7.5, 150 µ*M* NaCl, 2 m*M* β-ME, 0.4 m*M* ATPγS, 0.4 m*M* $MgCl_2$) and 1.5 µl reservoir solution (0.1 *M* NaH_2PO_4–NaOH pH 6.5, 1.7 *M* ammonium sulfate) against 500 µl reservoir solution.

The crystals were briefly soaked in crystallization solution containing 25%(*w*/*v*) glycerol prior to flash-freezing in a cryogenic nitrogen-gas stream. An X-ray diffraction data set from a native crystal was collected at 100 K on beamline BL17A at KEK Photon Factory, Japan at a wavelength of 1.0 Å. The crystal-to-detector distance was set to 299.6 mm and diffraction images were recorded using an ADSC Quantum 315 CCD detector with 1° oscillation and 20 s exposure per frame. A total of 360 frames were collected with an oscillation range of 180°. An X-ray diffraction data set from an SeMet-labelled crystal was collected at 100 K on the BL38B1 beamline at SPring-8, Japan at a wavelength of 0.97914 Å. The crystal-to-detector distance was set to 300 mm and diffraction images were recorded using an ADSC Quantum 315 CCD detector with 0.5° oscillation and 20 s exposure per frame. A total of 180 frames were collected with an oscillation range of 180°. The collected data set was processed using the *HKL*-2000 suite (Otwinowski & Minor, 1997). The programs *SHELXD* (Sheldrick, 2008), *HKL2MAP* (Schneider & Sheldrick, 2002) and *ARP*/*wARP* (Morris *et al.*, 2003) were used to perform initial phasing and autotracing, which succeeded in building the initial structure. The data-collection statistics are summarized in Table 1.

3. Results and discussion

Recombinant SeMet-labelled 130K HEL from ToMV was expressed at a very low level in commercially available *E. coli* B834 (DE3) and B834 (DE3) pLysS competent cells (Novagen). From our previous experimental results, *E. coli* Rosetta (DE3) cells produced a large amount of recombinant 130K HEL (Xiang *et al.*, 2012), suggesting that the presence of rare codons has a negative influence on protein expression. In this work, B834 (DE3) cells were transformed with the pRARE plasmid from Rosetta (DE3) [B834 (DE3) pRARE] and B834 (DE3) pRARE was transformed with the expression vector pDEST-trx-130K HEL for production of SeMet-labelled 130K HEL. SeMet-labelled 130K HEL was successfully expressed and purified to homogeneity using the same methods as for the native protein (Fig. 1). The native and SeMet-labelled 130K HEL crystals grew in about 20 d to approximate dimensions of 0.1 × 0.1 × 0.1 mm (Fig. 2*a*).

X-ray diffraction experiments with native 130K HEL were performed under liquid-nitrogen-cooled conditions at 100 K. Crystals were picked up in a nylon loop, soaked briefly in 25% glycerol and then successfully flash-cooled in a cryogenic nitrogen-gas stream. X-ray diffraction data for native 130K HEL were collected on beamline BL17A at Photon Factory, Tsukuba, Japan. The crystals diffracted X-rays beyond 2.05 Å resolution and belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 85.8, b = 128.3, c = 40.7 Å. Assumption of the presence of one 130K HEL molecule in the asymmetric unit gave a reasonable Matthews coefficient V_M of 2.2 Å^3 Da^{-1} and a solvent content of 45% (Matthews, 1968). Data-collection statistics are summarized in Table 1.

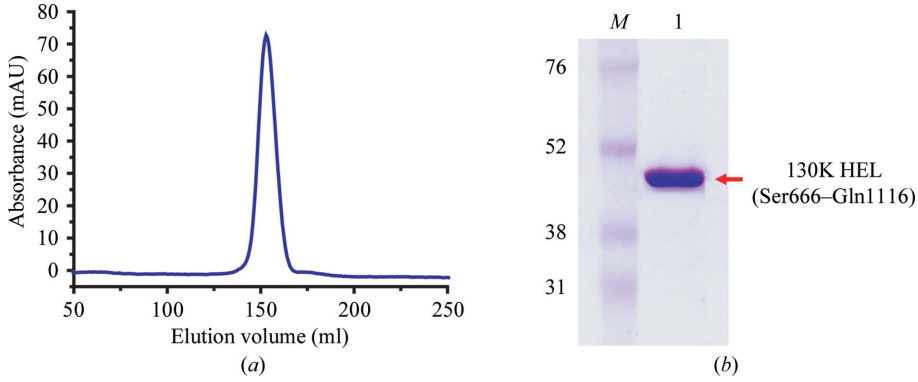

Figure 1
Purification of 130K HEL. (*a*) Superdex 75 pg gel-filtration chromatography of purified Ser666–Gln1116. (*b*) Commassie Brilliant Blue-stained 12.5% SDS–PAGE gel showing the purity of the 130K HEL used for crystallization. Lane *M*, standard molecular-weight protein markers (labelled in kDa); lane 1, purified 130K HEL.

