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Purification, crystallization and preliminary X-ray diffraction analysis of the Whirly domain of StWhy2 in complex with single-stranded DNA

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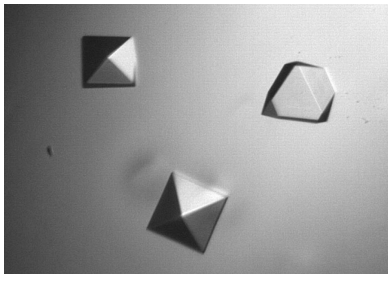


StWhy1 and StWhy2 are members of the Whirly family of single-stranded DNA (ssDNA) binding proteins. To understand the mode of binding of the Whirly proteins to single-stranded DNA, crystals of the Whirly domains of both StWhy1 and StWhy2 in complex with single-stranded DNA were obtained by the hanging-drop vapour-diffusion method. The diffraction patterns of the StWhy1–ssDNA complex crystals displayed severe anisotropy and were of low resolution, making them unsuitable for structure determination. In contrast, the crystals of the StWhy2–ssDNA complex diffracted isotropically to 2.20 Å resolution. The crystallization and data collection to 2.20 Å resolution of StWhy2 in the free form are also reported.

1. Introduction

Single-stranded DNA (ssDNA) binding proteins play a pivotal role in the metabolism of DNA. They are involved in replication, repair, recombination and transcription processes as well as in protection of the telomeres. In accordance with their important role, ssDNA-binding proteins are ubiquitously found in all realms of life.

The Whirlies belong to a family of ssDNA-binding proteins that are found mainly in the plant kingdom (Desveaux *et al.*, 2002*b*, 2005). They fulfil multiple roles including regulation of transcription (Desveaux *et al.*, 2000, 2004), protection of telomere ends (Yoo *et al.*, 2007) and possibly replication (Maréchal *et al.*, 2008). These roles are dependent on the ssDNA-binding activity of the Whirly proteins, which is located on the Whirly domain, a 160-amino-acid strongly conserved region that is necessary and sufficient for high-affinity DNA binding (Desveaux *et al.*, 2005). Elucidation of the crystal structure of StWhy1 (*Solanum tuberosum* Whirly 1) in the free form revealed a tetrameric quaternary structure for the Whirly domain resembling a whirligig (Desveaux *et al.*, 2002*b*). Despite the elucidation of the structure of StWhy1 in the free form, the mode of ssDNA binding to the tetramer has remained elusive.

Members of the Whirly family of proteins have been reported to bind different ssDNA sequences. StWhy1 binds the elicitor response element (ERE; Desveaux *et al.*, 2000) in the promoter region of the *PR-10a* defence gene as a prerequisite for transcriptional activation (Desveaux *et al.*, 2004). AtWhy1 (*Arabidopsis thaliana* Whirly 1), a close homologue of StWhy1, has been reported to bind the ERE sequence (Desveaux *et al.*, 2004) as well as a sequence-unrelated telomeric repeat (Yoo *et al.*, 2007). Finally, AtWhy2 and StWhy2 have been reported to bind ssDNA with little sequence specificity (Maréchal *et al.*, 2008; Vermel *et al.*, 2002). In order to understand the structural basis for ssDNA binding, we sought to determine the crystal structure of a complex between a Whirly protein and ssDNA. Here, we report the crystallization of StWhy1 and StWhy2 in complex with ERE_{32}, a 32-mer oligonucleotide based on the ERE sequence. Crystals of the StWhy1–ERE_{32} complex diffracted anisotropically to low resolution, whereas crystals of the StWhy2–ERE_{32} complex diffracted isotropically to 2.20 Å resolution, thereby allowing the elucidation of the crystal structure of a Whirly bound to single-stranded DNA. To gain further insight into the ssDNA-binding mechanism, we also obtained crystals of StWhy2 in the free form which diffracted to 2.20 Å resolution.

