Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray analysis of eukaryotic initiation factor 4E from *Pisum sativum*

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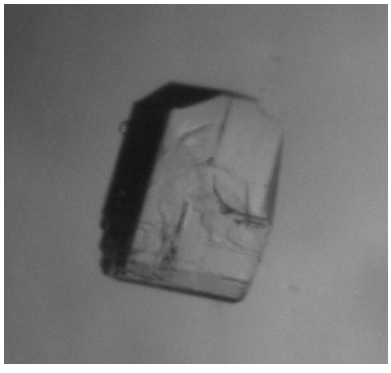
Received 12 June 2009
Accepted 12 July 2009

Crystals of an N-terminally truncated 20 kDa fragment of *Pisum sativum* eIF4E (ΔN-eIF4E) were grown by vapour diffusion. X-ray data were recorded to a resolution of 2.2 Å from a single crystal in-house. Indexing was consistent with primitive monoclinic symmetry and solvent-content estimations suggested that between four and nine copies of the eIF4E fragment were possible per crystallographic asymmetric unit. eIF4E is an essential component of the eukaryotic translation machinery and recent studies have shown that point mutations of plant eIF4Es can confer resistance to potyvirus infection.

1. Introduction

Studies of several different plant–virus interactions have revealed the importance of the eukaryotic translation factor 4E (eIF4E) and its paralogue eIF(iso)4E as factors that are required to support virus multiplication in susceptible plant genotypes. Much of this work has focused on members of the *Potyvirus* group of viruses, in which host plants carrying specific alleles of *eIF4E* [or in some cases *eIF(iso)4E*] exhibit recessive resistance to potyvirus infection. This appears to be the consequence of a failure in the physical interaction between eIF4E and the virus avirulence determinant virus-genome-linked protein (VPg; Robaglia & Caranta, 2006; Maule *et al.*, 2007). These natural resistance alleles of *eIF4E* encode polymorphic nonconservative amino-acid substitutions in highly conserved regions of the protein. Attempts to define the precise structural determinants for susceptibility and resistance in pepper (Yeam *et al.*, 2007; Charron *et al.*, 2008), lettuce (Nicaise *et al.*, 2003; German-Retana *et al.*, 2008) and tomato (Ruffel *et al.*, 2005) have relied upon homology modelling of the respective eIF4E sequences to the known structures of the wheat and mouse proteins (Monzingo *et al.*, 2007; Marcotrigiano *et al.*, 1997); the polymorphisms are seen to be clustered around two domains close to the m^7G cap-binding site. In this work, we describe the crystallization and preliminary X-ray analysis of an N-terminally deleted eIF4E from *Pisum sativum* (pea), a host for the potyvirus *Pea seed-borne mosaic virus*, for which resistance alleles *sbm1* and *sbm1*^{1} have been defined (Gao *et al.*, 2004). This structure will provide a platform for understanding the aspects of the eIF4E structure that specify potyvirus disease resistance in this legume crop.



2. Materials and methods

2.1. Protein expression, purification and crystallization

The *P. sativum* (pea) *SBM1* gene for eIF4E (UniProtKB/Swiss-Prot entry Q6TEC4) encodes a 228-amino-acid polypeptide with a total deduced molecular mass of 25 981 Da. Previous structural studies with other eIF4Es from mouse (Marcotrigiano *et al.*, 1997) and wheat (Monzingo *et al.*, 2007) have shown that full-length protein can be recalcitrant to crystallization, whilst N-terminally truncated versions have been successfully crystallized. We therefore designed a truncated version of the pea protein based on that used for the crystallization of the wheat protein (Monzingo *et al.*, 2007). Thus, the gene sequence covering residues 52–228 of the native protein (plus an added N-terminal Met residue) was cloned into the pET24a(+) (Novagen) expression vector. The resultant plasmid encoded a

178-amino-acid polypeptide with a total deduced molecular mass of 20 448 Da, hereafter denoted ΔN-eIF4E. In support of this strategy, disorder prediction with the *FoldIndex* server (http://bip.weizmann.ac.il/fldbin/findex; Prilusky *et al.*, 2005) suggested that the first 50 or so residues of pea eIF4E are likely to be disordered.

