PROTEIN STRUCTURE REPORT

Solution structure of the N-terminal domain of a replication restart primosome factor, PriC, in *Escherichia coli*

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Abstract: In eubacterial organisms, the *oriC*-independent primosome plays an essential role in replication restart after the dissociation of the replication DNA-protein complex by DNA damage. PriC is a key protein component in the replication restart primosome. Our recent study suggested that PriC is divided into two domains: an N-terminal and a C-terminal domain. In the present study, we determined the solution structure of the N-terminal domain, whose structure and function have remained unknown until now. The revealed structure was composed of three helices and one extended loop. We also observed chemical shift changes in the heteronuclear NMR spectrum and oligomerization in the presence of ssDNA. These abilities may contribute to the PriC-ssDNA complex, which is important for the replication restart primosome.

Keywords: protein structure; primosome; replication restart; protein-DNA interaction; PriC; NMR

Introduction

In bacterial organisms, DNA replication is occasionally halted by chemically or physically damaged DNA.¹ The damaged DNA is repaired or removed by

several protein systems, after which DNA replication is re-started by a complex of several proteins and the repaired fork DNA. This protein and DNA complex is called a replication restart primosome.^{2,3} In *Escherichia coli*, the restart primosome is considered two independent systems: a PriA-PriB-dependent system and a PriC-Rep-dependent system. Genetic analysis suggested that replication restart is complemented by both systems.³⁻⁶

PriC is a key component protein in the PriC-Rep-dependent replication restart system.^{2,7} Namely, a complex of PriC and DNA form a primosome by collaborating with Rep and DnaC. PriC recognizes the leading strand with a single-strand gap consisting of nine or more nucleotides in the repaired fork structure. Recently, PriC directly interacted with single stranded DNA binding protein,

Abbreviations: HSQC, heteronuclear single quantum coherence; NOE, nuclear overhauser effect.

Additional Supporting Information may be found in the online version of this article.

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SSB, which works as hub protein during DNA replication and DNA repair in *E. coli* genome. Their study also suggested that the PriC-SSB interaction is crucial for the following event, DnaB loading.⁸ Moreover, they suggested that two arginine residues (Arg 122 and Arg 155) were involved in PriC-SSB interaction.⁸ PriC provides DnaB helicase loading, after which DnaG primase is recruited there and synthesizes the primer RNA. Finally, from the primed sites, DNA chains are extended by a DNA polymerase III holoenzyme. These processes consist of a series of restart replications of genomic DNA.

Our recent limit-proteolytic analysis demonstrated that PriC is separated into two domains: the N-terminal domain (residues 1–97) and the C-terminal domain (residues 98–175).⁹ Each domain displayed an α -helical structure by CD spectrum. The C-terminal domain was able to bind single-stranded DNA through electrostatic interaction and oligomerize through hydrophobic interaction, suggesting that it is important for the PriC–Rep replication restart system. Moreover, the contribution of C-terminal domain to the PriC-SSB interaction was confirmed.⁸ Therefore, although the information of the C-terminal domain was gathered, in contrast, we have had no information on the structure or function of the N-terminal domain.

In this study, we determined the solution structure of the PriC N-terminal domain by NMR analysis. The structure was composed of three helices and an extended loop. We also observed chemical shift changes in the heteronuclear NMR spectrum and oligomerization in the presence of ssDNA.

Results and Discussion

NMR spectroscopy of PriC in *E. coli*

We prepared uniformly ¹⁵N-labeled full-length PriC (residues 1–175) and the N-terminal domain (residues 1–98), and recorded the ¹H-¹⁵N HSQC spectrum at 25°C and pH 6.0. The spectrum of the full-length PriC was too broad to allow us to analyze the structure by any attempt, such as several changes of the buffer conditions (data not shown). However, the spectrum of the N-terminal domain included separated and dispersed peaks [Fig. 1(A)]. Therefore, we assigned the ¹H, ¹⁵N, and ¹³C chemical shifts of the N-terminal domain using a double-labeled sample and heteronuclear three-dimensional NMR. We were able to assign most of the ¹H, ¹⁵N, and ¹³C signals in the N-terminal domain of PriC. (BioMagResBank accession number 11525).

