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STRUCTURE NOTE

Crystal structure solution of a ParB-like nuclease at atomic resolution

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INTRODUCTION

The DNA segregation apparatus of the eukaryotes is well characterized when compared to prokaryotes and archea, where the components of the machinery and mechanism of segregation is largely unknown. Par (partitioning) B nucleases† were initially identified as critical elements of a mechanism involved in the faithful partitioning of plasmid DNA during cell division in the absence of selection pressure.[1,2] Subsequently, a number of similar proteins have been identified in prokaryotes and archea carrying out the function of segregation of genomic DNA during cell division. ParB homologs are present in almost all eubacteria. Most of the available information on the segregation of DNA has originated from the studies carried out in *Escherichia coli*,[3] *Bacillus subtilis*,[4] and *Caulobacter crescentus*.[5] The studies implicate ParA and ParB proteins and their homologs in the DNA segregation process. Mutagenesis studies in *C. crescentus* have indicated ParB to be critical for events preceding cytokinesis. Null mutations of ParB blocked cell division.[6]

At present, no structural information is available on any of the Par proteins known to be involved in the segregation of DNA. To delineate the structural basis for the function of ParB proteins, we cloned a 26 kD ParB-like nuclease from *Pyrococcus furiosus*. The nuclease could be crystallized only after extensive purification followed by chemical modification. The present work describes the structure of the nuclease solved at atomic resolution. Based on the position of the ligands in the structure, a mechanism for the cleavage of the phosphodiester bond is proposed. The ability of the nuclease to degrade DNA was validated successfully.

MATERIALS AND METHODS

Protein production

A 26 kD ParB-like nuclease was amplified from the genomic DNA of *Pyrococcus furiosus* using PCR and cloned into pET 28a vector (Novagen, USA) as described before.[7] The gene was expressed with a N-terminal hexa Histidine tag. Protein was purified using Ni-affinity followed by anion exchange chromatography using DEAE Sepharose FF column (Amersham Biosciences, USA). DNA was removed by subjecting the protein to hydroxy-

†Atomic co-ordinates and structure factors for the structure reported in the current study have been deposited in the Protein Data Bank under accession code 1VK1.

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apatite chromatography. A final size exclusion chromatography on Sephadex G-75 column (Amersham Biosciences, USA) resulted in a pure, homogenous protein preparation when tested by SDS PAGE and Dynamic Light Scattering (Protein Solutions, USA).

Chemical modification

The nuclease was chemically modified by using a reductive methylation of surface lysines protocol described previously.[8] Excess chemicals were removed by size exclusion chromatography using a Sephadex G-75 column. After concentration, the methylated protein had a formulation similar to the native protein described previously. A 20 mg/mL methylated protein in 20 m*M* Tris-HCl, 300 m*M* NaCl, pH 8.0, was used for crystallization.

Specific activity

The nuclease activity of the ParB protein was determined by dilution method as described previously.[9] One unit of enzyme activity was defined as the amount of protein required to completely digest 1 μg of pMD-18T plasmid in 60 min at 37°C. The reaction buffer consisted of 50 m*M* Tris-HCl, pH 8.5, 10 m*M* $CaCl_2$, 100 m*M* NaCl, and 1 m*M* DTT. Protein concentration was estimated by measuring the absorbance at 280 nm.[10] An extinction coefficient of 28,590 M^{-1} cm^{-1} was used to calculate the amount of protein in mg mL^{-1}.

Crystallization

Native (nonmethylated protein) and methylated protein were set up for crystallization in microbatch mode as described[11] under identical conditions. A total of 288 commercial sparse matrix screen conditions (Crystal Screen I, Crystal Screen II, MEMFAC, and PEG Ion from Hampton Research, USA; Wizard I and II from DeCode Genetics, USA) were used in the initial screening experiments. All crystallization trials were incubated at 18°C and observed periodically. Diffraction quality crystals were produced from methylated protein using a precipitant solution consisting of 600 m*M* sodium di-hydrogen phosphate, 2.4*M* di-potassium hydrogen phosphate, 200 m*M* sodium chloride, 100 m*M* HEPES, pH 7.3.

