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Biochemical and Biophysical Research Communications 326 (2005) 766–776
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Crystal structure of a biologically functional form of PriB from *Escherichia coli* reveals a potential single-stranded DNA-binding site☆

Seijiro Shioi[a], Toyoyuki Ose[b], Katsumi Maenaka[b], Mitsunori Shiroishi[b], Yoshito Abe[a], Daisuke Kohda[b], Tsutomu Katayama[c], Tadashi Ueda[a,*]

[a] *Department of Immunology, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*
[b] *Division of Structural Biology, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*
[c] *Department of Molecular Biology, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

Received 28 October 2004
Available online 8 December 2004

Abstract

PriB is not only an essential protein necessary for the replication restart on the collapsed and disintegrated replication fork, but also an important protein for assembling of primosome onto ΦX174 genomic DNA during replication initiation. Here we report a 2.0-Å-resolution X-ray structure of a biologically functional form of PriB from *Escherichia coli*. The crystal structure revealed that despite a low level of primary sequence identity, the PriB monomer, as well as the dimeric form, are structurally identical to the N-terminal DNA-binding domain of the single-stranded DNA-binding protein (SSB) from *Escherichia coli*, which possesses an oligonucleotides-binding-fold. The oligonucleotide–PriB complex model based on the oligonucleotides–SSB complex structure suggested that PriB had a DNA-binding pocket conserved in SSB from *Escherichia coli* and might bind to single-stranded DNA in the manner of SSB. Furthermore, surface plasmon resonance analysis and fluorescence measurements demonstrated that PriB binds single-stranded DNA with high affinity, by involving tryptophan residue. The significance of these results with respect to the functional role of PriB in the assembly of primosome is discussed.

Keywords: Primosome; Primosomal protein B; Single-stranded DNA binding protein

Primosome is a multiprotein complex that synthesizes primer RNA for replication of bacterial chromosome and bacteriophage genome DNA. Replication of the single-stranded DNA (ssDNA) of phage ΦX174 to its duplex replicative form has been reconstituted using host-encoded proteins [1,2]. The ΦX174-type primosome is composed of DnaG primase associated with a preprimosomal complex that contains five proteins, PriA helicase, PriB, PriC, DnaT, and DnaB helicase. These proteins assemble sequentially on a specific site of ΦX174 DNA. Briefly, PriA binds to the primosome assembly site (PAS) on SSB (ssDNA-binding protein)-coated ΦX174 circular ssDNA (sscDNA) [3]. Then, PriB, DnaT, and PriC bind sequentially to the PriA–DNA complex [4,5]. In an ATP- and DnaC-dependent manner, DnaB is then loaded on the complex. This PriABC–DnaBT complex is capable of translocation along ssDNA in both the $3' \rightarrow 5'$ and $5' \rightarrow 3'$ directions by acting as a bidirectional DNA helicase [6] and forms

☆ *Abbreviations:* EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HPLC, high performance liquid chromatography; MAD, multiwavelength anomalous dispersion phasing; NHS, *N*-hydroxysuccinimide; OB-fold, oligonucleotides-binding-fold; *oriC*, chromosomal origin; PAS, primosome assembly site; rms, root mean square; ssDNA, single-stranded DNA; SPR, surface plasmon resonance; SSB, ssDNA-binding protein; HMtSSB, human mitochondrial SSB; *Mtu*SSB, SSB from *Mycobacterium tuberculosis*; *Eco*SSB, SSB from *Escherichia coli*.

* Corresponding author. Fax: +81 92 642 6667.
E-mail address: ueda@phar.kyushu-u.ac.jp (T. Ueda).

0006-291X/$ - see front matter © 2004 Published by Elsevier Inc.
doi:10.1016/j.bbrc.2004.11.104

the complete primosome through its association binding with DnaG primase, which directly interacts with DnaB [7].

In the case of *Escherichia coli* chromosomal origin (*oriC*), the so-called ABC primosome is used [8]. DnaA protein forms a multiprotein complex which can cause local unwinding of duplex DNA in the *oriC* region. DnaB helicase directly interacts with DnaA and is then loaded onto the exposed ssDNA region with the aid of the DnaC protein. Finally, DnaG forms a mobile complex with the loaded DnaB [9]. It has been recognized that the replication fork apparatus started from *oriC* often collapses or disintegrates spontaneously, for example, at sites of DNA damage on the template during the course of replication. In such cases, the apparatus can be reassembled at the site of collapse. This process is called replication restart, which in turn is intimately connected to the process of RecA-dependent recombinational repair of the damaged DNA [10–15]. Notably, the proteins required for the replication restart include factors of the ΦX174-type primosome, such as PriA and PriB. Biochemical studies using purified proteins have suggested that replication restart occurs at a three-way-junction DNA structure, the D-loop, which is an intermediate in recombinational repair [16–19]. In order to assemble the primosomal proteins necessary for replication restart, first, PriA binds to the specific site on the collapsed replication fork. By direct interaction with PriA, PriB disturbs the helicase activity to hold PriA on the site during primosome assembly [20]. In vitro PriB–PriA–DnaT interaction analysis has suggested that the efficiency of the PriA–DnaT interaction was dramatically reduced in the absence of PriB [21]. Therefore, since PriB is one of the important factors for the replication restart, its action during the assembling of the primosome has received much attention in recent studies. However, the sites at which PriB binds with ssDNA or PriA are unclear. In this study, we succeeded in determining the crystal structure of a biologically functional form of PriB from *E. coli* at a high resolution of 2.0 Å. The crystal structure of PriB was identical to that of the single-stranded DNA-binding protein (SBB), which was examined in detail for its interaction with ssDNA. Furthermore, we demonstrate the first approach to analyze features of PriB binding to ssDNA from a view of structural comparison with SBB.

