Crystal Structure of PriB, a Primosomal DNA Replication Protein of Escherichia coli*

Received for publication, June 17, 2004, and in revised form, August 31, 2004
Published, JBC Papers in Press, September 21, 2004, DOI 10.1074/jbc.M406773200

Jyung-Hurng Liu‡§, Tsai-Wang Chang¶, Cheng-Yang Huang§, Sue-Une Chen§, Huey-Nan Wu§, Ming-Chung Chang†‡, and Chwan-Deng Hsiao§§
From the ‡Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan 114, the §Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan 115, and the Departments of ¶Surgery and §Biochemistry, Medical College, National Cheng-Kung University, Tainan, Taiwan 701, Republic of China

PriB is one of the Escherichia coli φX-type primosome proteins that are required for assembly of the primosome, a mobile multi-enzyme complex responsible for the initiation of DNA replication. Here we report the crystal structure of the E. coli PriB at 2.1 Å resolution by multi-wavelength anomalous diffraction using a mercury derivative. The polypeptide chain of PriB is structurally similar to that of single-stranded DNA-binding protein (SSB). However, the biological unit of PriB is a dimer, not a homotetramer like SSB. Electrophoretic mobility shift assays demonstrated that PriB binds single-stranded DNA and single-stranded RNA with comparable affinity. We also show that PriB binds single-stranded DNA with certain base preferences. Based on the PriB structural information and biochemical studies, we propose that the potential tetramer formation is facilitated by the priC gene (13–15). The assembly of a primosome on the φX174 viral DNA, as described in the following sentences, is an ordered process (16). (i) PriA recognizes and binds to the pas. (ii) PriB joins PriA to form a PriA-PriB-pas DNA complex. (iii) DnaT then joins this complex to form a triprotein complex on the pas DNA; and (iv) DnaB is then transferred from a DnaB-DnaC complex to the PriA-PriB-DnaT-pas DNA complex to form a preprimosome assembly that consists of PriA, PriB, DnaT, and DnaB on the DNA. Finally, DnaG adds to this complex by interacting with DnaB and completing the primosome assembly (17). Although in vitro studies have demonstrated that PriC was not required for the stable preprimosome assembly on the 304-nucleotide pas DNA sequence (16), it is a component of the bound preprimosome that was isolated from the full-length φX174 viral DNA (18). The PriA-directed assembly of the φX-type primosome on the D-loop has been shown to be similar to that on the pas sequence of φX174 viral DNA (19).

During the formation of the preprimosome assembly, PriB interacts with and stabilizes the PriA-DNA complex (10, 16), facilitating the complex formation between PriA and DnaT (19). PriB was formerly known as the “n protein” because it can be inactivated by treatment with N-ethylmaleimide (NEM) (13). In vitro experiments indicate that the modified PriB protein decreased the primosomal replication activity of phage φX174 (13). Cells harboring priB mutations display defects in PriA-dependent replication restart (20–22) and ColE1-type plasmid replication (23, 24). PriB forms dimers in solution (13, 14), and each preprimosome may contain two PriB dimers (18). PriB can bind single-stranded DNA (ssDNA) in the presence or absence of single-stranded DNA-binding protein (SSB) in vitro (13, 25).

PriB is generally considered to be a structural component of the φX-type primosome. However, the function of this protein in primosome assembly is not fully understood at the molecular level. Recent studies on sequence comparisons and operon organization analyses have shown that PriB evolved from SSB via gene duplication with subsequent rapid sequence diversification (26). SSB has long been known for its importance in single-stranded DNA; ssRNA, single-stranded RNA; SSB, single-stranded DNA-binding protein; EcoSSB, Escherichia coli SSB; HsmtSSB, human mitochondrial SSB; pHMB, sodium p-hydroxymerc-cubenzoate; MAD, multi-wavelength anomalous diffraction; NEM, N-ethylmaleimide.

