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Crystal structure of the protein from *Arabidopsis thaliana* gene At5g06450, a putative DnaQ-like exonuclease domain-containing protein with homohexameric assembly

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Abstract

Arabidopsis thaliana gene At5g06450 encodes a putative DnaQ-like 3'-5' exonuclease domain-containing protein (AtDECP). The DnaQ-like 3'-5' exonuclease domain is often found as a proofreading domain of DNA polymerases. The overall structure of AtDECP adopts an RNase H fold that consists of a mixed β -sheet flanked by α -helices. Interestingly, AtDECP forms a homohexameric assembly with a central 6-fold symmetry, generating a central cavity. The ring-shaped structure and comparison with WRN-exo, the best structural homologue of AtDECP, suggest a possible mechanism for implementing its exonuclease activity using positively charged patch on the N-terminal side of the homohexameric assembly. The homohexameric structure of AtDECP provides unique information about the interaction between the DnaQ-like 3'-5' exonuclease and its substrate nucleic acids.

Keywords

3'-5' exonuclease; DnaQ-like exonuclease family; *Arabidopsis thaliana*; AtDECP; homohexamer; crystal structure

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Introduction

DNA damage occurs continuously in normal cellular processes and must be precisely managed to maintain genome integrity. Nucleases are enzymes that cleave nucleic acids and participate in DNA repair at checkpoints and initiate the signaling cascade to activate checkpoint leading to cell cycle arrest.¹ Nucleases can be classified into endonucleases and exonucleases depending on whether they cleave nucleic acids internally (endonuclease) or from the ends (exonuclease). In contrast to endonucleases which tend to interact with DNA via major grooves in a sequence-specific way, exonucleases interact with DNA minor grooves in a non-specific way.¹ Exonucleases are vital components of many cellular processes such as replication, recombination, repair, turnover, and processing. For example, mutations of nucleases such as Mre11 and WRN have been known to be detrimental and play crucial roles in cancer-prone autosomal disorders.¹⁻³ The DnaQ-like 3'-5' exonuclease family is often found in proofreading domains of DNA polymerases which allow the enzyme to check each nucleotide during DNA synthesis and to excise mismatched nucleotides in the 3' to 5' direction.⁴⁻⁵

At5g06450 (UniProtKB:Q9FNG3) has been annotated from the SCOP program⁶ as a putative DnaQ-like 3'-5' exonuclease domain-containing protein (AtDECP), which contains all three characteristic sequence motifs, ExoI, II, and III [Fig. 1(A)].⁷ The DnaQ-like 3'-5' exonuclease family is also known as the DEDD family after four acidic residues, Asp-Glu-Asp-Asp, present in the catalytic sites of its members and catalyzing the degradation of the 3'-ends of nucleic acids.^{3,8-9}

In this work, we determined the crystal structure of AtDECP, a putative DnaQ-like 3'-5' exonuclease domain-containing protein. The ring-shaped, homo-hexameric structure has a central cavity which is wide enough to accommodate dsDNA. The overall architecture of AtDECP shows high structural similarities to the DEDD members such as the exonuclease domain of WRN (WRN-exo) which is a member of RecQ DNA helicase family, RNase D, and Klenow fragment of DNA polymerase I from *E. coli*. The crystal structure of AtDECP provides unique information about the mode of interaction between DEDD and its substrate nucleic acids.

Materials and Methods

Cloning, protein expression, and purification

Native and SeMet-labeled AtDECP were cloned, expressed, purified and analyzed following the standard Center for Eukaryotic Structural Genomics pipeline protocols described in detail elsewhere.¹⁰⁻¹³ In short, the cDNA encoding AtDECP was cloned into a Pvp33K plasmid encoding a His₆-maltose binding protein (MBP) tag. Protein was expressed in *Escherichia coli* B834(DE3)p(LacI+RARE) cells using 2 L of self-inducing media. The cells were harvested and disrupted by sonication. The cell lysates were cleared by centrifugation and the supernatant fraction was purified via nickel affinity chromatography. TEV protease was used to cleave the affinity/solubility tag and the cleavage of the tag was verified by lack of product binding to a nickel column. Purified protein was applied to a final desalting step and protein was concentrated to 10 mg ml⁻¹.