Data collection

The crystals were frozen in liquid nitrogen using a solution containing mother liquor with 25% glycerol as cryo protectant. Derivatives were prepared by addition of a small grain of K_2PtCl_4 to the crystallization drop and subsequent incubation at room temperature for 2 h prior to mounting. Data was collected under cooling at −173°C (Table I) and processed with the HKL2000 suite.[12]

Table I
Data Collection and Refinement Statistics

Dataset	1	2
Derivatization	K_2PtCl_4	Native
X-ray source	RU-H3R	APS 22ID
Detector	R-AXIS 4	MAR CCD 225
Crystal-to-detector distance (mm)	122.5	100.0, 130.0
Wavelength (Å)	1.54	1.07
Number of images	360	360, 180
Oscillation width (°)	1.0	0.5, 1.0
Space group	C2	C2
Unit cell parameters		
a (Å)	115.24	115.15
b (Å)	40.08	40.10
c (Å)	66.02	65.67
β (°)	122.32	122.29
Resolution range (highest resolution shell, Å)	11.00–2.30 (2.38–2.30)	50.00–1.20 (1.24–1.20)
Completeness (%)	72.3 (7.0)	80.5 (21.5)
Redundancy	7.1 (2.6)	2.4 (1.1)
*R*sym (%)	8.5 (17.0)	4.0 (14.0)
I/σ	39.5 (9.8)	34.3 (4.1)
Refinement		
Resolution limits	50.00–1.20	
Number of refined atoms	2015	
*R*work (*R*free, %)	16.5 (17.5)	
Bond RMSD lengths (Å)/angles (°)	0.014/1.3	
Mean *B* value (Å^2)	14.7	
All-atom clash score	1.88	
Ramachandran favored	217/221 (no outliers)	

Structure solution and refinement

The initial phases were determined with the Sca2Structure pipeline[13,14] using the K_2PtCl_4-derivatized crystal (dataset 1) data. Phases were further improved with RESOLVE.[15] The phases were merged with the structure factor amplitudes of the native dataset 2 and an initial model was built with the program ARP/wARP.[16] Rebuilding and refinement were carried out with the programs XFIT[17] and REFMAC,[18] respectively. The structure was validated with MOLPROBITY[19] and PROCHECK[20] before deposition with PDB_EXTRACT to the Protein Data Bank.[21]

RESULTS

Structure of the ParB nuclease

The crystal structure of the ParB nuclease was solved by SAD phasing. Three heavy atom sites were identified by SOLVE using the Sca2Structure pipeline. An initial experimental electron density map calculated at 2.0 Å was of extremely high quality as 188 of the 221 residues (85%) could be fitted into the map by ARP/wARP. The structure was refined to 1.2 Å resolution with the final refined model having an *R*-value of 16.5% (*R*-free =

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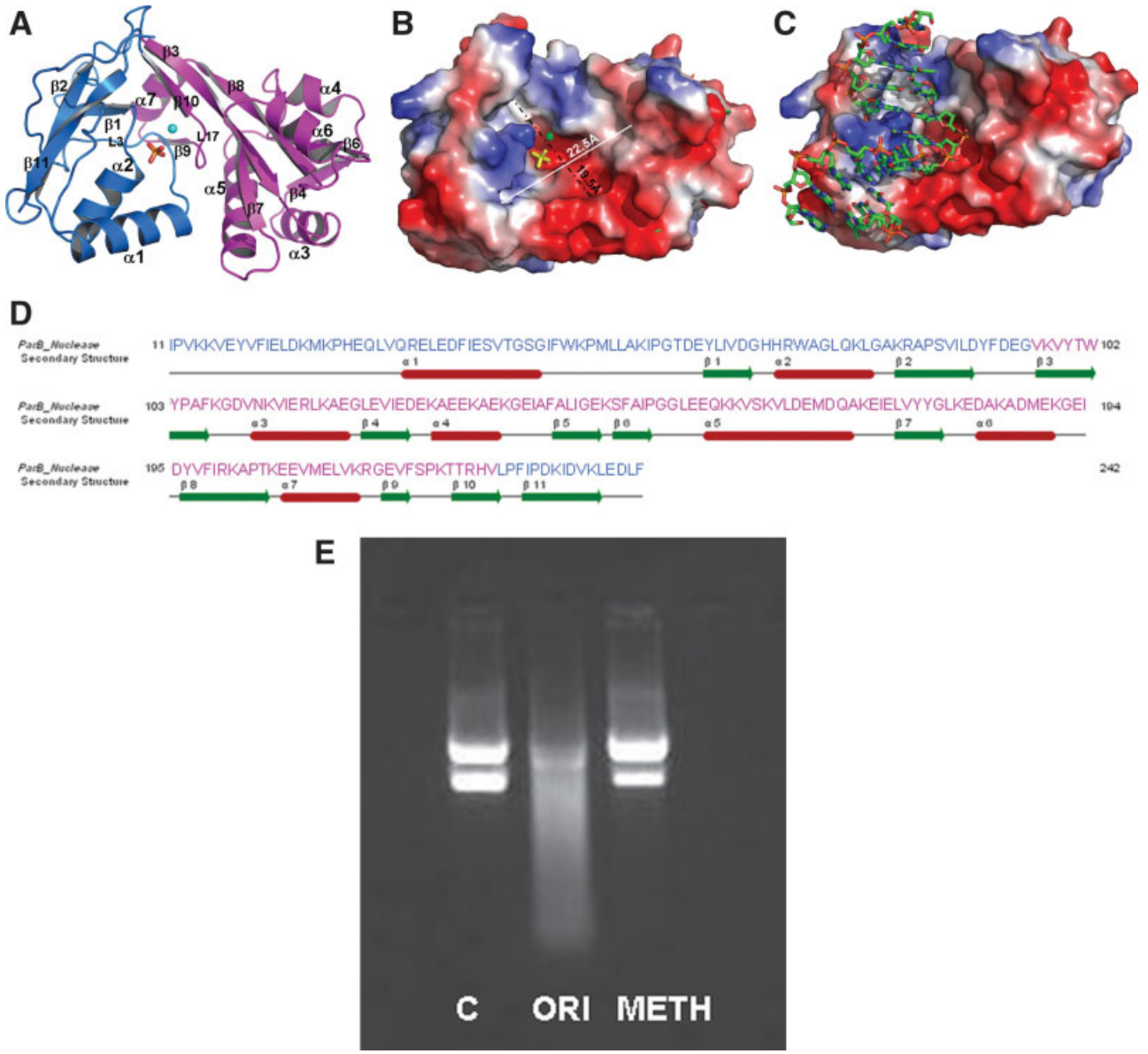

