Structure of a Four-way Bridged ParB-DNA Complex Provides Insight into P1 Segrosome Assembly*

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The plasmid partition process is essential for plasmid propagation and is mediated by par systems, consisting of centromere-like sites and two proteins, ParA and ParB. In the first step of partition by the archetypical P1 system, ParB binds a complexed centromere-like site to form a large nucleoprotein segrosome. ParB is a dimeric DNA-binding protein that can bridge between both A-boxes and B-boxes located on the centromere. Its helix-turn-helix domains bind A-boxes and the dimer domain binds B-boxes. Binding of the first ParB dimer nucleates the remaining ParB molecules onto the centromere site, which somehow leads to the formation of a condensed segrosome superstructure. To further understand this unique DNA spreading capability of ParB, we crystallized and determined the structure of a 1:2 ParB-(142–333):A3-B2-box complex to 3.35 Å resolution. The structure reveals a remarkable four-way, protein-DNA bridged complex in which both ParB helix-turn-helix domains simultaneously bind adjacent A-boxes and the dimer domain bridges between two B-boxes. The multibridging capability and the novel dimer domain-B-box interaction, which juxtaposes the DNA sites close in space, suggests a mechanism for the formation of the wrapped solenoid-like segrosome superstructure. This multibridging capability of ParB is likely critical in its partition complex formation and pairing functions.

Partition or segregation is the process whereby the genetic material in a cell is accurately moved and positioned to daughter cells during cell division. The segregation of prokaryotic and plasmid DNA is typically mediated by functionally homologous par systems comprising three essential elements, a cis-acting centromere-like (partition) DNA site and the ParA and ParB proteins (1–6). The Escherichia coli P1 plasmid partition apparatus has served as a prototype for the study of partition. The P1 plasmid is the prophage of bacteriophage P1 and exists as a stable, autonomously replicating unit (7). The P1 par system is encoded by a 2.5-kb region of the plasmid, the par operon, which contains the genes for the ParA (44 kDa) and ParB (38 kDa) proteins and the centromere-like site, also called the partition site parS, which is located downstream of parB (4–6). Biochemical and molecular biological studies compiled to date suggest a P1 partition model that includes several steps. In the first step, ParB binds parS to form the so-called partition complex or segrosome (8–11). It is believed that pairing between two plasmids occurs next. In this step, interactions between ParB-ParB and ParB-DNA would lead to a "trans" segrosome complex to mediate this pairing event (12). P1 ParA, a Walker A protein, then binds the partition complex (but only in its ATP-bound conformation) and drives plasmid separation (13, 14).

The centromere-like site parS, which is bound by ParB, is a surprisingly complicated DNA-binding site containing four copies of a heptamer sequence called the A-box (with consensus (G/T)TGAAAT) and two copies of a hexamer site, the B-box (with consensus TCGCCA) (9, 15–19). Both box motifs are recognized by ParB and are located at the ends (5′ and 3′) of the parS site. Thus, the parS site can be divided into three main regions, left, central, and right (see Fig. 1A). These left and right portions flank a central region, which is bound by the integration host factor (IHF). When IHF binds to the central site, it creates a large bend in the DNA (20). DNA bending by IHF can be partially replaced by intrinsically bent DNA, indicating that the role of IHF in this complex is just to bring the left and right sides of parS into close proximity, thus allowing ParB to bind the A-box and B-box elements across the bend to each arm (17). Importantly, IHF is not absolutely required for P1 partition, and the right side of parS (parS-small), which contains two A-boxes (A2 and A3) and one B-box (B2), can serve as a minimal centromere-like site or ParB nucleation site and is active for partition. However, partition in the absence of IHF is less efficient (20). Once the initial partition complex is formed between a dimer of ParB, IHF and the parS site (termed the “1 + B1” complex), the remaining ParB molecules in the cell all appear to load onto and the around the centromere site (~500 bp on each side), leading to the creation of a large nucleoprotein superstructure known as the segrosome (21, 22). Consistent with this, the cellular localization of ParB molecules to discrete foci coincident with the segrosome can be visualized by immunofluorescence spectroscopy (11).

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‡ The abbreviations used are: IHF, integration host factor; HTH, helix-turn-helix; MES, 4-morpholineethanesulfonic acid.

