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Phosphate binding in the active centre of tomato multifunctional nuclease TBN1 and analysis of superhelix formation by the enzyme

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Tomato multifunctional nuclease TBN1 belongs to the type I nuclease family, which plays an important role in apoptotic processes and cell senescence in plants. The newly solved structure of the N211D mutant is reported. Although the main crystal-packing motif (the formation of superhelices) is conserved, the details differ among the known structures. A phosphate ion was localized in the active site of the enzyme. The binding of the surface loop to the active centre is stabilized by the phosphate ion, which correlates with the observed aggregation of TBN1 in phosphate buffer. The conserved binding of the surface loop to the active centre suggests biological relevance of the contact in a regulatory function or in the formation of oligomers.

1. Introduction

Tomato multifunctional nuclease 1 (TBN1; UniProt accession No. Q0KFV0) is a 31.6 kDa, mainly α -helical glycoprotein which plays an important role in specific apoptotic functions, vascular-system development, stress response and tissue differentiation in plants (Matoušek *et al.*, 2007). Furthermore, TBN1 exhibits anticancerogenic properties (Matoušek *et al.*, 2008, 2010). The enzyme possesses endonuclease-like and exonuclease-like activity on double-stranded (ds) and single-stranded (ss) RNA and DNA and on structured RNA, with the production of 5'-mononucleotides and oligonucleotides, and specific 3'-nucleotidase activity (Podzimek *et al.*, 2011).

The structures of wild-type TBN1 (PDB entry 3sng) and the N211D mutant (PDB entry 4dj4) have recently been solved and a reaction mechanism has been suggested (Koval' *et al.*, 2013; Dohnálek *et al.*, 2011). The structural similarity to phospholipase C from *Bacillus cereus* (Antikainen *et al.*, 2003) led to our discovery of the phospholipase activity of TBN1 (Koval' *et al.*, 2013).

TBN1 is related to fungal P1 nucleases. The enzymes share a fold and active centre. In contrast to the multifunctionality of TBN1, P1 nuclease cleaves only single-stranded DNA and RNA (Romier *et al.*, 1998). The major differences between the P1 and TBN1 structures are in the overall shape and the electrostatic potential distribution (Koval' *et al.*, 2013).

As part of our effort to confirm the reaction mechanism and the binding modes of products and substrates, TBN1 was co-crystallized with 5'-mononucleotides. We solved a crystal

Table 1

Enzyme expression.

Source organism	<i>Solanum lycopersicum</i>
Forward primer	5'-GACATTGAAGGGGACTTCACTGACGGAATTTGG-3'
Reverse primer	5'-CCAAATTCGTCAGTGAAGTCCCTTCAATGTC-3'
Expression vector	pLV07
Expression host	<i>N. benthamiana</i>
Complete amino-acid sequence of the construct produced	WSKEGHVMTCRIAQGLLNDEAAHAVKMLPEYVN-GDLSALCVWPDQVRHWYKYKWTSLPHFIDTPD-KACNFDYERDCHDQHGKDMCVAGAIQNFTTQ-LSHYREGTSDRRYNMTEALLFLSHFMGDIHQPMHVGFSTDAGNSIDLRFHKSNNLHHVWDRE-IILTAAKDYYAKDINLLEEDIEGNFTDGIWSD-DLASWRECGNVFSCVNKFATESINIAKKGWYK-GVEAGETLSDDYFNSRLPIVMKRVAQGGIRLA-MLLNNVFGASQQEDSVV

Table 2

Enzyme crystallization.

Method	Vapour diffusion, hanging drop
Plate type	Qiagen EasyXtal 24-well
Temperature (K)	291
Protein concentration (mg ml ⁻¹)	2
Buffer composition of protein solution	10 mM 5'-AMP, 50 mM Tris-HCl pH 7.5, 0.3 M NaCl, 1%(v/v) glycerol, 10 mM 3-(1-pyridino)-1-propanesulfonate (NDSB-201)
Composition of reservoir solution	0.1 M bis-tris pH 6.5, 10%(w/v) PEG 8000
Volume and ratio of drop	1 µl, 1:1
Volume of reservoir (µl)	500

structure of the N211D mutant (N-glycosylation site removed) from a different crystallization condition in a new crystal form. Mononucleotide was not observed in the structure; however, an unexpected phosphate ion was localized in the active centre. The change in the crystallization conditions and the presence of phosphate ion did not change the packing of TBN1 in the crystals.