* This work was supported by research grants from Academia Sinica and National Science Council, Republic of China Grant NSC92-2311-B-00-088 (to C.-D. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1V1Q) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

‡ To whom correspondence may be addressed. Tel.: 886-6-235-3557 (ext. 5513); Fax: 886-6-275-4697; E-mail: mechang@ncku.edu.tw.
§§ To whom correspondence may be addressed. Tel.: 886-2-2788-2743; Fax: 886-2-2782-6085; E-mail: mbhsiao@ccvax.sinica.edu.tw.

The abbreviations used are: pas, primosome assembly site; ssDNA, (primosome assembly site) sequence that diverts the primosomes from host chromosomal replication to phage DNA production (5).
DNA replication (27). It raises an interesting question as to how PriB participates in DNA replication in a fashion different from that of its ancestor. In this article we present the crystal structure of the E. coli PriB at 2.1 Å resolution. Based on its structural features as well as results from biochemical and mutation studies, we discuss the potential role of PriB in dX-type primosome assembly.

**EXPERIMENTAL PROCEDURES**

**Cloning, Protein Expression, and Purification**—The wild-type priB gene was amplified by PCR from E. coli strain K12. PCR primers were designed to incorporate unique EcoRI and SalI restriction sites, permitting the insertion of the amplified gene into the pET21b vector (Novagen) for protein expression in E. coli. To facilitate the PCR experiment, Gly-103 in the recombinant PriB was substituted with valine by the manufacturer’s recommendation. The eluted protein was dialyzed against the crystallization buffer (0.1M NaCl and 20 mM Tris-HCl at pH 7.9) and concentrated to 10 mg ml⁻¹ for crystallization and scaling were performed using the HKL package (29) (Table I).

**Structure Determination and Refinement**—SOLVE (30) was used to locate the mercury sites and generate the initial MAD phases at 3 Å resolution. The initial phases were extended and further improved to 2.5 Å by RESOLVE (31). XthView (32) was used to examine electron density maps and molecular models. The native data set (2.1 Å) was used for further refinement using CNS (33). During refinement, non-crystallographic symmetry neglecting the loop regions was imposed initially but released after two monomers in the asymmetric unit were built. The final model of PriB contains all residues for monomer A and monomer B. The residues preceding position 1 of each monomer are free of disulfide bonds. The Ramachandran plot has no violation of accepted backbone torsion angles. The statistical data are shown in Table I. The atomic coordinates of PriB have been deposited in the Protein Data Bank under accession code 1V1Q.

**Preparation of 32P-Labeled Probes**—DNA oligonucleotides 35 bases long, (dT)₃₅ and (dA)₃₅, were purchased from Integrated DNA Technologies, whereas an RNA oligonucleotide, U₃₅, of the same length was obtained from Dharmacon. RNA I and RNA II carrying a T7 promoter sequence were synthesized using an in vitro transcription system (Riboprobe®, Promega). These probes were 5'-end labeled with T4 polynucleotide kinase (Promega) and [γ-³²P]ATP (6000 Ci mmol⁻¹; PerkinElmer Life Sciences). The probes were purified on denaturing polyacrylamide gel and resuspended in TF buffer (0.1 mM EDTA and 10 mM Tris-HCl at pH 8). The concentrations of the ³²P-labeled probes were estimated with isotope counting.
Electrophoretic Mobility Shift Assay—To analyze the formation of ssDNA- or single-stranded RNA (ssRNA)-PriB complexes, 0.01 pmol of [32P]-labeled (dT)35, (dA)35, or U35 was mixed with various amounts of recombinant PriB protein in a 10-μl-reaction mixture containing 40 mg ml−1 bovine serum albumin and 100 mM HEPES at pH 7 for 30 min at 25 °C. The resulting samples were resolved on a native 12% polyacrylamide gel at 4 °C in TBE buffer (89 mM Tris borate and 1 mM EDTA) and visualized by autoradiography. Complexed and free DNA bands were scanned and quantified. The PriB-ssDNA binding dissociation constants (Kd) were estimated from the protein concentration that binds 50% of the input DNA (34). In this report, each Kd is calculated as the average of at least three measurements ± S.D. To assess the binding ability of PriB to RNA I or RNA II, the reactions were carried out by mixing 1 pmol of [32P]-labeled RNA I or RNA II with various amounts of recombinant PriB protein in a 10-μl reaction volume for 30 min at 4 °C. The resulting complexes were resolved on a native 10% polyacrylamide gel at 4 °C and visualized by autoradiography.