For exonuclease activity assay and dsDNA binding assay, His₆-tagged AtDECP was cloned into a pET-15(b) plasmid (Novagen), expressed in *E. coli* BL21-CodonPlus (DE3)-RIPL cells (Agilent Technologies), and purified using four step purification; affinity chromatography (HiTrap chelating column, GE Healthcare), size-exclusion chromatography (Superdex200 16/600 prep grade, GE Healthcare), ion exchange chromatography (HiTrap Q column, GE Healthcare), and size-exclusion chromatography (Superdex200 16/600 prep grade, GE Healthcare). Purified protein was concentrated to 2 mg ml⁻¹.

Crystallization, data collection, and structure determination

Crystals of AtDECP were grown at 277 K by the hanging-drop vapor diffusion method from 10 mg ml⁻¹ protein solution in buffer (50 mM NaCl, 3 mM NaN₃, 0.3 mM TCEP, 5 mM Bis-Tris pH 6.0) mixed with an equal amount of well solution containing sodium citrate, polyethylene glycol 2000, and HEPES pH 8.0. Crystals typically grew as tiny thin half-moon-shaped plates (100 × 30 × 10 μm). Crystals were cryoprotected for further data collection at 277K by soaking in well solutions supplemented with increasing concentrations of glycerol up to a final concentration of 30% and were flash-frozen in liquid nitrogen at 100K.

X-ray diffraction data were collected at synchrotron beamline BioCARS 14-BM-D at the Advanced Photon Source of the Argonne National Laboratory. The diffraction images were integrated and scaled using HKL2000.¹⁴ The crystals of AtDECP belong to the space group *P*3₂21, with unit-cell parameters *a*=*b*=120.83, *c*=185.22 Å, α=β=90°, and γ=120°. The selenium substructure of SeMet-labeled AtDECP crystal was determined using Hyss¹⁵ and SHELXD¹⁶. The protein structure was phased and the initial phase information was further improved by electron-density modification using MAD data in autoSHARP. The automatic tracing procedure of ARP/wARP¹⁷ produced an initial model with approximately 80% residues placed, of which 60% had side chains assigned. The structure was completed using alternate cycles of manual building in Xfit¹⁸ and COOT¹⁹, and refinement in REFMAC5.²⁰ All refinement steps were monitored using an *R*_{free} value based on 5.0% of the independent reflections. *R* and *R*_{free} values of the final model were 18.3% and 23.3%, respectively, for the 34.92–2.10 Å data. The stereochemical quality of the final model was assessed using PROCHECK²¹ and MolProbity²². The data collection and refinement statistics are summarized in Table I.

Exonuclease activity assay and dsDNA binding assay

The exonuclease activity of AtDECP was tested by using MALDI-TOF mass spectrometer. 3'-overhang dsDNA, gapped dsDNA by one nucleotide, and 5'-overhang dsDNA were used as substrates in the exonuclease assay. For dsDNA binding assay, AtDECP was incubated with 3'-overhang, blunt-end, or 5'-overhang dsDNA with or without divalent metal ions (Mg²⁺ or Mn²⁺) at 37 °C for 1 hours, and the sample was electrophoretically eluted on 15% native polyacrylamide gel (PAGE). More detailed materials and methods are described in the Supplementary Information.

Data deposition

Atomic coordinates and structure factors have been deposited in the RCSB Protein Data Bank (accession code 1VK0).