2. Materials and methods

2.1. Protein preparation

For crystallization and diffraction experiments, a recombinant N211D mutant variant of tomato plant nuclease type I (R-TBN1-N211D) was used. The gene for wild-type TBN1 (UniProt sequence accession code No. Q0KFV0) was mutated in the plant expression vector pLV07 by PCR site-directed mutagenesis to replace Asn211 with Asp. The mutation leads to the hypoglycosylated version of the protein, with only two N-glycosylated sites. *Agrobacterium tumefaciens* LBA4404 was transformed by electroporation with the vector pLV07 and the protein was produced using leaf disc infiltration and by expression in *Nicotiana benthamiana* leaves (Matoušek *et al.*, 2009). Further, the protein was purified by precipitation in ammonium sulfate, ion-exchange chromatography (HiTrap Q FF, Amersham) and affinity chromatography (HiTrap Heparin HP, Amersham), and was desalted on a PD10 (Amersham)

Table 3

Data and structure-refinement statistics for TBN1-N211D-PO₄.

Values in parentheses are for the highest resolution shell.

X-ray source	BL14.1, BESSY II
Detector	MAR CCD 225
Wavelength (Å)	0.918
Crystal-to-detector distance (mm)	339.5
No. of frames	120
Exposure per image (s)	3
Oscillation angle (°)	1
Space group	<i>H3</i>
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = 115.9, <i>c</i> = 74.7
Resolution (Å)	35.0–2.8 (2.89–2.80)
No. of measured reflections	35101
No. of unique reflections	9202 (850)
Average multiplicity	3.8 (3.2)
Completeness (%)	99.8 (98.7)
Average <i>I</i> /σ(<i>I</i>)	17.2 (2.0)
<i>R</i> _{merge}	0.08 (0.50)
<i>R</i> _{work}	0.181
<i>R</i> _{free}	0.250
<i>R</i> _{all}	0.187
Average ADP (Å ²)	55.5
R.m.s.d., bond lengths (Å)	0.012
R.m.s.d., bond angles (°)	1.805
Solvent content (%)	52.9
Matthews coefficient (Å ³ Da ⁻¹)	2.61
No. of non-H atoms	2284
No. of monomers in asymmetric unit	1
No. of localized amino acids	265
No. of water molecules	13
Other localized ions	3 Zn ²⁺ , PO ₄ ³⁻
Localized glycosylation	4 GlcNAc, 2 β-D-mannose, 1 α-D-mannose

column (Lipovova *et al.*, 2008; Matoušek *et al.*, 2008). Protein expression details are summarized in Table 1.

The storage buffer for R-TBN1-N211D consisted of 50 mM Tris-HCl pH 7.5, 0.3 M NaCl, 1%(v/v) glycerol with 10 mM 3-(1-pyridino)-1-propanesulfonate (NDSB-201) to improve the protein solubility.

2.2. Crystallization

For crystallization, R-TBN1-N211D was concentrated to 2 mg ml⁻¹ in the storage buffer and 5'-AMP (Sigma-Aldrich) was added to a final concentration of 10 mM for co-crystallization. The hanging-drop vapour-diffusion method was used at 291 K with a 1:1 volume ratio of protein solution to reservoir solution in the drop (0.5 µl + 0.5 µl). The original crystallization reservoir solution, condition No. 2 from Crystal Screen 2 [0.1 M HEPES pH 7.5, 8%(v/v) ethylene glycol, 10%(w/v) PEG 8000; Hampton Research], was optimized to a final solution consisting of 0.1 M bis-tris pH 6.5, 10%(w/v) PEG 8000. Crystallization information is summarized in Table 2.

2.3. Structure solution and refinement

Crystals were equilibrated for 15 s in mother solution with the addition of glycerol to a concentration of 20%(v/v) as a cryoprotectant. The crystals were mounted in nylon Cryo-Loops (Hampton Research) and vitrified in liquid nitrogen.

