Structure and Function of DnaA N-terminal Domains

SPECIFIC SITES AND MECHANISMS IN INTER-DnaA INTERACTION AND IN DnaB HELICASE LOADING ON oriC

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DnaA forms a homomultimeric complex with the origin of chromosomal replication (oriC) to unwind duplex DNA. The interaction of the DnaA N terminus with the DnaB helicase is crucial for the loading of DnaB onto the unwound region. Here, we determined the DnaA N terminus structure using NMR. This region (residues 1–108) consists of a rigid region (domain I) and a flexible region (domain II). Domain I has an ααββαα motif, similar to that of the KH homology (KH) domain, and has weak affinity for oriC single-stranded DNA, consistent with KH domain function. A hydrophobic surface carrying Trp-6 most likely forms the interface for domain I dimerization. Glu-21 is located on the opposite surface of domain I from the Trp-6 site and is crucial for DnaB helicase loading. These findings suggest a model for DnaA homomultimer formation and DnaB helicase loading on oriC.

In Escherichia coli, DnaA initiates the replication of genomic DNA in a cell cycle-coordinated manner (1, 2). The initiation complex contains a homomultimer of DnaA protein and the replication origin, oriC, of the chromosome. DnaA binds cooperatively to the oriC region in a manner depending on tight binding to the DnaA box sequences within the oriC region. The initiation complex containing ATP-bound DnaA molecules unwinds the duplex of the AT-rich 13-mer repeats within oriC, forming an open complex. This leads to the loading of DnaB helicase onto single-stranded DNA (ssDNA) in the presence of the DnaC helicase loader. In this process, the interaction between DnaA and DnaB is crucial. DnaA creates a specific nucleoprotein structure within which ordered structural DNA changes and protein-protein interactions take place. DnaG primase then complexes with the ssDNA-loaded DnaB, which leads to DNA chain synthesis by the DNA polymerase III holoenzyme. The DNA-loaded β-clamp subunit of the holoenzyme complexes with Hda, a DnaA paralogue protein, and this complex promotes DnaA-ATP hydrolysis, yielding inactive ADP-DnaA (3–5).

DnaA is a 52-kDa basic protein that has four distinct functional domains (1, 2). NMR and crystal structure analyses have revealed that C-terminal domain IV of E. coli DnaA has a helix-turn-helix motif, which binds the DnaA box specifically (6–8). The C-terminal domain III contains ATP binding/hydrolysis motifs of the AAA + ATPase family (9–14). Erzberger et al. (9) proposed an oligomeric structure for DnaA based on crystal structures of the ATP- and ADP-bound forms of DnaA domains III–IV from the hyperthermophilic bacterium Aquifex aeolicus. In this model of the initiation complex, the ATP-DnaA molecules assemble in a head-to-tail manner, and the resultant oligomers form a spiral helix, consistent with the known features of the AAA + family proteins (11, 15). The structure-function relationships of the N-terminal domains I and II of DnaA remain obscure.

The amino acid sequence of domain I (residues 1–86 in E. coli DnaA; Fig. 1A) is highly conserved among DnaA homologs of eubacterial species, unlike that of domain II (residues 87–134 in E. coli DnaA) (2). The structure of domain II is suggested to be a flexible linker, part of which is dispensable for DnaA function. Domain I functions in DnaA oligomerization and DnaB helicase loading. A deletion analysis of domain I suggests that a region containing residues 1–77 carries a crucial site for DnaA oligomerization (16). The DnaA W6A mutant protein is inactive in initiation, whereas its affinities for oriC and ATP/ADP are sustained (17, 18). Chemical linking experiments suggest that the activity to form oligomers is somehow reduced in this mutant protein. The mechanism of interdomain I interaction remains unclear.

DnaA directs DnaB loading onto the ssDNA region in the presence of DnaC (19). Analyses of domain I-truncated mutants suggest that a region containing residues 1–62 is required for direct binding to DnaB during the loading process (20), whereas the region containing residues 24–86 is required for physical contact with DnaB (21). The specific residues required for DnaB loading remain to be determined. In addition, DnaB loading activity is prevented by anti-DnaA antibodies that specifically bind to the domain III N terminus (residues 111–148) (22, 23). DnaA interaction sites in
domain I and domain II may play specific roles in the steps for DnaB loading (20, 21). In eukaryotic genome replication, the origin recognition complex interacts with the MCM helicase (24), suggesting that the interaction of an origin-binding protein with a helicase is a conserved principal mechanism of the initiation process. However, the precise mechanisms of the initiator-helicase interaction and helicase loading remain to be elucidated. To reveal these mechanisms, the structure of the DnaA N-terminal domain and the structure-function relationship within this domain have to be determined.