Results and Discussion

Overall structure of AtDECP

The final model of AtDECP comprises eleven α -helices (α A to α K) and seven β -strands (β 1- β 7). The structure has an RNase H fold that consists of mixed β -sheet (β 1 $\downarrow$ - β 2 $\uparrow$ - β 5 $\uparrow$ - β 4 $\downarrow$ - β 3 $\uparrow$ - β 6 $\uparrow$ - β 7 $\uparrow$) flanked by α -helices. AtDECP monomers are composed of 200 amino acid residues and 6 residues (Pro60-Lys65) were disordered in the crystal and could not be modeled. Blast search results with AtDECP sequence show that 3'-5' exonucleases and WRN-exo from various organisms are the best homologous proteins to AtDECP. Representative residues for Exo I, II, and III motifs in AtDECP are also structurally well conserved with crystal structures of other DEDD proteins such as the exonuclease domain of WRN (WRN-exo), RNase D, and Klenow fragment of DNA polymerase I from *E. coli* with low sequence identity of 17%, 18%, and 15%, respectively [Fig. 1].

A distinctive feature of the AtDECP structure, as in the DnaQ-like 3'-5' exonuclease family, is that the protein adopts a homohexameric structure, with a buried surface area of 16,780 Å² [Fig. 1(C)] corresponding to approximately 37.4% of the entire hexamer solvent accessible area (44,900 Å²) calculated from the Protein Interfaces, Surfaces and Assemblies service (PISA).²³ The hexamer interface is mediated mainly through the formation of salt bridges (Lys107-Asp52, Arg124-Glu118, Arg132-Asp119, Lys107-Asp119, Arg124-Glu125, Arg47-Asp171, Arg47-Glu174, Arg38-Glu178, and Asp205-Arg161) and hydrogen bonds (Arg132-Val53, Arg124-Thr68, Lys107-Val114, Thr110-Phe152, Gly128-Trp71, Thr110-Gly154, Lys107-Thr155, and Ser204-Arg161) [Fig. 2(C)]. Notably, the homohexameric assembly structure forms central cavity with a narrowest diameter about 21 Å, which is wide enough to accommodate dsDNA. Even though the exact mechanism remains to be elucidated, this ring structure suggests a way in which AtDECP assembly implements specificity regarding DNA, which will be further discussed with a suggested DNA binding mode below.

Structural comparison with known DEDD

The DEDD family shares a conserved RNase H fold which is characterized as a central mixed β -sheet surrounded by several alpha helices. The best-studied DEDD is Klenow fragment of DNA polymerase I from *E. coli*, which cleaves dsDNA from 3'-end to 5'-end and confers proofreading function to ensure genome integrity during DNA replication.⁴ DEDD is characterized by the signature sequence of Asp-Glu-Asp-Asp and it is further classified into DEDDy or DEDDh exonucleases depending on the variation of ExoIII, which contains the last Asp of DEDD, as YX_{3~4}D or HX_{3~4}D, respectively.⁹ However, the significance of the differences in this motif is not well understood.²⁴ As a predicted member of the DnaQ-like exonuclease family, AtDECP contains a modified signature sequence of DYDE instead of DEDD in its catalytic core and Glu187 immediately precedes the last E of DYDE by 5 residues (EX₄E) [Fig. 1(A)]. While most DEDD proteins have been known to

show two-metal-ion-dependent nuclease activity, we could not observe metal ions at its active site of AtDECP, but many static water molecules exist at the active site and this may result from limitations of *in vitro* overexpression using *E. coli*.

When a structural homolog search was performed using the DALI server²⁵, a network service for comparing protein structures, WRN-exo (PDB ID:2FC0), a eukaryotic DEDD, turned out to be the closest structural homolog of AtDECP, with a root mean square deviation (r.m.s.d.) of 2.6 Å for 164 equivalent Ca positions, a Z-score of 18, and a sequence identity of only 17% [Fig. 1(A)]. WRN contains a unique 3'-5' exonuclease domain (WRN-exo) in the N-terminal region, followed by stretches of acidic amino acids, a conserved RecQ helicase domain, and C-terminal nuclear localization signal.²⁶ Even though WRN-exo was designated as a monomer in its crystal structure, the full length WRN has been known to form a multimeric assembly^{3,27}, which is consistent with the hexameric assembly of AtDECP. Our AtDECP crystal structure reveals an interesting homo-hexameric structure, which makes it apparently unique among DEDD family members, yet it is plausible that oligomeric status would be needed for the proper function in *Arabidopsis thaliana*.