Data collection was carried out at 100 K on beamline 14.1 at the BESSY II synchrotron-radiation source, Helmholtz-

Zentrum Berlin using a MAR CCD 225 detector and a mini-kappa goniometer (Mueller *et al.*, 2012). The data were processed using *XDS* (Kabsch, 2010*a,b*); the data statistics are summarized in Table 3. The structure was solved by molecular replacement using *MOLREP* (Vagin & Teplyakov, 2010) in the *CCP4* suite (Winn *et al.*, 2011) with PDB entry 4dj4 (Koval' *et al.*, 2013) chain *A* as a search model. The procedure resulted in a solution with an R_{cryst} of 0.395 and a contrast of 10.65 as defined in *MOLREP*.

Structure refinement involved iterative cycles of manual model building using *Coot* (Emsley *et al.*, 2010) according to $2mF_o - DF_c$ and $mF_o - DF_c$ Fourier maps and restrained refinement using *REFMAC5* (Murshudov *et al.*, 2011). R_{free} was used as a cross-validation statistic. The structure was validated using *MolProbity* (Chen *et al.*, 2010) and the set of validation tools in *Coot* (Emsley *et al.*, 2010). All figures were created using *PyMOL* (DeLano, 2004).

The structure was deposited in the PDB with accession code 4jdg. The structure-refinement statistics are summarized in

Table 3. The structure is referred to as TBN1-N211D-PO₄; the structure of the wild type (PDB entry 3sng) is referred to as TBN1-wt and a previous structure of the N211D mutant (PDB entry 4dj4) is referred to as TBN1-N211D.

3. Results and discussion

3.1. Structure of a mutant form of TBN1

TBN1 belongs to the phospholipase C/P1 nuclease superfamily (Pfam ID CL0368) and the S1/P1 nuclease family (Pfam ID PF02265) and shares its fold with other members of the family with known structure (Koval' *et al.*, 2013; Yu *et al.*, 2014; Romier *et al.*, 1998). The enzyme molecule consists of 11 α -helices, a 3_{10} -helix and a small β -sheet, and is stabilized by four disulfide bridges. Asn119 and Asn137 are N-glycosylated (Fig. 1). The modelled oligosaccharide chain connected to Asn119 consists of two units of *N*-acetylglucosamine (GlcNAc) and one unit of β -D-mannose; in the case of Asn137

Table 4
Zinc-cluster parameters of TBN1 (TBN1-N211D-PO₄, TBN1-N211D and TBN1-wt) and AtBFN2 from *A. thaliana* (AtBFN2-wt and AtBFN2-PO₄). The PDB code is given for each structure.

	TBN1-N211D-PO ₄ 4jdg	TBN1-wt 3sng	TBN1-N211D 4dj4 (chain A, B)	AtBFN2-wt 4cwm (chain A, B)	AtBFN2-PO ₄ 4cxv (chain A, B)
Zn–Zn2 (Å)	3.4	3.3	3.3, 3.3	3.4, 3.5	3.5, 3.6
Zn1–Zn3 (Å)	4.8	4.8	4.9, 4.9	5.0, 5.0	4.9, 4.9
Zn2–Zn3 (Å)	5.7	5.9	5.7, 6.0	5.7, 5.7	5.8, 5.8
Zn2–Zn1–Zn3 angle (°)	86.1	91.2	86.5, 92.2	84.1, 82.9	84.1, 84.9

Figure 1
Overall structure of TBN1-N211D-PO₄, shown in secondary-structure representation. α -Helices are in cyan, 3_{10} -helices in blue, β -sheets in magenta, unstructured loops in salmon, oligosaccharides (named by modified amino-acid residues) in green, phosphorus in orange and zinc ions in grey. The SDR-loop (residues 131–135) is shown in red.

Figure 2

(a) The active centre of TBN1-N211D-PO₄ with bound phosphate ion (phosphorus in orange). The omit $mF_o - DF_c$ Fourier map at the 3.5 r.m.s.d. level is shown in green. The zinc-coordinating residues are shown in green and Asp133' of the neighbouring molecule is shown in cyan. (b) The active centre of TBN1-N211D-PO₄ (cyan) superimposed (*LSQ* using zinc ions in *Coot*) with that of AtBFN2-PO₄ (magenta; PDB entry 4cxv, Yu *et al.*, 2014). Asp133' of the neighbouring TBN1-N211D-PO₄ molecule is shown in pale cyan. The phosphate ion is rotated by 48° around the P–O4 bond and the position of the P–O4 bond is shifted by 0.5 Å.

two units of *N*-acetylglucosamine (GlcNAc), one unit of β -D-mannose and one unit of α -D-mannose were modelled.