Structure of the PriC N-terminal domain

We carried out ¹H-¹⁵N heteronuclear NOE measurements based on the chemical shift assignments. Since the peak intensity ratios (Ion/Ioff) in the N-terminal domain are >0.7, the N-terminal domain appears to have a rigid structure in solution [Fig. 1(B)]. We calculated the structure of the N-terminal domain on the basis of a total of 908 NOE-derived inter-proton distances,

46 hydrogen bonds that originated from slowly exchanging amides, and 87 ϕ and ψ dihedral angle constants obtained from calculations using the TALOS Plus program. A total of 200 structures were calculated, and the 20 lowest-energy structures were selected and subjected to restrained energy minimization. The final 20 structures were superimposed on the mean coordinate position for the backbone atoms (N, C α , and C') of residues 12–40 and 45–87 of the PriC N-terminal domain [Fig. 2(A)]. The r.m.s.d. from the mean coordinate position was 0.79 Å for well-defined backbone atoms of residues. These calculated parameters are summarized in Table I. The structure is consistent with the ¹H-¹⁵N heteronuclear NOE results, suggesting that the overall structure was rigid except for some residues at the N-terminus and C-terminus [Fig. 1(B)].

The structure of the PriC N-terminal domain (residues 1–98) consisted of three α -helices [α 1 (residues 5–22), α 2 (residues 46–64), and α 3 (residues 71–93)], and the extended loop region [Fig. 2(B)]. Several hydrophobic or electrostatic residues were conserved in PriC homologs in eubacterial species [Fig. 2(C)]. Three helices interacted with each other through the conserved hydrophobic residues [Fig. 2(D)]. The extended loop region also interacted with the conserved hydrophobic residues. Moreover, since the side-chain distance between Glu89 in the α 3 helix and Arg33 in the loop was closed, as was that between Glu 52 in the α 2 helix and His 78 in the α 3 helix, they might form salt bridges [Fig. 2(D)]. These acidic or basic residues are also conserved [Fig. 2(C)], suggesting that the overall conformation of the N-terminal domain is evolutionarily conserved. We searched for proteins that are structurally similar to PriC N-terminal domain using the DALI server.¹² Many of three helix bundle proteins had a high Z-score for three helices in the PriC N-terminal domain (Supporting Information Table I). Therefore, PriC N-terminal domain is a typical three-helix bundle protein. However, the searched three-helix bundle proteins did not have a long loop, suggesting that the loop is unique structure of PriC N-terminal domain.

Oligomer formation of N-terminal domain by addition of ssDNA

In our recent article, it was suggested that the N-terminal domain of PriC was not able to bind ssDNA by gel shift assay and intrinsic tryptophan fluorescence quenching experiment.⁹ However, we observed subtle precipitation by adding ssDNA to the PriC N-terminal domain solution. Therefore, we examined the effect of oligo-dT25 on the PriC N-terminal domain using chemical shift perturbation. As the oligo-dT25 concentration increased (a molar ratio up to 1:2), some ¹H-¹⁵N cross peaks were shifted. The shifted peak of Ala4 was shown in Figure 3(A) as a representative. However, the other alanine residues peaks were not shifted [Fig. 3(A,B)].

Figure 1. A: ^1H - ^{15}N HSQC spectrum of 0.5 mM N-terminal domain of PriC (residues 1–98) in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (90/10%, v/v), at pH 6.0 and 25°C. Backbone NH resonances are labeled with their single letter codes and residue numbers. An enlargement of the selected region is shown in the inset. B: Sequence dependence of ^1H - ^{15}N NOE values. The values of some residues could not be obtained because of signal overlap.