The atomic coordinates and structure factors (code 2NTZ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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P1 ParB is a 333-residue protein that shows only limited sequence homology to other ParB homologues. However, all ParB homologues are modular in their arrangement, consisting of several flexibly attached domains. Indeed, P1 ParB is proteolytically labile and can be digested into multiple fragments (19, 23). The N-terminal region of P1 ParB, residues 1–141, is bound by ParA. This region is highly flexible as evidenced by the fact that limited exposure of ParB to proteases leads to rapid degradation of this part of the protein (23). A relatively stable P1 ParB proteolytic fragment that remains after tryptic digestion corresponds to residues 142–333. It has been shown that this region of ParB, called ParB-(142–333), contains all of the determinants required for parS binding and partition complex formation (19). Our recent crystal structures of ParB-(142–333), bound to the minimal centromere parS-small, revealed novel and unexpected DNA-binding characteristics of ParB that suggest how it can bind the complicated parS centromere-like site (24). Specifically, the structures showed that ParB-(142–333) forms an asymmetric dimer with extended N-terminal HTH domains that interact with the parS A-box elements. The two HTH domains emanate from a dimerized dimer domain that contacts the B-box motifs. The dimer domain consists of three β-strands and a C-terminal α-helix, which in the dimer combine to form a six-stranded β-sheet coiled coil. The HTH domain and dimer domain are linked by a short and highly flexible linker (residues 271–274) that permits essentially free rotation of the domains, thus explaining the ability of ParB to interact with box elements arranged in a variety of orientations, as found in parS. The structures, which showed that ParB can act as a bridging factor, indicated how ParB may bind across the bent full-length parS site and aid in plasmid pairing.

A poorly understood but critical aspect of the partition process is the creation, by ParB-DNA interactions, of a large condensed DNA superstructure called the segrosome. Formation of this complex involves the nucleation of the thousands of ParB molecules in the cell onto the DNA near and around the centromere-like site. Previous models had suggested that contacts between ParB and the two parS B-boxes would likely be important in the formation of solenoid-like partition complexes and would also likely play a role in the ability of ParB to spread onto DNA to form large nucleoprotein superstructures (19). Although, the ParB-(142–333)-parS-small structures revealed DNA bridging between two A-boxes and between an A-box and a B-box, a “double B-box interaction” was not observed (24). A model of the ParB-(142–333) dimer domain bound to two A-boxes showed a very close approach of the two DNA molecules, suggesting that this DNA-binding motif may only be capable of an interaction with a single B-box. Alternatively, such an interaction may be possible, but it was not observed in our ParB-parS-small structures due to the 2:1 stoichiometry of ParB dimer to B-box in these complexes (24). Thus, to determine whether ParB can bind two B-boxes simultaneously and gain further insight into the mechanism of P1 segrosome formation, we crystallized and determined the structure of a complex of ParB-(142–333) bound to a 16-mer DNA site corresponding to the A3-B2 right side region of full-length parS in a ratio of one ParB-(142–333) dimer to two DNA duplexes. The structure shows that, as in the previous structure, the ParB HTH domains interact simultaneously with two A-boxes located on two different DNA duplexes. However, in this structure, each face of the ParB dimer domain contacts a separate B-box from distinct DNA duplexes. This dimer domain-double B-box interaction represents a new type of protein-nucleic acid interaction and suggests a mechanism for how ParB may form condensed nucleoprotein complexes. Specifically, the structure shows that this bridging interaction juxtaposes the two bound DNA molecules in very close proximity and at sharp angles relative to each other. This remarkably tight bridging structure suggests a model for how ParB may wrap the DNA around itself to form the solenoid-like nucleoprotein superstructures that have been suggested to be important in partition (19). This unique multibridging feature of a protein-DNA complex observed in the ParB-16-mer structure may also serve as a model in understanding higher order protein-nucleic acid superstructures relevant to chromatin structure in higher organisms.