2. Materials and methods

2.1. Cloning, expression and purification

Recombinant StWhy1 was expressed and purified as described previously (Desveaux *et al.*, 2002*a*). Briefly, a pET-21a (Novagen) expression plasmid containing both StWhy1 (68–273) and a C-terminal hexahistidine tag was transformed into *Escherichia coli* strain BL21 (DE3). The cells were grown at 310 K in Luria–Bertani broth. When the cells reached an OD_{600} of 0.6, isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 1 m*M*. After further cell growth for 3 h at 310 K, the cells were harvested, resuspended in 20 m*M* sodium phosphate pH 7.5, 500 m*M* NaCl, 25 m*M* imidazole and lysed by alumina grinding. The recombinant protein was purified by applying the supernatant from the cell lysate onto a HiTrap Chelating nickel-affinity column (GE Healthcare).

StWhy1Δ (87–253) was obtained by PCR using an StWhy1 construct as a template and using the primers St1ΔFwd, GGAATTCCATATGGCATCTACGCCTAAGGT, and St1ΔRvs, CCGCTCGAGAGGCTTGAAGGAATTTAC. In order to clone StWhy2, total cDNA from *S. tuberosum* was obtained using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). The gene coding for StWhy2 (48–216) was then amplified by the polymerase chain reaction with *Pwo* polymerase (Roche) and the primers St2Fwd, GGAATTCCATATGGCTGATGCAGGAAAACGG, and St2Rvs, CCGCTCGAGAGGCCGATTAGTGAAGCG. The PCR products were subcloned into a pET-21a vector (Novagen) using *Nde*I/*Xho*I restriction sites. This cloning strategy added a methionine at the N-terminus of the proteins and a hexahistidine tag (LEHHHHHH) at the C-terminus. Both StWhy2 and StWhy1Δ were purified as described for StWhy1.

2.2. Crystallization of StWhy1–ERE_{32} and data collection

The oligonucleotide ERE_{32} (5′-TGTCATTTTTGTCATTTTTGTCATTTTTGTCA-3′) used for crystallization was obtained from IDT (Integrated DNA Technologies, Coralville, Iowa, USA). StWhy1 was mixed with ERE_{32} in a 1:2 tetramer:ERE_{32} molar ratio. The complex was allowed to form for 30 min at 293 K and was purified using a Superdex 200 16/60 size-exclusion column (GE Healthcare) pre-equilibrated in a buffer containing 25 m*M* citrate–phosphate buffer pH 5.5 and 300 m*M* NaCl. The purified complex was then concentrated to 10 mg ml^{-1} using Millipore 10K concentrators. The protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce). Crystals were typically obtained at 296 K by mixing 3 µl purified complex solution with 3 µl reservoir solution. The reservoir solution (500 µl) contained 0.5–1%(*v*/*v*) PEG 550, 500–900 m*M* NaCl and 25 m*M* sodium citrate/sodium phosphate buffer pH 5. Crystals appeared within a day but reached their maximal size after a week. Crystals of StWhy1–ssDNA were transferred into a cryoprotectant composed of the mother liquor with 20%(*v*/*v*) ethylene glycol. The crystals were then mounted in a cryoloop (Hampton Research) and flash-cooled in a stream of nitrogen gas at 100 K. 360 frames were recorded using an oscillation range of 0.5° and a crystal-to-detector distance of 350 mm. Diffraction data were collected using an ADSC Quantum 315 CCD detector on beamline X29 at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory (BNL, USA). The data were processed, indexed and scaled using *HKL*-2000 (Otwinowski & Minor, 1997).

2.3. Crystallization of StWhy2 in the free form and data collection

After nickel-affinity chromatography, StWhy2 was further purified using a Superdex 200 16/60 size-exclusion column (GE Healthcare) pre-equilibrated in a buffer containing 10 m*M* Tris–HCl pH 8.0 and 100 m*M* NaCl. The purified protein was then concentrated to 20 mg ml^{-1} using Millipore 10K concentrators. The protein concentration was determined using the BCA protein assay kit (Pierce). Crystals were obtained at 296 K by mixing 3 µl StWhy2 protein solution with 3 µl reservoir solution. The reservoir solution (500 µl) contained 21%(*v*/*v*) PEG 1000, 400 m*M* $(NH_4)H_2PO_4$ and 100 m*M* MOPS pH 7.0. Crystals appeared within a day but reached their maximal size after a week. A single crystal of StWhy2 in the free form was mounted in a cryoloop (Hampton Research) and flash-cooled in a stream of nitrogen gas at 100 K. 360 frames were recorded using an oscillation range of 0.5° and a crystal-to-detector distance of 325 mm. Diffraction data were collected as above but on beamline X25. The data were processed, indexed and scaled using *HKL*-2000 (Otwinowski & Minor, 1997).