RESULTS AND DISCUSSION

The Overall Structure of the PriB Monomer—The PriB crystal belongs to the orthorhombic space group P212121, with two molecules in an asymmetric unit. Two identical polypeptide chains form a dimer with a non-crystallographic 2-fold symmetry (Fig. 1A). A PriB monomer is a single domain protein (residues 1 to 104) topologically identical to the well characterized OB (oligonucleotide-binding) fold (35). Briefly, the PriB monomer structure has two pleated -sheets capped by a small α-helix located between the third and the fourth strands to form a β-barrel (Fig. 1B). The core of the β-barrel is filled with hydrophobic residues. Between the anti-parallel strands PriB has three β-hairpin loops termed L12 (residues 20–24), L23 (residues 37–44), and L45 (residues 81–88) that protrude from the central core. Structural comparison of the two monomers in the asymmetric unit reveals that the most significant structural variations between the monomers occur in the regions of the three loops (Fig. 1C). The C terminus (residues 101–104) is located at the tip of the β-barrel and protrudes from the main body of the barrel with considerable variation in its conformation as well. The root mean square deviation between the Ca positions (104 atoms in each monomer involved) in the two monomers is 2.54 Å. Neglecting the C termini and these loop regions decreases the root mean square deviation to 0.55 Å (79 atoms in each monomer involved).

Despite the relatively low sequence identity with other single-stranded DNA-binding proteins (SSBs) (Fig. 1D), the PriB monomer displays high structural similarity to the ssDNA-binding domains of E. coli SSB (EcoSSB), human mitochondrial SSB (HsmtSSB), and Mycobacterium tuberculosis SSB (MtuSSB) (36–38). The tracings of the Ca atoms of these proteins are shown in Fig. 1C and demonstrate clearly that the globular core of these proteins has similar conformation. However, unlike other SSBs, the three loop regions of PriB are distinct in their conformation and orientation with respect to the core. Sequence alignment reveals two gaps that significantly shorten the L23 and L45 hairpin loops of PriB (Fig. 1D). L45 of EcoSSB has been shown to be involved in interface interactions between dimers for the one of the tetramer formations (36). L45 of PriB, however, was not stabilized through crystal packing as shown in EcoSSB. Electron density for the tip of L45 is relatively weak and exhibited high atomic temperature factors.
Architecture of the PriB Dimer—Both HsmtSSB and EcoSSB function as homotetramers in solution (39, 40). In contrast to these SSBs, the PriB molecules form dimers in solution (13, 14) and crystal (Fig. 1A). The two monomers in the PriB dimer are related by a non-crystallographic 2-fold axis that is similar to that of the EcoSSB or HsmtSSB dimer (Figs. 1A and 2). The dimeric interactions of PriB are fairly strong. The intermolecular contacts involve side chains and six short CO···NH main chain H-bonds from $\beta_1$ of each monomer, which build up the continuity of the three-stranded $\beta$-sheets of each subunit. Consequently, a six-stranded anti-parallel pleated sheet extends over the dimer. In addition to the interactions from the intermolecular anti-parallel strands, the arm region (Arg-34 to Trp-47; $L_{12}$ and parts of the connecting $\beta_1$ and $\beta_1$) bends to one side of the paired monomer with intimate contacts involving van der Waals interactions and five hydrogen bonds (Fig. 1A). This close state structural feature is unique and has never been reported for crystal structures of SSBs, even for those that bind with ssDNA (37, 40, 41).