**EXPERIMENTAL PROCEDURES**

**Crystallization and Data Collection**—To crystallize a ParB dimer bound simultaneously to two A-boxes and two B-boxes, we utilized several oligonucleotides that encompass the rightmost side of parS, which contains one A-box (A3) and one B-box (B2). We mixed these oligonucleotides with the ParB-(142–333) dimer at a ratio of 2 DNA duplexes to 1 ParB-(142–333) dimer. Based on modeling, we predicted that the packing may be improved by the closer approach of the dimer domain-double B-box interaction compared with our previous crystallization studies with ParB and parS-small oligonucleotides. Indeed, after crystallization trials with 35 such duplexes, we obtained 20 different crystal forms, one of which diffracted to beyond 3.5 Å resolution. These crystals were grown by combining ParB-(142–333) with the blunt 16-mer, 5’-CGT-GAAATCGCCACGA-3’ (see Fig. 1A) in a 1:2 stoichiometry and mixing equal volumes of the macromolecular solution with a reservoir consisting of 7% 2-methyl-2,4-pentanediol, 0.1 M NaCl, 0.1 M MES, pH 6.5, and 15 mM MgCl₂. These crystals are trigonal: P3₂₁, a = b = 144.4 Å, c = 78.8 Å. For cryoprotection, glycerol was added to the drop to a final concentration of ~20%, and the crystals were flash frozen in a nitrogen cryostream. Data for both a selenomethionine-ParB-(142–333)-16-mer crystal and a native crystal were collected at the Advanced Light Source (ALS) Beamline 8.2.1 at 100 K, processed with MOSFLM and scaled with SCALA.

**Structure Determination and Refinement**—To determine the structure of the ParB-16-mer complex by molecular replacement, the individual DNA-binding modules from the 2.98-Å resolution ParB-DNA structure (24) were broken into separate search models and used in the program Evolutionary Programming for Molecular Replacement (EPMR) (25). We started with the HTH domain-A-box search model. After EPMR correctly positioned this fragment, it was used as a static model to start a second EPMR search for a second HTH domain-A-box complex. After both HTH domain-A-box complexes were located, they served as a static starting model, and the dimer domain fragment was located again using EPMR. The crystallographic asymmetric unit consists of one ParB-(142–333) dimer and two
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Table 1: Selected crystallographic data

| Property                        | Value 1 | Value 2 | Value 3 |
|---------------------------------|---------|---------|---------|
| MAD phasing statistics          |         |         |         |
| Wavelength (Å)                  | 0.9797  | 1.0200  | 0.9796  |
| Resolution (Å)                  | 129.10–3.62 | 129.10–3.57 | 129.10–3.62 |
| Overall Rmerge(%)               | 2.5 (53.4) | 6.0 (55.3) | 5.4 (51.4) |
| Overall Rwork (%)               | 6.2 (1.5) | 4.7 (1.3) | 6.1 (1.4) |
| No. of total reflections        | 35,766  | 39,766  | 50,701  |
| No. of unique reflections       | 10,910  | 11,455  | 10,915  |
| Multiplicity                    | 4.7     | 3.5     | 5.4     |
| Overall figure of merit         | 0.490   |         |         |

Refinement statistics

| Feature                      | Value 1        | Value 2        | Value 3        |
|------------------------------|----------------|----------------|----------------|
| Space group                  | P3,21          |                |                |
| a = b = c = 144.42, 78.88   |                |                |                |
| Resolution (Å)               | 129.10–3.35    |                |                |
| Overall Rmerge(%)            | 4.9 (33.3)     |                |                |
| No. of total reflections     | 54,307         |                |                |
| No. of unique reflections    | 15,703         |                |                |
| Percent complete             | 98.2 (94.0)    |                |                |
| Rwork/Rmerge(%)              | 28.8/33.6      |                |                |
| Root mean square deviation   |                |                |                |
| Bond angles (°)              | 1.80           |                |                |
| Bond lengths (Å)             | 0.012          |                |                |

16-mers, and when all three solutions were combined and the two B-boxes were located (in difference Fourier maps), the DNA formed the two continuous 16-mer DNA sites. The model was subjected to rigid body refinement (26).

The correctness of the model was further confirmed by utilizing phases derived from multiple wavelength anomalous diffraction data obtained from a selenomethionine-ParB-(142–333)-16-mer crystal (Table 1). Briefly, the positions of the selenomethionines were first obtained from the molecular replacement model (after several rounds of refinement with multiple wavelength anomalous diffraction data) (26). The selenium positions were then used for phases, and after density modification, an improved electron density map was calculated. This unbiased map was used to rebuild short regions of the model and also revealed density for N- and C-terminal residues, not present in the original model, for one ParB subunit. This final model was then refined by simulated annealing and xzy refinement in CNS resulting in an Rfree, of 33.6% to 3.35 Å resolution (Table 1) (26). The final model contains one ParB dimer (residues 144–270:275–332 of one subunit and 150–269:275–326 of the other subunit) and all 16 bp of both parS-16-mer duplexes and shows excellent stereochemistry (28).