In the present study, we determined the structure of the E. coli DnaA N terminus (residues 1–108) using NMR analysis. We found a unique residue, Glu-21, that is crucial and specific for DnaB loading. Furthermore, we have demonstrated that domain I carries weak affinity for ssDNA carrying the AT-rich 13-mer within oriC but not for poly(dT) ssDNA. Based on these findings, we propose a novel model of DnaA homo-oligomer formation and DnaB helicase loading, in which a hydrophobic surface containing Trp-6 forms homodimers between the DnaA domain I regions within the larger DnaA multimer, thereby exposing Glu-21 for DnaB binding.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation of the DnaA N terminus—**A 5′-region of the wild-type dnaA corresponding to amino acid residues 1–128 was amplified by PCR. The amplified DNA fragment was ligated into the vector pET-22b(+) (Novagen), and introduced into E. coli BL21(DE3). For the preparation of labeled protein, the transformed cells were cultured in M9 minimal medium containing 13NH4Cl and 13C-labeled glucose. The DnaA N terminus (residues 1–128) was overexpressed from E. coli by induction with isopropyl 1-thio-β-D-galactopyranoside, and the cells were suspended in chilled buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM KCl, 2 mM dithiothreitol, and 10% sucrose), incubated on ice for 1 h in the presence of 0.2 mg/ml lysozyme, and frozen in liquid nitrogen. The frozen cell suspension was melted on ice and centrifuged at 17,000 rpm for 40 min. The resultant supernatants were dialyzed against buffer A (50 mM Tris-HCl, 2 mM dithiothreitol, and 10% sucrose) and loaded onto Toyopearl DEAE-650 M (Tosoh Co.) and Toyopearl CM-650M. The DnaA N terminus was collected in the following fractions after both chromatography. The collected proteins were precipitated by salting-out, using 80% ammonium sulfate, dissolved in and dialyzed against buffer A, and stored at −20 °C.

**NMR Measurements and Structural Calculations—**The NMR spectra were recorded at 25 °C on a Varian Unity INOVA 600 spectrometer. NMR samples (0.8 mM) of the DnaA N terminus were dissolved in buffer B (50 mM sodium phosphate (pH 6.5), 20 mM EDTA, 40 mM KCl, 2 mM dithiothreitol, and 10% sucrose) containing 100% D2O or 90% H2O, 10% D2O. A series of three-dimensional double and triple resonance experiments (HNHA, HNCA, H(N(CO)CA; HNHB, HNCB, H(CO)NH, HNCO, HN(CA)CO, (H)CC(CO)NH, HCC-HOCSY, HCC-COSY, and 15N-edited TOCSY) were recorded for the spectrum assignments of the DnaA N terminus.

For the structural determination, the stereospecific assignments of the methyl groups of the leucine and valine residues were obtained from the 1H-13C HSQC spectrum recorded on a 13C biosynthetically directed labeled sample (26). The pulse sequence used to measure the 1H-13N heteronuclear NOEs (nuclear Overhauser enhancements) was described by Grezeieck and Bax (27). The NOE data from the DnaA N terminus (residues 1–108) were obtained from 1H NOESY and 13N- or 1H3C-edited NOESY experiments carried out with a mixing time of 150 ms. NMRpipe (28) was used to process the data and to assign the resonance peaks. The assignments of the resonance peaks of each amino acid residue were carried out using Olivia (fermi.pharm.hokudai.ac.jp). CYANA (version 2.1) (30) was used to calculate the structures. A total of 107 ϕ and ψ values were estimated on the basis of the HN, C′, Co, and Cβ chemical shifts using TALOS (31). 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 1. A total of 200 structures were finally obtained, and the mean coordinates were obtained by averaging the coordinates of the 20 structures with the lowest energy. Ramachandran analysis was performed using the PROCHECK program (32). Structure figures were generated using PyMOL (www.pymol.org) and MOLMOL (34). The domain I structure and poly(C)-binding protein 2 (PCBP2) were superimposed using the Insight II package (Accelrys Inc.). The coordinates used for the ensemble of NMR structures have been deposited in the PDB under accession code 2E0G. The tables have been deposited in BioMagResBank (www.bmrb.wisc.edu) under accession number 10027.

ssDNA and Salt Titration—The 28-mer ssDNA (5′-GATCTGTCTATTGTGATCTTATTAG-3′) bearing the oriC 13-mer repeats and the 28-mer poly(dT) were purchased (Invitrogen) and purified before conducting NMR titration. We recorded the 3H-15N HSQC (heteronuclear single quantum correlation) spectra of 0.1 mM 15N-labeled protein in the presence of various concentrations of KCl (up to 160 mM) or of the indicated ssDNA (up to 0.6 mM). The respective concentrations of the proteins and the oligonucleotides or salt were corrected for dilution. The overall volume changes amounted to less than 10%.