Figure 1

*Overall structure of ParB nuclease. (**A**) A cartoon representation of the ParB nuclease structure. The nuclease is made up of seven helices, eleven β-sheets, and numerous loops. The structure could be divided into two domains (colored blue and magenta) according to CATH analysis. (**B**) A surface electrostatic potential representation of the structure showing the cleft region. The phosphate ligand is buried in a tunnel formed inside the positively charged (blue) cleft. Negative potentials are colored red. The phosphate ligand is shown as sticks and the Ca is represented as a sphere. (**C**) Putative active site of the ParB-like nuclease with DNA from Eco RV structure (1RVA) superimposed over the cleft region. (**D**) The primary amino acid sequence of the ParB nuclease annotated with secondary structural elements. Residues making up Domain I are colored blue, while residues forming Domain II are colored magenta. (**E**) pMD18-T plasmid was treated with purified ParB nuclease (ORI) and the methylated variant (METH) for 60 min at 37°C in a 50 mM Tris-HCl, pH 8.5 buffer, containing 10 mM $CaCl_2$, 100 mM NaCl, and 1 mM DTT. A control (C) was set up under identical conditions without the protein. The plasmid was degraded by the ParB nuclease.*

17.5%). The Ramachandran plot analysis revealed 217 of the 221 residues were in the most favored region. The data collection and refinement statistics are listed in Table I. Density for the first ten amino acids at the N-terminus was not observed, which is consistent with the PONDR® VL-XT analysis of the primary sequence indicating the region to be disordered.

The overall structure consists of seven helices and eleven β-sheets with nineteen loops [Fig. 1(A)]. The structure was classified as mixed αβ with two-layer sandwich architecture according to CATH.[22] Although the topology is similar to other proteins, the structure of the ParB-like nuclease represents a new superfamily. The CATH analysis identified two domains for the protein

with interdigitated secondary structural elements [Fig. 1(A,D)]. Domain I is 101 amino acids long and is made up of two sequence segments. The first segment consists of residues 11 to 96, while the second segment stretches from residues 227 to 242. Domain II is made up of 122 amino acids and includes residues from 97 to 226 [Fig. 1(D)]. Opposite surfaces of the protein are concave in shape, with one surface being more concave than the other. The phosphate ligand is sitting deep inside a tunnel found in a large positively charged cleft region [Fig. 1(B)]. The cleft is 22.5 Å in length and 19.5 Å in width and these dimensions are large enough to accommodate binding of a double-helical DNA moiety. Two loops, L3, and L17 near the edge of the cleft, are juxtaposed to the tunnel and most likely participate in the regulation of access of the molecules to the tunnel [Fig. 1(A)]. The location of the putative active site could be identified by superimposing the structure of Eco RV endonuclease in complex with DNA (PDB code: 1RVA) over the ParB-like nuclease. Cαs of 43 residues overlapped with an r.m.s.d. of 2.3 Å. Interestingly, the DNA superimposes over the cleft region of the ParB-like nuclease, suggesting the involvement of the region in binding DNA [Fig. 1(C)]. The minor groove of the DNA is oriented such that the scissile phosphodiester bond is inside the tunnel of the ParB-like nuclease. In the ParB-like nuclease structure, the phosphate ligand is occupying a similar position inside the tunnel. The active site is expected to be located within the tunnel.