Fluorescence Polarization Studies of B-box Binding by ParB Dimer Domain Mutants—Fluorescence polarization measurements were collected with a PanVera Beacon 2000 fluorescence polarization system. Samples were excited at 490 nm, and fluorescence emission was measured at 520 nm. A 5'-fluoresceinated parS-small deoxy-oligonucleotide duplex (F-ATT-TCAAGGTTAATCGCCA, where F = 5'-fluorescein label) was used in the binding studies, which were carried out by titrating either the ParB-(142–333) wild-type protein or mutants (K287A, R298A, and R306A) into a 0.990-mL reaction buffer (25 mM Tris, pH 7.5, 100 mM NaCl, 2.5% glycerol) containing 1 nm fluoresceinlabeled DNA. The resulting data were fit to a simple bimolecular binding model by nonlinear regression (29).

Plasmid Stability Assays—Wild-type and plasmid stability assay was used to test the ability of the ParB-(142–333) mutant proteins, R298A and R306A, to stabilize the pBEF246 mini-P1 plasmid, which is deleted for parOP, para, and parB but retains parS. In this assay, ParA and ParB are provided in trans from the plasmid pEF5 and the para and parB genes under the control of the β-lactamase promoter called blapF (13). Vector pBR322 is used as a control. The C-terminal regions of the ParB-(142–333) mutants were swapped into pEF5 to place them in the context of the full-length protein, and the resulting mutations were confirmed by sequencing. The stability assay was that reported by Fung et al. (13). Briefly, fresh colonies of DH5 containing pEF5 or pBR322 and pBEF246 were grown for several generations in LB medium with ampicillin and chloramphenicol and then diluted several thousand-fold into medium containing only ampicillin and allowed to grow overnight. The cultures were sampled at the beginning and the end of the growth in ampicillin medium by plating on LB/amp plates. The colonies were then transferred by toothpicks onto medium with chloramphenicol to see whether the original cells contained pBEF246. The retention values reported are percentages of cells with mini-P1 at the end compared with the beginning (over ~18–20 generations). The data reported are the average of three experiments.

Results and Discussion

Overall Structure of ParB-(142–333)-16-Mer parS Site—To obtain a structure of a ParB-(142–333) dimer bound simultaneously to two B-boxes and two A-boxes, we crystallized ParB-(142–333) dimer-DNA in a ratio of 1:2 using a 16-mer DNA site containing one A-box and one B-box based on the parS-small A3–B2 region and solved the structure by molecular replacement (Fig. 1A). Because previous ParB-DNA structures revealed that the DNA-binding domains are flexibly attached, the individual domains and their bound DNA sites were used separately as search models in molecular replacement (see “Experimental Procedures”). After the two HTH domain-A-
Box fragments and the dimer domain were located, refinement commenced. After minimal positional refinement, a difference Fourier revealed clear density for the bound B-boxes, which are bound by both “faces” of the dimer domain. The tracing was also aided by phases obtained from multiple wavelength anomalous diffraction data collected on a crystal grown using selenomethionine-substituted ParB-(142–333). This experimental map also revealed clear density for both B-boxes in the crystallographic asymmetric unit, consistent with the initial difference Fourier map. Thus, the crystallographic asymmetric unit consists of one ParB-(142–333) dimer and two 16-mer DNA duplexes in which the A-boxes of each site are bound by an HTH domain from each ParB-(142–333) subunit and where the B-box motifs are each bound to one side or face of the dimerized dimer domain (Fig. 1B).

**ParB-A-box Interactions**—The HTH domain of ParB is solely responsible for mediating contacts with the A-box. The majority of the contacts are provided by the residues from the HTH motif, which is composed of α2–α3 (residues 168–189). Comparisons of the HTH motif of ParB to HTH motifs found in other proteins reveal that the ParB HTH is essentially a canonical prokaryotic HTH motif, in that it docks onto the DNA such that the N-terminal region of the recognition helix is inserted into the major groove. Interestingly, most of the thirteen contacts made by the HTH domain to the A-box are to the phosphate backbone. These phosphate contacts are tightly clustered on either side of the A-box, thus permitting precise docking of the domain onto the DNA (Fig. 2A). The side chains of Thr-183 and Arg-184 from the recognition helix provide the only two base interactions. However, only the contact from Arg-184, which is to G(4) appears specific. Three residues outside the HTH motif contribute to the anchoring phosphate contacts at either end of the DNA site. The side chain of Lys-162 from the C terminus of ParB contacts phosphate 8, and the side chain of Thr-206 and the amide nitrogen of Phe-207, both from the N terminus of ParB, interact with phosphate group 1 (see Fig. 2).