The active centre of TBN1 is formed by three Zn²⁺ ions. The zinc cluster forms an almost right-angled triangle (cluster parameters and a comparison are given in Table 4). The positions of the Zn²⁺ ions were confirmed by the anomalous signal. The zinc cluster is conserved in this group of zinc-dependent nucleases (Table 4), including the multifunctional

nuclease TBN1 (Koval' *et al.*, 2013), the multifunctional nuclease AtBFN2 (Yu *et al.*, 2014) and the single-strand-specific P1 nuclease (Romier *et al.*, 1998).

Compared with the previous structures of TBN1 (Koval' *et al.*, 2013), coordination of the zinc ions is conserved except for residue Asp70 (Zn2) and the newly occurring coordination by Asp133' (Zn3) from a symmetry-related molecule (symmetry operator $-y + 2/3, x - y + 1/3, z + 1/3$). While in the previous structures TBN1-wt and TBN1-N211D the O ^{δ 1} atom of Asp70 formed a weak coordination to Zn2 with a distance of 2.7 Å, in the reported structure this coordination is disappearing, with a distance of 3.1 Å. The side chain of Asp70 is the most flexible part of the active centre according to its high atomic displacement parameter. The O ^{δ 1} atom of Asp133' coordinates Zn3 with a distance of 2.3 Å and participates in the binding of the phosphate ion in the zinc cluster.

A peak in the difference $mF_o - DF_c$ Fourier map within the zinc cluster was interpreted as a phosphate ion (Fig. 2a). It is most likely that the phosphate ion originates from impurities in the added 5'-AMP (purity 97%, Sigma-Aldrich, St Louis, USA). However, 5'-AMP was not observed in the structure. It was not possible to confirm the presence of the phosphate ion from the anomalous signal. There were also no peaks of anomalous density observed at the sulfur sites.

The active centre of TBN1-N211D-PO₄ exhibits differences when compared with that of TBN1-N211D (Fig. 3). The distance between the carboxyl group of Asp185 and C ^{α} of Trp26 is increased, which opens space for the free rotation of the carboxyl group (both shown positions are stable in the refinement of TBN1-N211D-PO₄). Moreover, the distance of 3.0 Å suggests the possibility of the formation of a hydrogen bond between the carboxyl group of Asp185 and delocalized electrons of the 26–27 peptide bond and the N-terminal amine of Trp26.

A phosphate ion in the zinc cluster was observed in the structure of multifunctional nuclease type 2 from *Arabidopsis*

Figure 3

Superimposed active centres of TBN1 structures: TBN1-N211D (cyan) and TBN1-N211D-PO₄ (green) (*LSQ* using C ^{α} atoms of all residues in *Coot*). Zinc ions are shown as spheres, the zinc-binding residues and phosphate ion are shown as sticks and water molecules of TBN1-N211D are shown as small red spheres. Blue dashed lines show the distances between the Trp26 main chain (C and C ^{α}) of TBN1-N211D-PO₄ and Asp185 OD1 of TBN1-N211D-PO₄ (3.0 Å) and magenta dashed lines show those between the Trp26 main chain (C and C ^{α}) of TBN1-N211D and Asp185 OD1 of TBN1-N211D-PO₄ (2.6 Å). The Trp26 main-chain atoms are shifted by 0.5 Å between TBN1-N211D-PO₄ and TBN1-N211D. The zinc ions are shifted by 0.25 Å for Zn1, 0.26 Å for Zn2 and 0.4 Å for Zn3.

thaliana (AtBFN2; Yu *et al.*, 2014; PDB entry 4cxv; Fig. 2*b*; referred to as AtBFN2-PO₄). Here, the phosphate ion is rotated towards Zn3. Two O atoms of the phosphate ion are located in the plane defined by the zinc ions in TBN1-N211D-PO₄. The bonding distances of the phosphate ion in both structures are listed in Table 5 and the zinc-cluster parameters are compared in Table 4. A similar position of a phosphate ion in the zinc cluster was observed in a complex of phospholipase C from *B. cereus* with the substrate analogue (3*S*)-3,4-di-*N*-hexaoxyloxybutyl-1-phosphocholine (Antikainen *et al.*, 2003; PDB entry 1p6d; Fig. 4).