DALI analysis[23] for identification of structural neighbors revealed the nuclease to be structurally similar to a chromosome partitioning protein SPO0J (PDB code: 1VZ0) from *Thermus thermophilus*.[24,25] Although the primary sequence identity was 19%, with a *Z* score of 3.9, the SPO0J main chain carbons overlapped the Cαs of the Domain I of the nuclease with an r.m.s.d. of 2.0 Å (data not shown).

Nuclease activity

ParB nucleases show endo and exo nuclease activities against double- and single-stranded DNA.[26] The ability of the protein to degrade DNA was validated by using double-stranded plasmid DNA as substrate [Fig. 1(E)]. The purified nuclease and the chemically modified variant were used for the assay. pMD18-T plasmid was treated with the proteins for 60 min at 37°C. A control was set up under identical conditions without the protein. At the end of the incubation time, the reaction mixture was analyzed by agarose gel electrophoresis. Although the nuclease could degrade the DNA under the ssay conditions described in Materials and Methods, the chemical modification of the protein compromised the nucleic acid degradation ability of the protein under identical conditions. The methylated protein could not degrade DNA [Fig. 1(E)]. Even though optimum temperature for the nuclease activity was 37°C, the nuclease retained 60% of its activity at 90°C (data not shown). The ability of the nuclease to cleave DNA at high temperatures was not surprising, since the organism from which the protein was cloned, *Pyrococcus furiosus*, is capable of growing at temperatures as high as 100°C. Thus, the nuclease seems to posses a physiological role in the organism.

The specific activity of the ParB nuclease was 920 U mg^{-1} under the assay conditions described in Materials and Methods.

DISCUSSION

Although the primary amino acid sequence of the ParB-like nuclease shows very low homology to other nucleases, the overall architecture of the active site shares features essential for the breakage of the DNA backbone with known structures of restriction enzymes.[27–31] The ligand 302/NA is in an environment and position similar to that of metal ions observed in the 3D structures of a number of restriction enzymes. At the time the coordinates were deposited in the PDB, the ligand was assigned as a Na^{+}. The 8-fold co-ordination of the ligand is characteristic of a divalent Ca^{2+} and unlike a divalent Mg^{2+}, which shows 6-fold co-ordination. Therefore, the ligand is actually more likely to be a Ca^{2+} rather than Na^{+}. Assay of nuclease activity performed using the native protein in the presence of EDTA showed that metal ions were essential for the activity (data not shown). Although Ca^{2+} could be substituted by Mg^{2+} in the assay, the activity was significantly lower in presence of Mg^{2+} (not shown). The phosphate ion was seen bound in a position similar to that of a phosphodiester-bonded phosphate attached to the active site.

During the recovery and purification of the protein, it was observed that the protein bound unusually high amounts of DNA. The initial size exclusion chromatography elution profiles indicated a highly aggregated protein sample bound to nucleic acids. Similar nucleoprotein complexes involved in the partitioning of DNA have been reported for ParB proteins previously.[32] The protein could be recovered in a monomeric form after complete removal of the bound DNA using hydroxyapatite chromatography. Only the monomeric form of the protein free of nucleic acids crystallized after the chemical modification. Extensive soaking or cocrystallization experiments with DNA failed to reveal electron density for DNA.

We have determined the crystal structure solution of a ParB-like nuclease at atomic resolution. Functional studies clearly demonstrated the ability of the ParB nuclease to degrade DNA. Although the overall mechanism of segregation of DNA remains unknown, the structure of the ParB-like nuclease protein determined in this study pro-

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vides new tools for deciphering the events of DNA segregation at the atomic level. In addition, the nuclease structure determined in this study offers an excellent target for designing new powerful antimicrobial drugs to not only help fight infectious diseases but also contain the spread of drug-resistant plasmids.

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