The only structure that shows some homology to P1 ParB is that of KorB, the ParB homologue from plasmid RP4 (30). This structure includes only the HTH-containing DNA-binding domain, and the dimer domain of KorB does not interact with DNA. KorB recognizes a centromere-like site, consisting of 12 different 13-bp inverted repeats, which is very different from the P1 centromere parS. The structure of the KorB DNA-binding domain was solved bound to a 17-bp DNA site containing one centromere-like repeat (30). Comparison of P1 ParB-(142–333) with the KorB DNA-binding domain structures reveals that three helices from the HTH domain, including the two helices of the HTH, are structurally conserved between these proteins (24, 30). The C-α atoms of residues from these helices, corresponding to α1–α3 of P1 ParB, superimpose with the root mean squared deviation of 1.3 Å. Outside these helices, however, there are no structural homologies between the two proteins.
teins. Moreover, the HTH domains of each protein recognize their cognate DNA sites in very different manners. Specifically, the only base contacts observed in the P1 ParB HTH-A-box interaction are provided by residues from the recognition helix. In contrast, KorB utilizes residues outside its recognition helix, namely Thr-211 and Arg-240, to specify bases in its DNA site. Also distinct from the P1 ParB HTH-A-box interaction, these base contacts ensure high specificity in the KorB-operator interaction (30). Therefore, although the HTH of related ParB-like proteins may be conserved structurally, it appears that the motif will likely interact in unique manners with its respective cognate DNA site in each case. This suggests a partial explanation for how related ParB-like proteins with apparently structurally similar HTH domains can recognize such remarkably diverse DNA sites. Also contributing to diversity are additional DNA-binding regions, such as the dimer domain of ParB (see below).

The DNA bound by KorB appears mostly B-DNA in nature. Interestingly, the P1 ParB-bound A-box, which is A/T-rich, displays a distinctly narrowed minor groove (3 Å width compare with 6 Å for B-DNA). Because narrow minor grooves are a characteristic of DNA A/T tracts, indirect readout may play a role in the ParB interaction with the A/T-rich A-box (31). In that regard, it is notable that the DNA surrounding the parS site is extremely A/T-rich. These combined findings suggest that the HTH domain may play a role in nonspecific spreading onto the A/T-rich DNA surrounding parS. Such nonspecific spreading interactions could contribute to the formation of higher order nucleoprotein complexes.

The ParB Dimerized Dimer Domain; New DNA-binding Motif—Dimerization of the ParB dimer domain creates an extensive hydrophobic core comprising Leu-281, Phe-284, Phe-304, Leu-307, and Leu-323. Mutational analyses found these residues are critical for partition (32). Moreover, these and other studies revealed that ParB dimerization is essential for its DNA-binding function (19, 32). Our ParB-DNA structures show that this is because this region must dimerize to form a functional DNA-binding module. Notably, this dimerized module, which contacts the B-box, represents a completely new class or category of DNA-binding motifs. Based on the homology of the Dimer region to other P1-like ParB proteins, it
is likely that this DNA-binding module is found in P1-like ParB proteins and mediates critical species-specific DNA-binding interactions (see below). Whether this motif is found in DNA-binding proteins outside the P1 ParB family remains to be determined. However, structural homology search programs failed to find any protein, DNA-binding or otherwise, with significant structural homology to the P1 ParB-dimerized dimer domain. Only weak topological homology is observed between the ParB dimer domain monomer subunit and the N domain from λ integrase (33). In particular, the λ integrase N domain contains a three-β-strand:one-α-helix topology similar to the monomeric unit of the P1 ParB dimer domain. However, the sheets of each protein are arranged in completely different orientations relative to the helix, and thus, the β-β-α units of the two proteins cannot be superimposed.