Table 1
Diffraction data statistics.

Values in parentheses are for the last observed resolution shell.

Data set	StWhy2 in the free form	StWhy2–ERE_{32}
Beamline	X25, NSLS	X29, NSLS
Wavelength (Å)	1.08	1.08
Space group	*F*432	*F*432
Unit-cell parameter (Å)	a = 164.58	a = 167.16
Resolution (Å)	50–2.20 (2.28–2.20)	50–2.20 (2.28–2.20)
No. of unique reflections	10208	10665
Redundancy	37.3 (19.5)	35.9 (9.9)
Completeness (%)	100.0 (99.9)	99.8 (98.7)
$R_{\rm merge}$†	0.076 (0.92)	0.051 (0.56)
$I/\sigma(I)$	16.9 (3.8)	23.1 (3.8)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an individual measurement of the reflection with Miller indices hkl and $\langle I(hkl)\rangle$ is the mean intensity of that reflection.

2.4. Crystallization of StWhy2–ERE_{32} and data collection

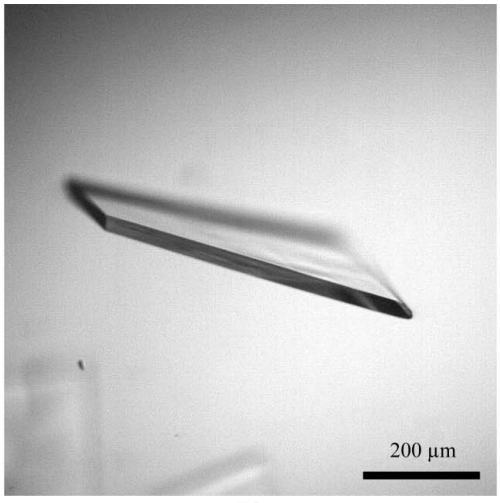
Purified StWhy2 was mixed with ERE_{32} in a 1:2 tetramer:ERE_{32} molar ratio. The complex was allowed to form for 30 min at 293 K and was purified using a Superdex 200 16/60 size-exclusion column (GE Healthcare) pre-equilibrated in a buffer containing 10 m*M* Tris–HCl pH 8.0 and 100 m*M* NaCl. The purified complex was then concentrated to 10 mg ml^{-1} using Millipore 10K concentrators. The protein concentration was determined using the BCA protein assay kit (Pierce). Crystals were obtained at 296 K by mixing 3 µl purified complex solution with 3 µl reservoir solution. The reservoir solution (500 µl) contained 13.5%(*v*/*v*) PEG 6000, 1 *M* LiCl and 100 m*M* Tris–HCl pH 8.0. Crystals appeared within a week but reached their maximal size after a month. To reduce the salt concentration, a single crystal of StWhy2–ERE_{32} was quickly soaked in a solution containing 13.5%(*v*/*v*) PEG 6000, 0.5 *M* LiCl and 100 m*M* Tris–HCl pH 8.0. The crystal was then mounted in a cryoloop (Hampton Research) and flash-cooled in a nitrogen-gas stream at 100 K. 360 frames were recorded using an oscillation range of 0.5° and a crystal-to-detector distance of 275 mm. Diffraction data were collected as described for StWhy1–ERE_{32}. The data were processed, indexed and scaled using *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