This plasmid was transformed into *Escherichia coli* strain Rosetta-2 (DE3) pLysS (Novagen; Studier & Moffatt, 1986) and a 50 ml overnight culture of the cells harbouring the pET-ΔN-eIF4E construct was used to inoculate a 5 l culture of Luria–Bertani medium containing 50 µg ml^{-1} kanamycin. The cells were grown at 310 K to an $OD_{600\,nm}$ of around 0.8. Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.4 m*M* and the culture was left shaking for 3 h at 294 K. Harvested cells were resuspended in buffer *A* (20 m*M* HEPES pH 7.6, 150 m*M* NaCl, 2 m*M* EDTA and 5 m*M* DTT) containing a Complete EDTA-free protease-inhibitor cocktail (Roche) and lysed by three passes through a French press at 6.9 MPa. The cell lysate obtained by centrifugation at 43 000*g* for 30 min was applied onto a 3 ml 7-Methyl-GTP Sepharose 4B column (GE Healthcare) connected to an ÄKTA FPLC system (GE Healthcare). The column was then washed with 20 column volumes of buffer *A* and the bound protein was eluted over four column volumes in buffer *A* containing 0.1 m*M* m^{7}GTP (Sigma–Aldrich). This procedure was repeated a further three times for the same cell lysate and fractions containing ΔN-eIF4E (confirmed by SDS–PAGE) were pooled before the NaCl concentration was brought to 300 m*M* by adding 5 *M* NaCl as necessary. Protein samples were concentrated to a volume of approximately 3 ml using an Amicon Ultra-4 10 kDa cutoff concentrator (Millipore) and subsequently applied onto a Superdex-75 HiLoad HP gel-filtration column (GE Healthcare) pre-equilibrated with buffer *B* (20 m*M* Tris-HCl pH 7.6, 300 m*M* NaCl, 2 m*M* EDTA and 5 m*M* DTT) and eluted at a flow rate of 0.2 ml min^{-1}. Fractions containing pure ΔN-eIF4E were pooled and concentrated to around 10 mg ml^{-1} using an Amicon Ultra-4 10 kDa cutoff concentrator (Millipore) and m^{7}GTP was added to a final concentration of 1 m*M*.

Prior to crystallization, dynamic light scattering (DLS) was used to monitor the solution properties of the protein. For this purpose, approximately 30 µl of sample was centrifuged through a 0.1 µm Amicon Ultrafree filter (Millipore) to remove particulate material before introduction into a 12 µl microsampling cell. The latter was then inserted into a DynaPro Titan molecular-sizing instrument at 298 K (Wyatt Technology). A minimum of 15 scattering measurements were taken and the resulting data were analysed using the *DYNAMICS* software package (Wyatt Technology).

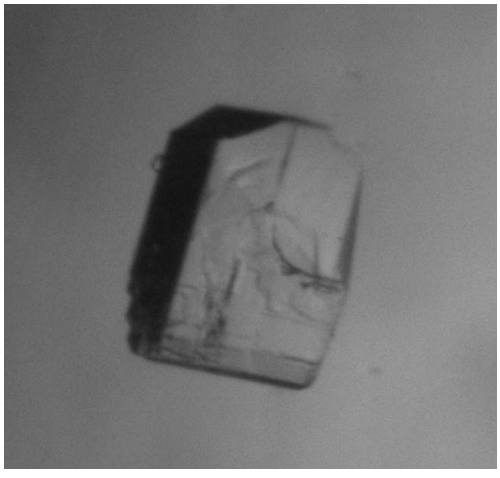


Figure 1
Single crystal of *P. sativum* ΔN-eIF4E with approximate dimensions of 450 × 350 × 150 µm.

Table 1
Summary of X-ray data for *P. sativum* ΔN-eIF4E.

Values in parentheses are for the outer resolution shell.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 73.61, b = 136.32, c = 74.41, β = 92.65
Wavelength (Å)	1.542
Resolution range (Å)	21.93–2.20 (2.32–2.20)
Unique reflections	72233 (10355)
Completeness (%)	97.4 (95.4)
Redundancy	3.7 (3.5)
R_{merge}†	0.067 (0.241)
$\langle I/\sigma(I)\rangle$	14.6 (5.8)
Wilson B value (Å^2)	23.5

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations l of reflection hkl.