The PriB dimer is further stabilized by a symmetrical pair of intermolecular disulfide bridges formed by Cys-48 of one monomer to Cys-80 of the other monomer. These intermolecular interactions have not been observed in other SSBs. In addition to the disulfide bridges, side chains of Met-50 and Met-90 from both subunits form a methionine cluster (Fig. 1A) that locates between the two intermolecular disulfide bridges. Together, the methionine cluster and the disulfide bonds constitute the hydrophobic core of the extended $\beta$-barrel in the PriB dimer. Because of the close contact of the arm region of one subunit to the $\beta$-barrel of the other (Fig. 1A), the intermolecular interactions of PriB are much stronger than those of EcoSSB or HsmtSSB. The total surface area buried during dimerization in PriB is calculated to be $\sim 3545 \, \text{Å}^2$ as compared with that of 2335 and 2894 $\text{Å}^2$ for EcoSSB and HsmtSSB, respectively. In addition, over two-thirds (69%) of the buried surface in PriB is contributed by hydrophobic interactions. In comparison, the percentage of the buried hydrophobic surface area is 52 and 54% in EcoSSB and HsmtSSB, respectively.

PriB Fails to Form Homotetramers—PriB does not form a tetrameric structure in crystal (this work) or solution (13, 14). In EcoSSB or HsmtSSB the homotetramer is a dimer of dimers with a 222 symmetry and has molecular 2-fold dyads along three intersecting axes (Fig. 2A). Two $\beta$-strands, $\beta_1$ and $\beta_4$, are important for the homotetramer formation (36, 37). Residues in $\beta_1$ and $\beta_4$ of PriB share only low sequence similarity to those of EcoSSB or HsmtSSB (Fig. 1D). In EcoSSB, a hydrophobic cluster formed by four symmetry-related isoleucine residues (Ile-9) stabilizes the six-stranded $\beta$-sheet-mediated tetramer interface (42). Similarly, four symmetric His-18 residues in HsmtSSB substitute for those isoleucine residues in EcoSSB. In PriB, however, the corresponding residue is Val-6. The low steric extent of the valine side chains probably cannot stabilize the potential tetramer interface (Fig. 2A).

In addition, the charge distribution of PriB at the potential tetramer formation surface is considerably different from that in EcoSSB or HsmtSSB (Fig. 2B). In EcoSSB and HsmtSSB, a pair of charged residues (Lys-7 and Glu-80 in EcoSSB and Arg-16 and Glu-95 in HsmtSSB) form a cluster of intermolecular salt bridges at the tetramer formation surface and lock the orientation of subunits. In PriB, the corresponding Arg-4 and Glu-95/Glu-98 are unable to constitute a similar intermolecular salt bridge cluster such as that of EcoSSB or HsmtSSB. The abundance of negatively charge residues (Glu-58, Glu-95, and Glu-98) on the potential tetramer formation surface may also prevent homotetramer formation in PriB.

Possible Roles of the Potential Tetramer Formation Surface of PriB—A priB triple mutant (G56E, H57N, and E58K) has been reported to be defective in the cellular replication of ColE1-related plasmids (23). Coincidentally, Glu-58 is located at the potential tetramer formation surface of PriB (Fig. 2B). Therefore, the negatively charged area containing Glu-58, Glu-95, and Glu-98 on the potential tetramer formation surface can possibly be a determinant for protein-protein interaction between PriB and other proteins during primosome assembly.

Direct interaction between PriB and EcoSSB has been shown, and this protein complex retains ssDNA-binding ability (13). Interestingly, bacterial SSBs from different species can form cross-species heterotetramers through their six-stranded $\beta$-sheet surfaces (43). This finding suggests that the similar six-stranded $\beta$-sheet surfaces of the PriB dimer could interact with an EcoSSB dimer. However, the formation of this heterotetramer has not been reported, and this possibility requires further investigations.

Base Preference of Single-stranded DNA Binding of PriB—To assess the in vitro ssDNA binding ability of PriB, purified proteins were incubated with different amounts of $^{32}$P-labeled ssDNA in electrophoretic mobility shift assay-type experiments (Fig. 3, A and B). EcoSSB has two major ssDNA-binding modes that occlude 35 ± 2 and 65 ± 3 nucleotides per tetramer (44, 45). Considering the structural similarity between the putative oligonucleotide-binding site in PriB (this work) and that in EcoSSB (40), we hypothesize that the length of ssDNA that can be occluded by the PriB dimer is also $\sim 35$ nucleotides. Therefore, synthetic 35-mer ssDNAs were chosen for these assays. We used (dA)$_{35}$ and (dT)$_{35}$ as models for testing the preference of PriB between purine and pyrimidine, respectively (Fig. 3, A and B).
and B). Compared with other polynucleotides, it is well known that EcoSSB exhibits its greatest affinity for poly(dT) (46, 47). Coincidentally, PriB shows a higher preference for (dT)$_{35}$ ($K_d = 1.2 \pm 0.4 \mu M$) than for (dA)$_{35}$ ($K_d = 16 \pm 3 \mu M$). However, the molecular recognition factor on PriB that contributes to this preference is still unknown.