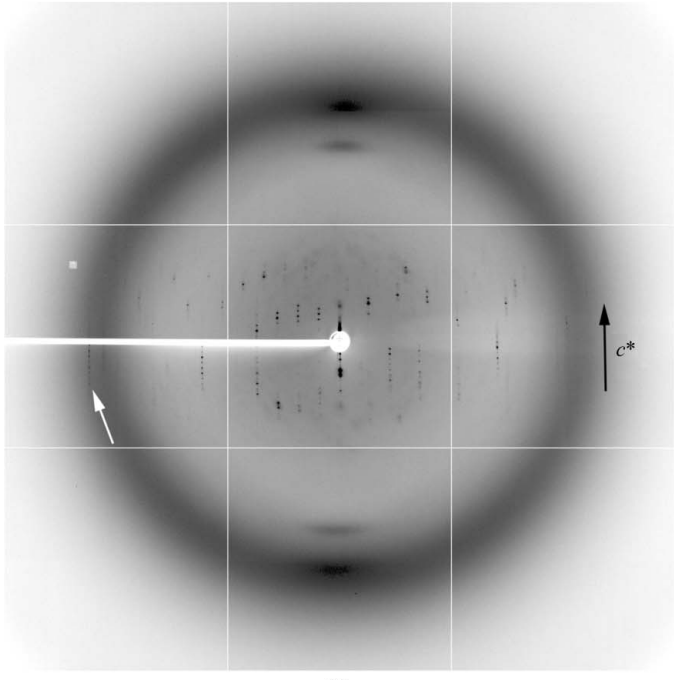
The StWhy1–ERE_{32} complex initially appeared to be a promising candidate for crystallization since the crystal structure of StWhy1 had been solved in the free form (Desveaux *et al.*, 2002*a*,*b*) and the interaction of StWhy1 with ERE had been characterized both *in vitro* and *in vivo* (Desveaux *et al.*, 2004). We obtained our initial crystal-

lization conditions for the StWhy1–ERE$_{32}$ complex by sparse-matrix screening. Refinement of the conditions led us to obtain thin plate-shaped crystals that appeared in less than 1 d (Fig. 1*a*). A data set could be collected from these crystals but was unusable owing to severe anisotropy, with diffraction limited to 3 Å along the *a** and *b** dimensions and to 6 Å along the *c** dimension (Fig. 1*b*). Screening different sequences and lengths of oligonucleotides did not reduce the anisotropy (data not shown). Truncated StWhy1Δ protein lacking the flexible N- and C-terminus tails yet retaining high ssDNA-binding affinity also did not improve the data anisotropy or increase the resolution (data not shown). To overcome the data anisotropy, we decided to crystallize the Whirly domain of StWhy2, a homologue of StWhy1, both in the free form and as a complex with ssDNA.

Initial crystallization trials for StWhy2 in the free form and for the StWhy2–ERE$_{32}$ complex took place using the high-throughput batch crystallization facility at the Hauptman–Woodward Institute in Buffalo (Luft *et al.*, 2003) and resulted in 17 and 20 hits, respectively. Several crystallization conditions were successfully reproduced using hanging drops, yielding bipyramidal crystals for both StWhy2 in the free form and StWhy2–ERE$_{32}$. Importantly, the crystals of the StWhy2–ERE$_{32}$ complex (Fig. 2*a*) diffracted isotropically to 2.20 Å resolution (Fig. 2*b*), whereas the same conditions yielded no crystals for the StWhy1–ERE$_{32}$ complex. Interestingly, StWhy2 in the free form and StWhy2–ERE$_{32}$ gave morphologically identical crystals that belonged to space group *F*432, even though they were crystallized in different chemical environments. Crystals were harvested, washed three times with their respective mother liquor, dissolved and then analyzed by 15% SDS–PAGE followed by silver staining (Fig. 3). This confirmed that the protein was present in the crystals of StWhy2 in the free form and that the StWhy2–ERE$_{32}$ crystals contained both the protein and the oligonucleotide.