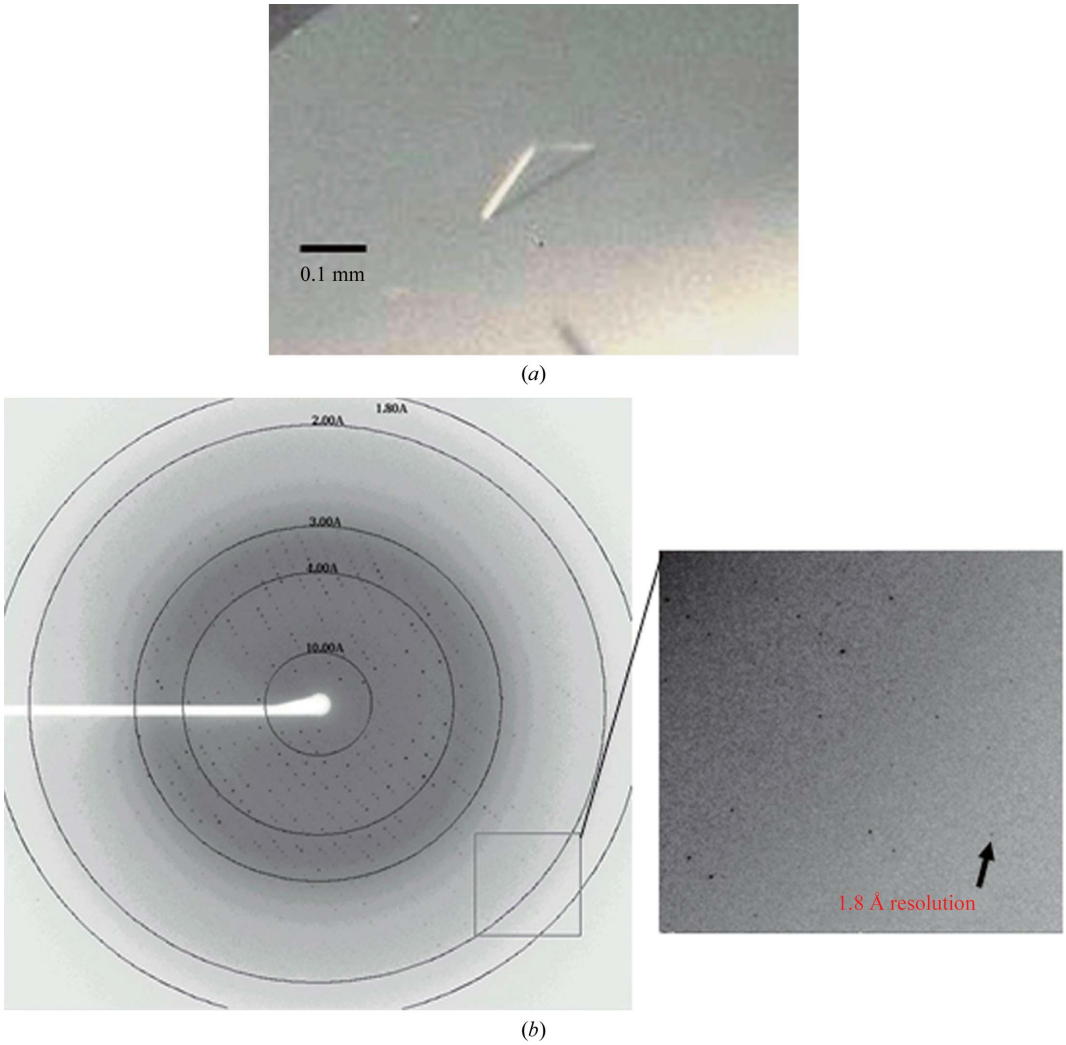

Figure 2
Crystallization of Se-Met labelled 130K HEL. (*a*) A typical crystal with dimensions of 0.1 × 0.1 × 0.1 mm. (*b*) X-ray diffraction pattern showing diffraction beyond 1.8 Å resolution.

X-ray diffraction data from SeMet-labelled 130K HEL were collected on beamline BL38B1 of the SPring-8 synchrotron-radiation source, Harima, Japan. The crystals diffracted X-rays beyond 1.75 Å resolution (Fig. 2*b*). The SeMet-labelled crystals belonged to the same space group as the native crystals, with almost identical unit-cell parameters (Table 1).

Single-wavelength anomalous dispersion (SAD) data were collected from a SeMet-labelled crystal at 100 K at a wavelength of 0.97914 Å, which was chosen after an absorption-scan measurement to maximize the anomalous signal of the Se atom. SeMet-labelled crystal diffraction images were indexed and scaled using the *HKL*-2000 suite (Otwinowski & Minor, 1997). SAD phasing and the calculation of the initial map were performed using *SHELXD* (Sheldrick, 2008) and *HKL2MAP* (Schneider & Sheldrick, 2002). Subsequently, autotracing using *ARP*/*wARP* (Morris *et al.*, 2003) succeeded in building an initial structure. Manual model building and further refinement with *Coot* (Emsley & Cowtan, 2004) are in progress.

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