In addition to representing a new fold for a DNA-binding module, the manner in which the dimer domain motif interacts with DNA is unique and presents a mechanism for how ParB may mediate DNA condensation. Specifically, residues located on the β-strand tips and connecting loops of the dimer domain mediate base contacts while the N termini of the helices anchor backbone (Figs. 1 B and 2 B). In the dimer, the β-fingers are arranged on opposite ends of the module, and in the ParB-16-mer structure, each of these β-fingers engages a separate B-box element. In this interaction, each face of the dimer domain interacts with B-boxes that could be arranged as direct repeats on a single looped DNA molecule, identical to the arrangement observed in full-length parS. However, each β-finger motif could also interact with sites on separate DNA molecules. In each case, this interaction would lead to the juxtaposition of the two DNA sites in surprisingly close proximity and at sharp angles relative to each other. Thus, this DNA-binding module is perfectly suited to function in the formation of the wrapped solenoid-type nucleoprotein superstructures, which have been predicted to be key in segregosome assembly.

Unlike the HTH domain, the dimer domain makes specific base contacts to a C/G-rich box called the B-box. The dimer domain is optimally docked for B-box recognition by hydrogen bonds and helix dipole interactions from the N terminus of one helix of the coiled coil (Fig. 2 A). Anchored in this manner, residues from one end of the β-sheet reach deep into the major groove to make base-specific contacts. G(12′) of each B-box is contacted by Arg-298, which is located in the turn between β2 and β3. (Fig. 2). The remaining base contacts are provided from the other subunit; Arg-306 reads both O6 atoms of G(10) and G(11′) via its NH1 and NH2 groups, and both bases of bp9 are specified by Asp-288 and Lys-287 (Fig. 2). Asp-288 contacts the C(9) N4, whereas Lys-287 contacts the O6 atom of G(9′) (Fig. 2 B). These extensive ParB-B-box interactions lead to a widening of the B-box DNA major groove to 13 Å (compared with 11 Å for B-DNA).

Swapping experiments between the homologous P1 and P7 par systems revealed that the region corresponding to P1 ParB residues 281–290 provides critical species specificity in partition site recognition in this subclass of par systems (34–37). Thus, this region was termed the “discriminator recognition sequence.” Besides P7, the Yersinia pestis pMT1, Salmonella typhimurium pSLT, and Salmonella flexneri pWR501 par systems have proteins and partition sites homologous with the P1 system. Our structures reveal the basis for the discrimination. Specifically, the structures show that P1 ParB discriminator recognition sequence residues Lys-287/Asp-288 specify the 5′ B-box bp, whereas Arg-298 and Arg-306, which are outside the discriminator recognition sequence, recognize the 3′ B-box bp C(11′)C (12). Consistent with these findings, all of the partition sites of P1-like systems have C(11′)C(12) bp and ParB proteins with arginines, corresponding to P1 ParB Arg-298 and Arg-206. However, only the discriminator recognition sequence regions of the pSLT and pWR501 ParB proteins have the identical Lys-287/Asp-288 pair found in P1 ParB and bind B-boxes with the same 5′ sequences.

**Contributions of Dimer Domain Residues in Specific B-box Interactions**—Our combined ParB-DNA structures indicate that the dimer domain represents a new category of DNA-binding motifs, which appears to bind G/C-rich DNA sites with high specificity. In fact, despite the limited resolution (3.35 Å) of our ParB-16-mer structure, the electron density is most clearly delineated for the residues of the dimer domain that interact with B-box bases (Fig. 3 A). To further test the contribution of individual base-specifying residues Lys-287, Arg-298, and Arg-
306 to B-box binding, the residues were substituted singly to alanine. The DNA-binding affinities of the resulting mutant proteins for B-box DNA were examined by fluorescence polarization and compared with wild-type proteins (29).

All of the mutant proteins behaved like the wild-type protein during purification, and circular dichroism experiments revealed that the secondary structures of these proteins are identical to that of the wild-type protein (data not shown). Fluorescence polarization experiments were carried out with a fluoresceinated parS-small site (see “Experimental Procedures”). The results, which reveal ~10-fold reduction in binding upon mutation of these residues, clearly indicate their importance in DNA binding. Moreover, these reductions in binding are on the order of ~1.5 kcal/mol within the range of one to two hydrogen bonds, as observed in our structures. Representative binding isotherms are shown in Fig. 3B. The resulting $K_d$ values were for wild type (▴) 49 ± 7 nM, for K287A (■) 335 ± 72 nM, for R298A (▲) 403 ± 72 nM, and for R306A (✦) 519 ± 88 nM.