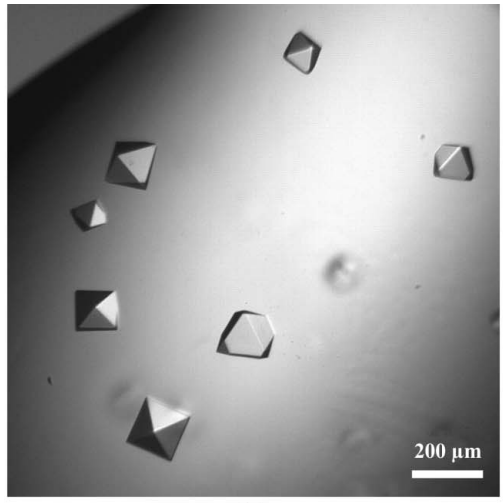


(*a*)

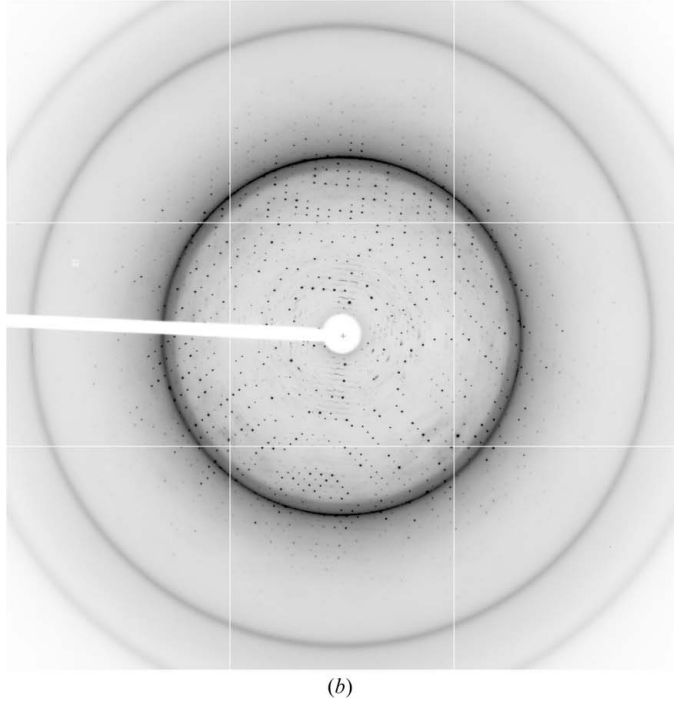


(*b*)

Figure 1
(*a*) Crystals of StWhy1–ERE$_{32}$. (*b*) Diffraction pattern of StWhy1–ERE$_{32}$ showing high anisotropy along the *c** direction (black arrow). The white arrow highlights diffraction at 3.2 Å resolution.



(*a*)



(*b*)

Figure 2
(*a*) Crystals of StWhy2–ERE$_{32}$. (*b*) Diffraction pattern of StWhy2–ERE$_{32}$ exhibiting low anisotropy.

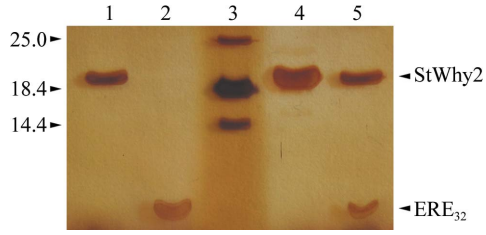


Figure 3
SDS–PAGE analysis of dissolved crystals. Lane 1, purified StWhy2; lane 2, ERE_{32}; lane 3, molecular-weight markers (kDa); lane 4, washed and dissolved crystals of StWhy2 in the free form; lane 5, washed and dissolved crystals of StWhy2–ERE_{32}. Proteins and DNA were stained with silver nitrate.

The Whirly domain of StWhy2 shares 51% sequence identity and 73% sequence similarity to the Whirly domain of StWhy1. We therefore used the structure of StWhy1 (PDB code 1l3a) as a molecular-replacement template for StWhy2 both in the free form and in the ERE_{32} complex. The molecular replacement was successful and electron density corresponding to DNA bases was observed in an $F_o - F_c$ electron-density map for the StWhy2–ERE_{32} complex but not for StWhy2 in the free form. Structure refinement is ongoing and should provide the structural basis for the binding of the Whirly proteins to ssDNA.

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