Based on the chemical shift perturbations of these shifted peaks, we calculated the K_d value to be 1.25 mM [in concentration of the DNA fragment, Fig. 3(B)]. Therefore, since the PriC N-terminal domain has weak affinity for oligo dT25, the ssDNA binding to the N-terminal domain could not be detected by gel shift assay.⁹ We also plotted the chemical shift changes as a function of the number of PriC N-terminal domain residues at 0.2 mM ssDNA [Fig. 3(C)]. The residues causing significant (more than 0.03 ppm) chemical shift changes occurred in the N-terminal (residues 3–10) or the loop region (residues 34–38) [Fig. 3(C,D)]. There are some polar residues, which could be involved in the interaction

between the PriC N-terminal domain and ssDNA. Furthermore, the chemical shifts of aromatic residues, Phe 34 and Phe 38 in loop region, also perturbed. These residues are conserved in PriC homologs [Fig. 2(C)]. In particular, Phe 34 is on the surface of PriC N-terminal domain structure. The structural studies of oligonucleotide binding (OB)-fold proteins suggested that nucleotides in ssDNA interacted with aromatic side chain exposed to the solvent in OB-fold protein via stacking interaction.¹³ Therefore, aromatic ring of Phe34 possibly contribute to PriC-ssDNA interaction. Since three tryptophan residues (Trp 74, 93, and 98) were located apart from the DNA binding site, we could not

Table I. Summary of Structural Statistics Obtained for the PriC N-Terminal Domain

Number of distance and dihedral angle constraints	
Total NOEs	908
Intraresidue NOEs	297
Sequential NOEs (residue i to $i + 1$)	307
Medium-range NOEs (residue i to $i + 2, 3, 4$)	172
Long-range NOEs	124
Hydrogen bond restraints	46×2
Dihedral angle restraints ($\phi + \psi$)	$87 + 87$
Root mean square deviation (rmsd) against the mean structure	
rmsd for backbone atoms (residues 12–40, 45–87) (Å)	0.79 ± 0.14
rmsd for heavy-chain atoms (residues 12–40, 45–87) (Å)	1.58 ± 0.13
R.m.s. deviation from experimental constraints	
Distance (Å)	0.0022 ± 0.0002
Angle ($^{\circ}$)	0.0324 ± 0.0073
R.m.s. deviation from idealized covalent geometry	
Distance (Å)	0.0008 ± 0.00003
Angle ($^{\circ}$)	0.3005 ± 0.0013
Impropers ($^{\circ}$)	0.0967 ± 0.0028
CNS energy terms (kcal/mol)	
E_{bond}	0.91 ± 0.06
E_{angle}	38.9 ± 0.35
E_{imp}	1.17 ± 0.07
Ramachandran analysis (%)	
Residues in the most favored regions	88.5
Residues in the additional allowed regions	10.2
Residues in the generously allowed regions	0.9
Residues in the disallowed regions	0.4

Statistics are derived from a set of 20 low-energy structures, each obtained by simulated annealing and derived from a unique starting model. Ramachandran analysis was performed by the PROCHECK program.¹¹

previously observe ssDNA binding to the PriC N-terminal domain using the intrinsic tryptophan fluorescence quenching experiment.⁹

In addition, we examined whether the DNA induced oligomer formation in the PriC N-terminal domain using a chemical crosslink by glutaraldehyde. Mixtures of protein and variable lengths of DNA were incubated with 0.2% glutaraldehyde. The oligomerization was evaluated by SDS-PAGE staining with coomassie brilliant blue (CBB). In the presence of DNA, especially that >15 mer, we observed the oligomer of the PriC N-terminal domain [Fig. 3(E)]. This result suggested that the PriC N-terminal domain has homo-oligomerization ability by the addition of ssDNA. Protein is mainly crosslinked by glutaraldehyde between the proximal amino groups (within 7Å) in the lysine side chain or the N-terminus. The PriC N-terminal domain has Lys 2 and Lys 9, whose chemical shifts were perturbed when ssDNA was added to the PriC solution [Fig. 3(C,D)]. Therefore, it was found that the N-terminus of the PriC N-terminal domain has the potential to form an oligomer in the presence of ssDNA. Possibly, the PriC N-terminal domain might bind to and align with the long ssDNA at the N-terminus.

To confirm further, the complex between PriC N-terminal domain and ssDNA, we used gel filtration chromatography. We found that the Cy3-modified ssDNA (oligo-dT35) peak in the presence of excess PriC N-terminal domain was eluted faster than that in the absence of PriC N-terminal domain (Supporting Information Fig. 1). Therefore, we con-

firmed the PriC N-terminal domain and ssDNA complex, although the interaction between PriC N-terminal domain and ssDNA was weak.