Homo-hexameric assembly and potential interaction with nucleic acids

There have been many reports of ring-structured dsDNA binding proteins such as PCNA which interact with dsDNA via their central cavity.²⁸⁻²⁹ The crystal structure of WRN-exo in complex with dGMP, the best structural homolog of AtDECP, showed that it exploits Trp145 located in cavity to bind to dGMP, which also implicates that dsDNA would interact with the protein using the central cavity.³ The central cavity of AtDECP is ~21 Å wide in the narrowest part and it is wide enough to accommodate dsDNA. Considering the features of WRN-exo that recognizes dsDNA in a non-specific way, AtDECP may interact with minor groove of dsDNA. However, AtDECP contains Asp121 in the equivalent position of Trp145 in WRN-exo and the α -helix α B, which is directly connected to the structurally disordered part and faces the central cavity, blocking the way to the active site from the central cavity [Fig. 1]. So, for the proper exonuclease activity of AtDECP through the central cavity, a conformational change of α B needs to occur, which is not a rare event in dynamic regions.

Since AtDECP has been annotated as a member of DEDD from sequence-based prediction and further supported by our structural analysis, we implemented exonuclease activity assay using MALDI-TOF MS analysis, which detects cleaved nucleotides, and dsDNA binding assay after co-incubation of AtDECP with different types of dsDNA. However, we could not observe either cleaved nucleotides or gel shift from AtDECP-dsDNA interaction (Supplementary Information). In many DEDD members, 3'-5' exonuclease activity has been shown in coordination with other DNA-related activities from domains in the same protein; polymerase activity or helicase activity for Klenow fragment or WRN-exo, respectively. In addition, many nucleases form protein complexes with other proteins for proper function.⁹ Furthermore, it has been shown that the DNA end-binding Ku70/80 protein specifically stimulates the exonuclease activity of WRN-exo.^{3,30-31} In this context, AtDECP may also need additional interacting partners to accomplish its function. In addition, more

experiments with other nucleic acids such as single-stranded DNA or RNA would be needed to elucidate the actual function of AtDECP.

Interestingly, the electrostatic potential surface is unevenly distributed. One side of the ring-structured hexamer with C-terminus on top is dominantly negative while the other side contains unique positively charged patch leading to the active site which starts from the structurally disordered part [Fig. 2(A-B)]. Considering that the C-terminal sequence of the structurally disordered part PETETK which is directly connected to α B, the positive charge in the patch would be more intensified and stabilize interacting nucleic acids. To our surprise, the positively charged patch generated from one AtDECP monomer reaches to the active site from the other monomer in the interface. Thus, the interface from each monomer would cooperatively contribute to the catalytic activity and this may account for the importance of homohexameric assembly of AtDECP. During purification of His₆-tagged AtDECP, we consistently observed both hexameric and monomeric AtDECP fractions from size-exclusion chromatography after initial Ni-NTA affinity chromatography. Interestingly, the monomeric fraction almost disappeared after subsequent ion-exchange chromatography and hexameric fraction clearly appeared (Supplementary Information), which shows *in vitro* monomer-hexamer transition of AtDECP and implicates the possibility of the physiological hexameric assembly reconstitution in time of enhanced functional needs.

In addition, Trp177 in α J located around the entrance of active site is strictly conserved among representative 3'-5' exonucleases such as RNase D and WRN-exo domains from various organisms [Fig. 1(A) and 2(D)]. In the case of Klenow fragment, the equivalent residue of Trp177 in AtDECP is Phe which is also able to stabilize DNA bases via stacking interaction. Based on our structural data analysis, we suggest that the catalytic activity of AtDECP could be enhanced by formation of the homohexameric assembly which cooperatively provides unique environment around the canonical DEDD active site. The unique homohexameric structure of AtDECP would be further used to extend our knowledge regarding DNA binding proteins that form oligomeric assembly for physiological function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DEDD	Asp-Glu-Asp protein or DnaQ-like 3'-5' exonuclease family
dsDNA	double-stranded DNA
MBP	maltose binding protein
PDB	Protein Data Bank

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Figure 1.