The function of the ssDNA binding ability of PriB in primosome assembly remains poorly understood. The SSB-like fold may help PriB in displacing SSB from ssDNA (25). It is known that DnaT interacts with the PriA-PriB-DNA complex during primosome assembly (16, 19) and that DnaT is capable of binding ssDNA (48). Therefore, we postulate that PriB binds ssDNA that has been unwound or presented by PriA and then positions or maintains the bound ssDNA in a specific conformation that allows DnaT recognition and binding. This hypothesis is consistent with the data showing that in the presence of excess DnaT, PriB is not required for the in vitro primosome assembly (19).

RNA Binding Activity of PriB—Recent studies based on sequence comparison and analysis of operon organization show that PriB has evolved from a single-stranded DNA-binding protein via gene duplication with subsequent rapid sequence diversification (26). Gene duplication followed by functional diversification of the paralogs is one of the principal routes of evolutionary innovation (26, 49, 50). It has been reported that the binding affinity of EcoSSB for ssRNA is much weaker than that for ssDNA (51, 52). To assess the PriB function that differs from its ancestor, we used an electrophoretic mobility shift assay to test the ability of PriB to bind ssRNA (U$_{35}$). Interestingly, we found that PriB bound U$_{35}$ ($K_d = 1.5 \pm 0.3 \mu M$) with comparable affinity as that against (dT)$_{35}$ ($K_d = 1.2 \pm 0.4 \mu M$) (Fig. 3, A and C). This finding suggests that PriB may have evolved different a preference from its ancestor (26).

The biological function of the PriB ssRNA-binding ability remains unclear. Although it might bind RNA primers synthesized during DNA replication, this possibility seems low. Evidence has shown that the dX-type primosome can retain its integrity throughout replication (18), and maintenance of the dX-type primosome during replication may provide chances for PriB to access RNA primers. However, during Okazaki fragment priming the RNA primer presumably forms heteroduplexes with its DNA template and is not favorable for PriB binding (2). PriB has also been shown to be involved in ColE1-type plasmid replication (23). ColE1-type plasmid replication is regulated by interacting RNA primer (RNA II) with its antisense RNA (RNA I) (53). Interestingly, we found that PriB can bind both RNAI and RNAII (Fig. 3, D and E). We are currently investigating the contribution of PriB to the antisense RNA replicative regulation of ColE1-type plasmid.

Residues and Surface Involved in the Binding of Single-stranded Oligonucleotide—Because PriB has a structural resemblance to SSBs, PriB may use similar strategies to recognize ssDNAs or ssRNAs. The PriB dimer contains a pair of 2-fold related grooves that may define the path of the bound single-stranded oligonucleotide (Fig. 4A). The green area is contributed from one monomer, and the cyan area is from the other. The locations of functional residues are assigned according to their three-dimensional relationships and colored based on Fig. 4A.