3.2. Crystal composition of TBN1 in known structures

In the reported structure of the deglycosylated mutant TBN1-N211D-PO₄, the molecules of TBN1 are packed into a new crystal form (Table 6) with some conserved crystal motifs in comparison to the previously solved structures of the wild type (PDB entry 3sng; referred to as TBN1-wt) and the deglycosylated N211D mutant (PDB entry 4dj4; referred to as TBN1-N211D). The molecules form superhelices in the crystals in all of the structures of TBN1 known to date (Fig. 5), which is caused by an interaction of the active centre of one molecule with a surface loop Ser132'-Asp133'-Arg134' (SDR-loop) of a neighbouring molecule (residues of the SDR-loop in symmetry-related molecules are marked with a prime). The superhelices are generated by crystal symmetry around the 3₁ axis.

However, the manner in which the superhelices pack with each other to form the crystal varies among the known structures. In TBN1-wt the orientation of two interacting superhelices is antiparallel and they are related by a crystallographic twofold. In TBN1-N211D and TBN1-N211D-PO₄ the orientation of the superhelices is parallel and there is no

Table 5

Bond distances (in Å) of phosphate-ion O atoms to zinc-cluster atoms and active-centre residues in TBN1-N211D-PO₄ and AtBFN2-PO₄ (PDB entry 4cxv; Yu *et al.*, 2014).

Residue numbers according to TBN1-N211D-PO₄ are in parentheses. Atom labelling is according to TBN1-N211D-PO₄.

	TBN1-N211D-PO ₄				AtBFN2-PO ₄			
	PO ₄ ³⁻ (601)				PO ₄ ³⁻ (401-A)			
	O1	O2	O3	O4	O1	O2	O3	O4
Zn1 (401)	2.3		2.3		3.3		1.8	
Zn2 (402)			2.7	2.2			2.2	2.1
Zn3 (403)	2.6				2.1			
Asp OD1 (70)		4.0	3.0	3.5		2.4	3.3	3.9
Asp OD2 (70)		3.8	2.2	4.3		3.2	3.3	3.0
Arg NE (73)		5.2		4.3				
Arg NH (73)		5.4		4.3				
Lys NZ (48) [†]						3.6		3.1

[†] The residue in AtBFN2-PO₄ in the place of Arg73 in TBN1-N211D-PO₄.

additional symmetry involved besides crystallographic translational symmetry; however, the mutual position of the superhelices differs (Fig. 6).

The putative asymmetrical enzyme dimers formed by the interaction of the SDR-loop with the active centre show only minor differences in their arrangement across the known structures. The mutual orientation of the two molecules within a dimer varies by less than 10°. Details of the interaction interfaces of the dimers and their variations are shown in Table 7.

Thus, in contrast to the conservation of the superhelix motif, the contact of the SDR-loop with the active centre differs in the structures (Fig. 7). Coordination of Zn3 is completed by Ser132' in TBN1-wt; however, Asp133' takes this role in TBN1-N211D and TBN1-N211D-PO₄. The interaction of Arg134' with Phe86 and Asp88 in the base-binding site

Figure 4
Comparison of the active centres of (a) TBN1-N211D-PO₄ with phosphate ion and the SDR-loop of a symmetry-related molecule (cyan) and (b) phospholipase C from *B. cereus* (Antikainen *et al.*, 2003) with (3*S*)-3,4-di-*N*-hexaoxyloxybutyl-1-phosphocholine (cyan; PDB entry 1p6d). Zinc-coordinating residues are shown as green sticks. P atoms are shown in orange. All of the zinc-binding residues are conserved except Asp70, which is substituted by Asn55 in phospholipase C. Arg73, which is involved in the catalytic mechanism (Koval' *et al.*, 2013), in TBN1-N211D-PO₄ and Phe66 in the same position in phospholipase C are shown in magenta. Notice the similarity in phosphate ion position and orientation.

Table 6

Parameters of the crystal structures TBN1-wt, TBN1-N211D and TBN1-N211D-PO₄.