Overall structure of AtDECP. (A) Sequence alignment with AtDECP and other proteins similar in amino acid sequence (name in green) and three-dimensional structure (name in blue). *C. melo*, *P. prop*, *M. trun*, and *H. spns* represent *Cucumis melo*, *Paludibacter propionigenes* WB4, *Medicago truncatula*, and *Homo sapiens*, respectively. (B) Monomer structure of AtDECP. α -helices, β -strands, and loops are colored in cyan, green, and pink, respectively. The representative amino acid residues in the active site are shown as stick model (carbon and oxygen in yellow and red color, respectively). The structurally disordered loop region is denoted by a dotted line. (C) Homohexameric structure of AtDECP. Each monomer is represented in different colors; red, orange, magenta, pale cyan, blue, and cyan, respectively.

Figure 2.

Electrostatic potential surface and the positively charged patch of AtDECP. Electrostatic potential surface of N-terminal face containing positively charged patch (A) and C-terminal face with dominant negative charges (B). The electrostatic potential surface was calculated using APBS tools³² in PyMol³³. (C) Interface between AtDECP monomers. Amino acid residues involved in the interaction are shown in stick model and oxygen, nitrogen, and carbon atoms from each monomer are represented in red, blue, green, and marine, respectively. Salt bridges and hydrogen bonds are denoted by dotted lines in red. (D)

Magnified view of the positively charged patch and active site. The structurally disordered loop region is denoted by a dotted line.

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Table I

Statistics for data collection and model refinement

Data collection ^a		AtDECP	
Space group		<i>P3₂21</i>	
Cell dimensions			
a, b, c (Å)		120.83, 120.83, 185.22	
α, β, γ (°)		90, 90, 120	
Data set	Se λ1 (edge)	Se λ2 (peak)	Se λ2 (remote)
X-ray wavelength (Å)	0.9800	0.9792	0.9641
Resolution (Å) ^b	50.00–2.15 (2.20–2.15)	50.00–2.10 (2.15–2.10)	50.0–2.10 (2.15–2.10)
Total / unique reflections	945,075 / 164,314	1,014,369 / 176,555	1,016,566 / 176,282
Completeness (%)	100 (100)	100 (99.9)	100 (99.9)
<i>R</i> _{merge} (%) ^c	6.0 (41.8)	6.0 (51.0)	9.2 (89.0)

Refinement	Data set: Se λ2 (peak)
Resolution (Å)	34.92–2.10
<i>R</i> _{work} ^d / <i>R</i> _{free} ^e	0.183 / 0.233
No. of protein atoms / mean B-factor (Å ²)	9,546 ^f / 30.26
No. of solvent atoms / mean B-factor (Å ²)	645 / 33.96
Ramachandran plot analysis (for Chain A)	
Most favored regions	196 (100%)
Additional allowed regions	0 (0%)
Disallowed regions	0 (0%)
R.m.s. deviations from ideal geometry	
Bond lengths (Å)	0.023
Bond angles (°)	1.777

^aData collected at the synchrotron beamline BIOCARS 14-BM-D of the Advanced Photon Source.^bNumbers in parentheses indicate the highest resolution shell of 15.^c $R_{merge} = \sum_h \sum_i |I(h)_i - \langle I(h) \rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the observed intensity of reflection h , and $\langle I(h) \rangle$ is the average intensity obtained from multiple measurements.^d $R_{work} = \sum ||F_o| - |F_c|| / \sum |F_o|$, where $|F_o|$ is the observed structure factor amplitude and $|F_c|$ is the calculated structure factor amplitude.^e R_{free} = R-factor based on 5.0% of the data excluded from refinement.^fNumber of non-hydrogen protein atoms included in refinement.