Crystallization trials were carried out by vapour diffusion in a sitting-drop format with 96-well MRC plates (Molecular Dimensions) using a variety of in-house and commercially available screens (Hampton Research, Molecular Dimensions and Qiagen). Drops consisted of 1 µl protein solution mixed with 1 µl precipitant solution and the reservoir volume was 50 µl; the protein concentration was approximately 10 mg ml^{-1}. Improved crystals were subsequently obtained by refining the successful conditions in a hanging-drop format using 24-well VDX plates (Molecular Dimensions) over a reservoir volume of 1 ml.

2.2. Cryoprotection and X-ray data collection

Prior to data collection, a crystal was transferred directly from the hanging drop to a cryoprotectant solution for a maximum of 15 s, after which the crystal was mounted in a LithoLoop (Molecular Dimensions) and flash-cooled to 100 K in a stream of gaseous nitrogen produced by an X-Stream cryocooler (Rigaku-MSC). Diffraction data were collected using a MAR 345 image-plate detector (MAR Research) mounted on a Rigaku RU-H3RHB rotating-anode X-ray generator (operated at 50 kV and 100 mA) fitted with Osmic confocal optics and a copper target (Cu $K\alpha$; λ = 1.542 Å). The diffraction data were processed using *MOSFLM* (Leslie, 2006) and *SCALA* (Evans, 2006).

3. Results and discussion

ΔN-eIF4E was purified with an approximate yield of 30 mg protein from 5 l culture and was judged to be greater than 98% pure from SDS–PAGE analysis. DLS analysis gave a monomodal distribution with a polydispersity of 22.3%. From these results, the molecular size was estimated at 22.6 kDa, which is close to the value expected for a ΔN-eIF4E monomer (20.45 kDa).

Preliminary crystals grew within 5 d after setup from 16–20%(*w*/*v*) PEG 3350, 100 m*M* HEPES pH 7.0 at 293 K. Improved crystals were subsequently obtained after three weeks at 277 K from 18%(*w*/*v*) PEG 3350 in 100 m*M* HEPES pH 7.0 and had maximum dimensions of approximately 450 × 350 × 150 µm (Fig. 1).

A single crystal of ΔN-eIF4E was cryoprotected using 20%(*v*/*v*) ethylene glycol, 18%(*w*/*v*) PEG 3350 in 100 m*M* HEPES pH 7.0 prior to X-ray data collection: a total of 360 × 0.5° oscillation images were recorded in a continuous sweep to a maximum resolution of 2.2 Å. Indexing was consistent with a primitive monoclinic lattice, with unit-cell parameters a = 73.61, b = 136.32, c = 74.41 Å, β = 92.65°.

Subsequent processing revealed systematic absences corresponding to space group $P2_1$ and yielded a data set that was 97.4% complete to 2.2 Å resolution. Data-collection and processing statistics are summarized in Table 1. Solvent-content estimations suggested that between four and nine ΔN-eIF4E molecules were possible per asymmetric unit, giving solvent-content values in the range 39.4–73.0% (Matthews, 1968). Analysis of the data set with *SFCHECK* (Vaguine *et al.*, 1999) did not find any evidence for pseudo-translational symmetry, indicating that none of the molecules in the asymmetric unit were related by translational symmetry alone. However, a self-rotation function calculated on data in the resolution range 10–5 Å using *POLARRFN* (Collaborative Computational Project, Number 4, 1994) revealed a number of peaks on the $\kappa = 90^\circ$ and 180° sections indicative of fourfold and twofold axes of noncrystallographic symmetry, respectively.

We have obtained a preliminary molecular-replacement solution for the *P. sativum* ΔN-eIF4E structure using the known structure of the wheat orthologue (PDB code 2idr; Monzingo *et al.*, 2007) as a template. This model contained eight copies of the molecule per asymmetric unit (with a corresponding solvent content of 46.1%), which are arranged as a pair of loosely associated tetramers, each displaying approximate fourfold (C_4) noncrystallographic symmetry. Details of the structure solution and the resultant model will be reported elsewhere.

We would like to thank the BBSRC for financial support through responsive mode funding (ref. BB/E006949/1) and the Core Strategic Grant to the John Innes Centre.

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