In this study, we determined the structure of the N-terminal domain of PriC. Our recent domain characteristics study suggested that the C-terminal domain contributes mainly to the ssDNA binding ability.⁹ However, we showed in this study that the N-terminal domain has a role in weak ssDNA binding and self-oligomerization induced by ssDNA. The weak binding and oligomerization ability, together with the C-terminal domain, may contribute to the retention or stabilization of the PriC-ssDNA complex before replication restart. In fact, PriC-ssDNA complex was unstable without the N-terminal domain.⁹ Possibly, the C-terminal domain interacts with SSB and lagging strand DNA, and the N-terminal domain weakly interacts with leading strand DNA (Supporting Information Fig. 2). Furthermore, oligomerization induced by ssDNA binding might be important events in recruiting DnaB helicase, because DnaB is a hexamer. To elucidate further, the function and structure of PriC, our future research will focus on the structure of the C-terminal domain of PriC.

Materials and Methods

PriC protein preparation

The N-terminal domain (residues 1–98) with a His₆-tag at the N-terminus or the full length

Figure 2. Structure of PriC N-terminal domain. A: Superposition of 20 simulated annealing of the PriC N terminal domain (residues 1–98, PDB code 2rt6). B: Ribbon model of the PriC N terminal domain represented as three α -helices [α 1 (residues 5–22), α 2 (residues 46–64), and α 3 (residues 71–93)] and long loop region (residues 24–45). C, D: Conserved residues in PriC homologs in eubacterial species are displayed on the sequence (C) and structure (D). Red, blue, and green represent acidic (aspartic acid and glutamic acid), basic (arginine, histidine, and lysine), and hydrophobic (alanine, cysteine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine, and valine) amino acids, respectively. Sequence logos were generated by the WEBLOGO server¹⁰ using PriC homologs in 26 eubacterial species (*E. coli*, *G. hollisae*, *M. morganii*, *P. stuartii*, *S. amazonensis*, *S. boydii*, *V. alginolyticus*, *A. actinomycetemcomitans*, *Brenneria* sp., *C. koseri*, *D. dadantii*, *E. cloacae*, *E. bacterium*, *E. amylovora*, *H. aegyptius*, *K. pneumoniae*, *P. agglomerans*, *P. multocida*, *P. carotovorum*, *P. asymbiotica*, *P. mirabilis*, *P. haloplanktis*, *Rheinheimera* sp., *S. enterica*, *S. marcescens*, and *Y. enterocolitica*).

(residues 1–175) of the PriC protein was obtained from the *E. coli* expression system constructed and purified according to our previous article.⁹ The ¹³C-, ¹⁵N-labeled N-terminal domain of PriC was expressed in M9 medium containing ¹³C glucose and ¹⁵NH₄Cl. The Ni²⁺-NTA agarose column was used for purification. The protein was dialyzed against distilled water, lyophilized, and stored at –20°C until NMR analysis. The yield of the labeled protein was about 10 mg/L of culture.

NMR measurements and structural calculations

The NMR spectra were recorded at 25°C on a Varian Unity INOVA 600 spectrometer. NMR samples (0.5

mM) of the N-terminal domain of PriC were dissolved in distilled water containing 150 mM NaCl and 10% D₂O. The pH was adjusted to 6.0 by DCl or NaOD. A series of three-dimensional double- and triple-resonance experiments (HNHA, HNCA, HN(CO)CA, HNCBCA, CACBCONH, HNCO, HN(CA)CO, (H)CC(CO)NH, HCCH-TOCSY, HCCH-COSY, and ¹⁵N-edited TOCSY) were recorded for the spectrum assignments of the N-terminal domain of PriC. The pulse sequence used to measure the ¹H–¹⁵N heteronuclear NOEs (Nuclear Overhauser Enhancements) was described previously.¹⁴ The NOE data from the PriC N-terminal domain (residues 1–98) were obtained from ¹⁵N- or ¹³C-edited NOESY