	TBN1-wt	TBN1-N211D	TBN1-N211D-PO ₄
PDB code	3sng	4dj4	4jdg
Protein form	Wild type	N211D mutant	N211D mutant
Glycosylation	Full	Hypoglycosylated	Hypoglycosylated
Additional ligands	SO ₄ ²⁻ , bis-tris, Cl ⁻	Na ⁺ , Cl ⁻	PO ₄ ³⁻
Crystallization solution	1.0 M (NH ₄) ₂ SO ₄ , 0.1 M bis-tris pH 5.5, 1% (w/v) PEG 3350	0.16 M MgCl ₂ , 0.08 M Tris pH 8.5, 19% (w/v) PEG 3350, 3.3% (w/v) 1,4-butanediol	0.1 M bis-tris pH 6.5, 10% (w/v) PEG 8000; additive 10 mM 5'-AMP
Resolution (Å)	35–2.16	30–2.48	35–2.80
Space group	<i>P</i> ₃ ₁ ₂ ₁	<i>H</i> ₃	<i>H</i> ₃
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = 101.0, <i>c</i> = 71.5	<i>a</i> = <i>b</i> = 113.4, <i>c</i> = 138.4	<i>a</i> = <i>b</i> = 115.9, <i>c</i> = 74.7
No. of monomers per asymmetric unit	1	2	1

Figure 5

Superhelices of TBN1 in (a) TBN1-N211D-PO₄, (b) TBN1-N211D and (c) TBN1-wt. Top row, two neighbouring helices viewed perpendicular to the superhelix axes; arrows indicate the direction of the superhelices. Bottom row, packing of all superhelices in contact with the central (red) superhelix viewed along the superhelix axes. Colours distinguish different superhelices; shades of one colour distinguish the molecules in a superhelix.

Table 7
Variation of the dimer interface of TBN1, the key intermolecular interaction leading to the formation of superhelices, in crystals in known structures calculated by *PISA* (Krissinel & Henrick, 2007).

The percentage with respect to the whole TBN1 molecule is given in parentheses. BASA stands for buried accessible surface area.

	TBN1-N211D-PO ₄		TBN1-N211D		TBN1-wt	
	Active site	SDR-loop	Active site	SDR-loop	Active site	SDR-loop
No. of residues	28 (10.5%)	21 (7.9%)	26 (10.4%)	20 (7.5%)	30 (11.2%)	21 (7.9%)
No. of non-H atoms	95 (4.4%)	89 (4.1%)	99 (4.9%)	87 (4.1%)	100 (4.7%)	79 (3.7%)
BASA (Å ²)	792 (6.3%)	837 (6.6%)	762 (6.4%)	833 (6.7%)	715 (5.6%)	802 (6.3%)

Figure 6
Illustration of differences in superhelix packing details: comparison of the crystal contacts in TBN1-N211D (cyan) and TBN1-N211D-PO₄ (red) which involve Arg175. Crystals are superimposed by the molecules labelled A. A and B refer to chains in the TBN1-N211D structure. The distance between the C α atoms of Asp207 in the left panel is 11.6 Å. The distance between the C α atoms of Arg175 in the right panel is 9.7 Å.

Figure 7
Comparison of the interaction of the SDR-loop (right, in sticks, residues denoted by primes) with the active centre (left) in the TBN1-wt (a), TBN1-N211D (b) and TBN1-N211D-PO₄ (c) structures. The main chain is shown in cartoon representation with colours and diameters according to the values of the atomic displacement parameters of the C α atoms (low, blue and thin; high, red and thick).

remains the same as in the previously reported structures of TBN1. The currently reported structure provides an observation of the most orderly localized SDR-loop. In the TBN1-wt and TBN1-N211D structures the SDR-loop has high atomic displacement parameters and is even not localized in chain B of TBN1-N211D. Hence, we propose that the presence of the phosphate ion in the active centre may stabilize the interaction, but it is not crucial for the formation of the contact and the superhelices.

Every molecule in TBN1-wt is surrounded by four other protein molecules, whereas every molecule in TBN1-N211D and TBN1-N211D-PO₄ is surrounded by six other molecules. However, the contact causing the formation of the superhelices, *i.e.* the contact involving the SDR-loop, is the most significant (buried accessible surface area of about 800 Å² as calculated by PISA; Krissinel & Henrick, 2007), in contrast to other relatively weaker contacts (buried accessible surface area of about 100 Å²); these weaker contacts mediate the mutual packing of superhelices into the crystal.