**Importance of B-box-interacting Residues on Plasmid Segregation**—Our structural and biochemical data indicate that the dimer domain residues Lys-287, Arg-298, and Arg-306 are important for B-box binding. However, to begin to address the importance of these residues in plasmid segregation in cells, we carried out plasmid stability assays utilizing ParB proteins that carried the R298A and R306A mutations (see “Experimental Procedures”). The R298A and R306A mutant proteins were chosen for these assays, because unlike Lys-287, they are entirely responsible for specifying given bp in the ParB-B-box interaction. Specifically, Arg-298 contacts G(12′), and Arg-306...
Structure of Multibridged ParB-DNA Complex

ParB-DNA Structures and Mechanism of Segrosome Assembly—Our previous two structures of ParB bound to parS-small sites indicated that the flexible linker (residues 271–274) permits rotation of the HTH domain relative to the dimerized dimer domain, readily explaining how ParB can bind multiple arrangements of box elements on the IHF-bent parS site. Our current ParB-16-mer structure provides additional views of this remarkable flexibility, supporting our hypothesis that the linker allows essentially free rotation of each domain relative to the other (Fig. 4). One important prediction from this finding is that, when the first ParB molecule binds the bent parS site, it likely can bind in multiple arrangements. For example, one ParB dimer may bind to A1 on the right arm and either A2, A3, or B2 on the left arm. Also possible would be several permutations in which ParB binds B1 on the right arm and either A2, A3, or B2 on the left arm. This initial binding event appears to take place with high affinity and is followed by the loading of the additional ParB molecules in the cell onto the looped site via both specific and nonspecific contacts to the DNA. Specific contacts would be mediated by HTH domain interactions with the remaining A-box elements and dimer domain contacts to unbound B-box motifs. Finally, nonspecific contacts would permit ParB spreading and DNA condensation. In fact, ParB is observed to spread ∼500 bp up and downstream of parS. The nonspecific binding would be relatively low affinity compared with the specific interactions; however, they may be aided by tethering contacts between ParB N-domains, which have been shown to form transient higher order oligomers (23). The formation of these oligomers would act to anchor ParB molecules near the DNA to permit low affinity binding.

The flexible attachment of the two DNA-binding motifs would also be essential to further link and bridge DNA elements on the same or different P1 plasmids. The use of DNA bridging to facilitate DNA condensation is also observed in the barrier-to-autointegration factor (BAF), which binds DNA in a sequence-independent manner and functions in retroviral integration. The recent structure of BAF bound to a 7-mer DNA site revealed that BAF is a dimer with HTH DNA-binding domains located on each end of the dimer (38). The placement of the HTH domains allows them to bridge between DNA similar to how the HTH domains of ParB bridge between A-boxes. Unlike BAF, which can only bridge between two DNA sites, ParB has the remarkable ability to bridge between potentially four distant DNA sites using two DNA-binding motifs with distinct specificities. This ability of ParB to contact DNA elements with two distinct sequences, A/T-rich for the HTH domain and G/C-rich for the dimer domain, suggests a model for assembly of the nucleoprotein segrosome. In this model, DNA condensation takes place when ParB binds between distant regions of the DNA chain. Subsequent binding by additional ParB dimers to other regions of the DNA would then collapse the DNA or colocalize independent DNA molecules (19). In this process, the HTH domain and the dimer domain would fulfill different roles. Specifically, because the regions surrounding parS are notably A/T-rich, the HTH domains would likely function in spreading and long distance DNA condensation by binding and bridging somewhat nonspecifically to the numerous A/T-rich sites dispersed within the region encompassing the parS site, whereas the dimer domain would contact the less numerous B-box-like regions surrounding the parS site, aiding in the collapse and colocalization of highly condensed nucleoprotein regions.

In conclusion, the structure of the ParB-16-mer complex has revealed a unique four-way bridged protein-DNA complex. Within this complex are two distinct DNA-binding elements that not only harbor distinct DNA sequence preferences but also mediate different DNA bridging and condensation properties. These properties suggest a model for overall segrosome formation involving distant spreading and bridging interactions primarily mediated by the ParB HTH domains and subsequent collapse of DNA into highly condensed superstructures, the latter achieved predominantly by the novel dimer domain-DNA interaction. Finally, this mechanism of DNA condensation may aid in understanding higher order protein-nucleic acid superstructures relevant to chromatin structure in higher organisms.

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