**Fig. 3.** A–C, the oligonucleotide-binding ability of PriB. 0.01 pmol of 5'-end $^{32}$P-labeled (dT)$_{35}$ (panel A), (dA)$_{35}$ (panel B), or U$_{35}$ (panel C) was incubated with various amounts of PriB in a 10-μl reaction mixture for 30 min at 25 °C. Lanes 1–7 correspond to 0, 3.6, 11, 18, 25, 36, and 220 pmol of PriB, respectively. Protein-DNA or Protein-RNA complexes were separated from free DNA or RNA electrophoretically on a 12% native polyacrylamide gel. D and E, RNA I and RNA II binding ability of PriB. 1 pmol of 5'-end $^{32}$P-labeled RNA I (panel D) or RNA II (panel E) was incubated with various amount of PriB in a 10-μl reaction mixture for 30 min at 4 °C. Lanes 1–6 correspond to 0, 4, 8, 16, 32, and 64 pmol of PriB, respectively. Protein-RNA complexes were separated from free RNA electrophoretically on a 10% native polyacrylamide gel.
The biological roles of Cys-12 and Cys-27 are not clear. It is noticeable that there is no cysteine residue in EcoSSB or in HsmtSSB. One could speculate that the region containing Cys-12 or Cys-27 may be a potential protein-protein interface for PriB to interact with other proteins during primosome assembly. According to the current knowledge of primosome assembly, PriA and DnaT are both candidates that can physically interact with PriB (16, 19). DnaT has no cysteine in its polypeptide sequence (48) and, therefore, is unlikely to interact with PriB through intermolecular disulfide bridging. PriA has two cysteine residues, Cys-439 and Cys-445. These cysteine residues are required for the formation of the PriA-PriB complex (19, 54). However, it is still unclear how these cysteine residues participate in the PriA-PriB complex formation. Given that PriB functions as an ssDNA-binding protein, the protein-protein interaction that occurs between PriA and PriB may affect the ssDNA-binding of PriB during primosome assembly.

The bacteriophage T4 replisome assembly provides a good example of how protein-protein interaction can affect protein-ssDNA interaction. During bacteriophage T4 replisome assembly, loading of the helicase (gp41) onto the gp32 (T4 SSB)-coated ssDNA requires gp59 to remove gp32 and replace it with gp41. Cross-linking studies between gp32 and gp59 revealed an interaction between Cys-166 of gp32 and Cys-42 of gp59. Because Cys166 lies in the DNA-binding core domain of gp32, this interaction may affect the association of gp32 with DNA (55).

Cys-12 of PriB may be directly involved in the oligonucleotide binding of PriB because it sits on the putative oligonucleotide-binding surface. In the NF-xb p50 subunit Cys-62 is located on a DNA recognition loop, and its reduced form is essential for the DNA binding activity of NF-xb (56). Therefore, a redox change on the sulphhydryl group of Cys-12 might alter the oligonucleotide-binding affinity of PriB. Interestingly, the in vitro primosomal replication activity of PriB can be inactivated by the sulphhydryl modification agent NEM (13). This finding infers that the redox state of free cysteine residues may influence the role of PriB in primosome assembly. However, these speculations need to be confirmed by further biochemical studies.

Conclusions—In the present study, we determined the three-dimensional structure of PriB from E. coli at 2.1 Å by x-ray crystallography. We also demonstrated that PriB binds ssDNA and ssRNA with comparable affinity. Thereby, the present study provides the first indication that PriB may possess functions different from that of SSBs. In addition, we propose several regions that may be involved in protein-protein interactions during primosome assembly. However, many questions, such as how PriB recognizes and binds to PriA-DNA complex, how PriB interacts with other primosome components, and how PriB switches its ability to bind ssRNA, remain unanswered. The high-resolution structure of PriB presented here offers a starting point for further studies on primosome assembly. The structure also provides the first framework for understanding the structure of other PriB from the same superfamily.

Acknowledgments—We thank Drs. Ming F. Tam and Hsou-Min Li for discussion and critical reading of the manuscript. We gratefully acknowledge access to the synchrotron radiation beamline 17B2 at the National Synchrotron Radiation Research Center (NSRRC) in Taiwan.

References

1. Schekman, R., Weiner, A., and Kornberg, A. (1974) Science 186, 987-993
2. Kornberg, A., and Baker, T. (1992) DNA Replication, 2nd Ed., pp. 275-306, W. H. Freeman & Co., New York
3. Wickner, S., and Hurwitz, J. (1974) Proc. Natl. Acad. Sci. U S A. 71, 4120-4124
4. Minden, J. S., and Marians, K. J. (1985) J. Biol. Chem. 260, 9316-9325
5. Marians, K. J. (1999) Prog. Nucleic Acids Res. Mol. Biol. 63, 39–67