4. Conclusion

The third reported crystal structure of the enzyme TBN1 confirms that the superhelices, which are the main motif in the crystal composition, persist in all of the different crystal forms. The binding of the surface SDR-loop of one molecule to the active centre of a neighbouring molecule, present in all known structures of this enzyme, suggests that this intermolecular contact is not a crystallization artefact, as might be perceived. Instead, it may have a biological role. In the presented structure, a phosphate anion is bound to the zinc cluster in the active centre, which leads to better localization (lower atomic displacement parameters) of the SDR-loop. Phosphate binding to the zinc cluster has been observed for other related enzymes, but here it takes a special place. The previous results (Koval' *et al.*, 2013) are now corroborated by the crystallographic data. The superhelices can also form without phosphate, but we propose that the presence and binding of phosphate stabilizes their formation, which is most likely to lie behind enzyme aggregation in certain conditions. The observed interactions may represent an important regulatory process controlled by phosphate abundance and phosphate starvation, which is known to be correlated with senescence processes.

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References

- Antikainen, N., Monzingo, A., Franklin, C., Robertus, J. & Martin, S. (2003). *Arch. Biochem. Biophys.* **417**, 81–86.
- Chen, V. B., Arendall, W. B., Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S. & Richardson, D. C. (2010). *Acta Cryst. D* **66**, 12–21.
- DeLano, W. (2004). *Abstr. Pap. Am. Chem. Soc.* **228**, 030-CHED.
- Dohnálek, J., Koval', T., Lipovová, P., Podzimek, T. & Matoušek, J. (2011). *J. Synchrotron Rad.* **18**, 29–30.
- Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). *Acta Cryst. D* **66**, 486–501.
- Kabsch, W. (2010a). *Acta Cryst. D* **66**, 133–144.
- Kabsch, W. (2010b). *Acta Cryst. D* **66**, 125–132.
- Koval', T., Lipovová, P., Podzimek, T., Matoušek, J., Dušková, J., Skálová, T., Štěpánková, A., Hašek, J. & Dohnálek, J. (2013). *Acta Cryst. D* **69**, 213–226.
- Krissinel, E. & Henrick, K. (2007). *J. Mol. Biol.* **372**, 774–797.
- Lipovova, P., Podzimek, T., Orctova, L., Matousek, J., Pouckova, P., Soucek, J. & Matousek, J. (2008). *Neoplasma*, **55**, 158–164.
- Matoušek, J., Kozlová, P., Orctová, L., Schmitz, A., Pešina, K., Bannach, O., Diermann, N., Steger, G. & Riesner, D. (2007). *Biol. Chem.* **388**, 1–13.
- Matoušek, J., Podzimek, T., Poučková, P., Orctová, L., Souček, J., Škvor, J., Lipovová, P. & Matoušek, J. (2008). *Anticancer Res.* **28**, 3399.
- Matousek, J., Podzimek, T., Pouckova, P., Stehlik, J., Škvor, J., Lipovova, P. & Matousek, J. (2010). *Neoplasma*, **57**, 339–348.
- Matoušek, J., Podzimek, T., Poučková, P., Stehlik, J., Škvor, J., Souček, J. & Matoušek, J. (2009). *Oncol. Res.* **18**, 163–171.
- Mueller, U., Darowski, N., Fuchs, M. R., Förster, R., Hellmig, M., Paithankar, K. S., Pühringer, S., Steffien, M., Zocher, G. & Weiss, M. S. (2012). *J. Synchrotron Rad.* **19**, 442–449.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst. D* **67**, 355–367.
- Podzimek, T., Matoušek, J., Lipovová, P., Poučková, P., Spiwok, V. & Šantrůček, J. (2011). *Plant Sci.* **180**, 343–351.
- Romier, C., Dominguez, R., Lahm, A., Dahl, O. & Suck, D. (1998). *Proteins*, **32**, 414–424.
- Vagin, A. & Teplyakov, A. (2010). *Acta Cryst. D* **66**, 22–25.
- Winn, M. D. *et al.* (2011). *Acta Cryst. D* **67**, 235–242.
- Yu, T.-F., Maestre-Reyna, M., Ko, C.-Y., Ko, T.-P., Sun, Y.-J., Lin, T.-Y., Shaw, J.-F. & Wang, A. H.-J. (2014). *PLoS One*, **9**, e105821.