Figure 3. ssDNA binding's effect on PriC N-terminal domain. **A:** Part of the ^1H - ^{15}N HSQC spectrum of the PriC N terminal domain showing the chemical shift changes in the 0 mM (black), 0.05 mM (yellow), 0.1 mM (orange), 0.15 mM (pink), and 0.2 mM (red) 25-mer ssDNAs (oligo dT25). **B:** NMR titration of the ssDNA to the N-terminal domain of PriC. The change in the NMR chemical shift of the amide proton peak of Ala 4 (closed squares) and of Ala 28 (closed circle) in ^1H - ^{15}N HSQC is indicated. **C:** Differences in the chemical shift changes between the absence and presence (0.2 mM) of ssDNA are displayed for the back-bone amides as a function of sequence number. All chemical shift changes in the ^1H - ^{15}N HSQC spectra were calculated according to the formula $[\Delta\delta(^1\text{H})^2 + (\Delta\delta(^{15}\text{N})/7)^2]^{1/2}$. **D:** Ribbon model (left) and surface model (right) of PriC N terminal domain indicating residues that experience ssDNA-derived chemical shift changes. The red residues are those that underwent chemical shift changes of more than 0.03 ppm. The gray residues were those for which we could not trace the perturbation of the chemical shift because of Pro or signal overlap. Lysine residues in possible oligomerization sites are indicated by stick models (blue). **E:** Oligomerization of PriC. PriC protein (20 μM) and various lengths of oligo dT (7mer, 15mer, and 35mer, each at a concentration of 10 μM) were incubated with 0.2% glutaraldehyde. The mixture was analyzed by 16% SDS-PAGE staining with CBB. T, D, and M represent trimer, dimer, and monomer, respectively.

experiments carried out with a mixing time of 150 ms. NMRPipe¹⁵ was used to process the data and to assign the resonance peaks. The resonance peak of each amino acid residue was assigned using Olivia (fermi.pharm.hokudai.ac.jp). CYANA (version

2.1)^{16,17} was used to calculate the structures. A total of 87 ϕ and ψ values were estimated on the basis of the HN, C α , C β , and C' chemical shifts using TALOS Plus.¹⁸ The distance constraints of the hydrogen bonds were incorporated based on the

results of an experiment involving slowly exchanging amide protons. The restraints used for the structural calculations are summarized in Table I. A total of 200 structures were obtained, and the mean coordinates were obtained by averaging the coordinates of the 20 structures with the lowest target function (final average target function 1.69 ± 0.09). The distance constraints from the 20 structures were converted to the CNS format using h2orefine.py script.^{19,20} The final 200 structures were calculated and refined by the CNS 1.21 program.^{21,22} Ramachandran analysis was performed by the PROCHECK program.¹¹ Structure figures were generated using PyMOL (www.pymol.org). The coordinates used for the ensemble of NMR structures have been deposited in the PDB under accession code 2rt6. The tables have been deposited in BioMagResBank (www.bmrb.wisc.edu) under accession number 11525.

ssDNA titration

The 25-mer ssDNA (oligo dT25) was purchased (Invitrogen) and purified before NMR titration. We recorded the ^1H - ^{15}N HSQC spectra of 0.1 mM ^{15}N -labeled protein in the presence of various concentrations of the indicated ssDNA (up to 0.2 mM). The overall volume changes amounted to <5%. The titration curve was analyzed by a nonlinear iterative fitting procedure using xcrvfit (<http://www.bionmr.ualberta.ca/bds/software/xcrvfit>).

Gel filtration chromatography

Cy3-modified oligodT35 (15 μM) were incubated with the eluted buffer (50 mM MES, pH 6.5) in the presence or absence of 240 μM PriC N-terminal domain. Five microliter of the mixture solutions were loaded onto a column (4.6×250 mm, SRTTM SEC-300, SEPAX-Technologies, Newark, DE). The DNAs and proteins were eluted with the eluted buffer at a flow rate of 0.1 mL/min. The eluted DNAs were monitored at 570 nm fluorescence excited by 515 nm.

Chemical crosslinking by glutaraldehyde

The PriC N-terminal domain (20 μM) and various ssDNAs (see Figure Legends, each concentration was 10 μM) were incubated with 0.2% glutaraldehyde in buffer (20 mM Tris-HCl [pH 7.5] containing 100 mM NaCl). After 7 min incubation at room temperature, the reaction was stopped by the addition of 10 mM glycine. The mixture was analyzed by 16% SDS-PAGE staining with CBB.

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