Materials and methods

Cloning of the E. coli priB gene. The *priB* gene was PCR-amplified from genomic DNA of the *E. coli* K-12 strain MG1655. Primers complementary to the 5′ (5prib) and 3′ (3prib) ends of the prib gene (listed at the Genobase (http://www.ecoli.aist-nara.ac.jp) under Accession No. JW4159) were used. The flanking restriction sites, *Nde*I and *Bam*HI, were added using the following primers: 5′priB primer, 5′-TGATGAC CAACCGTCTGGTGTT-3′; 3′priB primer, 5′-GGCTAGTCTCCAG AATCTATCA-3′. The digested fragment (*Nde*I/*Bam*HI) was inserted between the *Nde*I and *Bam*HI sites of the pET22b(+) vector (Novagen, Madison, WI) just downstream of the T7 promoter. The nucleotide sequencing was determined by using an ABI PRISM 310 Genetic Analyzer.

Expression and purification of selenomethionyl PriB. BL21(DE3) cells transformed with pET22-priB were grown at 37 °C in LeMaster medium [22,23] supplemented with sereno-L-methionine (Wako) to a cell density corresponding to $A_{600} = 0.5$. Protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to 1.0 mM, and the culture was allowed to continue for 5 h at 37 °C. The cells were suspended in buffer A [50 mM Hepes (pH 7.0), 10% (w/v) sucrose, 10 mM DTT, and 1 mM PMSF] and disrupted by sonication. Insoluble debris was removed by centrifugation for 15 min at 17500 rpm. To decompose DNA and RNA, spermidine–HCl (Wako) was added to the supernatants to a final concentration of 20 mM, and the mixture was again centrifuged for 20 min at 17500 rpm. For salting out, ammonium sulfate (0.3 g/ml) was added to the supernatant. The precipitate was resuspended in buffer B [25 mM Hepes (pH 7.0), 2.5 mM DTT, and 20% glycerol] containing 50 mM NaCl and then applied to a SP-Toyopearl 650M column (70 × 1.6 cm) equilibrated with buffer B containing 100 mM NaCl. The column was washed with three column volumes of buffer B containing 100 mM NaCl, and the proteins were eluted with a linear gradient consisting of 400 ml buffer B containing 100 mM NaCl and 400 ml buffer B containing 500 mM NaCl. The PriB-containing fractions, detected by SDS–PAGE, were pooled and precipitated by addition of ammonium sulfate (0.35 g/ml). The precipitate was collected and dissolved in buffer C [50 mM Tris–HCl (pH 8.0), 2.5 mM DTT, 20% glycerol, and 0.5 M NaCl]. The mixture was then chromatographed on a Sephadex G-75 superfine column (1.5 × 30 cm) equilibrated with buffer C. The PriB-containing fractions were pooled and concentrated to 25 mg/ml in 20 mM AcONa (pH 5.0), 5 mM DTT, 150 mM NaCl, and 20% glycerol. The protein concentration was determined by the Bradford method [24] using bovine serum albumin as a standard. The purified proteins were stored at −20 °C until use.

Crystallization of the selenomethionyl PriB. The selenomethionyl PriB crystals were grown by vapor diffusion using the hanging drop method. A 1 μl droplet of protein was mixed with an equal volume of reservoir solution and incubated at 25 °C over a period of 1 week. The reservoir solution contained 100 mM Tris–HCl (pH 8.0), 25% (v/v) 2-methyl-2,4-pentanediol (MPD), and 50 mM $MgCl_2$. The reservoir volume was 150 μl.

Data collection. Se-MAD datasets were collected on a beamline BL38B2 at SPring-8 using a Quantum4R CCD detector. Four wavelengths were selected for the data collection based on the fluorescence spectrum for the Se K edge, corresponding to the maximum f' (0.9790Å, peak), minimum f'' (0.9792 Å, edge), high energy reference point (0.9000 Å, high_remote), and low energy reference point (0.9000 Å, low_remote). All datasets were collected at 100 K after crystals were soaked in anti-freezing buffer containing 15–20% MPD. The crystal of Se-Met PriB belongs to the space group $C2$ with unit cell parameters of $a = 103.5$, $b = 70.3$, $c = 74.8$ Å, $\beta = 131.59°$, and there are four protomers in an asymmetric unit (V_M [25] = 2.18 A3/Da, $V_{solv} = 43.07\%$). All reflection data were integrated and scaled using Denzo and Scalepack software [26]. The statistics of data collection and processing are shown in Table 1.

Structure determination and refinement. The structure of PriB was determined using the multi-wavelength anomalous diffraction (MAD) method. Five out of eight Se sites were determined by program SHELX-97 [27] and used to refine atomic parameters for the MAD-phasing using SOLVE [28] at 2.0 Å resolution. The electron density of the initial phases was further improved using the program RESOLVE [29]. The initial model was built using program O [30] based on the results of auto model building programs using both RESOLVE [31] and ARP/wARP [32]. The molecular dynamic refinement was performed using the program CNS [33] with a 9.0–2.0 Å resolution region