Construction, Purification, and Functional Assays of the Site-specifically Mutated DnaA—Mutant DnaA proteins were constructed by a site-directed mutagenesis method as we described previously (12, 13). The sequences of the mutagenic primers used are listed in supplemental Table 1. The overexpression, purification, and assays of nucleotide binding activities and
minichromosomal replication activities using a crude replica-
tive extract and a system reconstituted with purified proteins,
P1 nuclease assay, and ABC primosome assay were performed
as we described previously (12, 13, 35).

The form I* formation assay was performed as described by
Marszalek
et al. (23) with minor modifications. Briefly, DnaA
was incubated at 30 °C for 30 min in buffer (25 µl) containing 20
mM Tris-HCl (pH 7.5), 0.1 mg/ml bovine serum albumin, 8 mM
dithiothreitol, 10 mM Mg(OAc)₂, 125 mM potassium glutamate,
2 mM ATP, 2.32 g of single strand-binding protein (SSB), 2.5
ng of HU protein, 150 ng of DnaB, 92 ng of DnaC, 180 ng of
DNA gyrase A subunit, 225 ng of DNA gyrase B subunit, and
200 ng of M13KEW101 minichromosome. The reaction was
stopped in the presence of SDS and EDTA followed by 0.7%
agarose gel electrophoresis.

A reconstituted, staged regulatory inactivation of DnaA
(RIDA) reaction system was used basically as described previ-
ously (5, 36), except that we used Hda tagged with His₆ at the C
terminus. Briefly, the clamps (100 pmol as dimer) were incu-
bated at 30 °C for 20 min in buffer containing 20 mM Tris-HCl
(pH 7.5), 10% glycerol, 8 mM dithiothreitol, 0.01% Brij-58, 8 mM
Mg(OAc)₂, 120 mM potassium glutamate, 2 mM ATP, 0.1 mg/ml bovine serum albumin, 56 fmol of the
C-terminally His-tagged Hda, and [α-³²P]ATP-DnaA (0.25 pmol).
Nucleotides bound to DnaA were recovered by filter retention,
separated by thin-layer chromatography, and quantified by a
BAS2500 bioimaging analyzer (Fuji Film).

RESULTS

The Structure of the DnaA N Terminus—To determine the
structure of the DnaA N terminus using NMR analysis, we
labeled amino acid residues 1–108 with ¹⁵N and recorded the
¹H-¹⁵N HSQC spectrum at 25 °C and pH 6.5. The spectrum
included separated and dispersed peaks. Therefore, we
assigned the ¹H, ¹⁵N, and ¹³C chemical shifts of the DnaA N
terminus using a double-labeled sample and heteronuclear
three-dimensional NMR. We were able to assign most signals
in the DnaA N terminus except for residues 1–3 (BioMagRes-
Bank accession number 10027).

We carried out ¹H-¹⁵N heteronuclear NOE measurements
based on the assignment of the chemical shifts. Because the
peak intensity ratios (I₉/I₉₀) in the domain I region (residues
1–78) are more than 0.8 (Fig. 1C), domain I appears to have a

5 M. Su'etsugu and T. Katayama, unpublished.
rigid structure in solution. In contrast, the peak intensity ratios of more than 0.02 ppm were concentrated in the N-terminal half of domain I monomers. We focused on the hydrophobic interaction between DnaA domain I and II. The hydrophobic interaction is enhanced at higher salt concentrations. We observed that the chemical shift changes of several peaks of domain I occurred as the KCl concentration was increased to a level near the physiological salt concentration (Fig. 3). The $^1$H-$^1$N HSQC spectra of the $^{15}$N-labeled DnaA N terminus overlap in the presence of 40 mM or 160 mM KCl (Fig. 3A). Residues causing chemical shift changes of more than 0.02 ppm were concentrated in the N-terminal $\alpha$1-helix and the loop between $\beta$1 and $\beta$2 (Fig. 3, B and C). Representatives of such residues are Leu-5, Trp-6, Leu-10, and Leu-33, which are exposed on the surface and form a hydrophobic patch (Fig. 3C). DnaA Trp-6 is crucial for weak interactions between DnaA molecules detected in chemical cross-linking experiments (17, 18). Therefore, the observed chemical shift changes supported the idea that a pair of domain I monomers forms a homodimer using the hydrophobic patch as an interface (Fig. 3D).

### Domain I Has a Weak Affinity for the oriC 13-Mer ssDNA but Not for Poly(dT) ssDNA—The KH domain was first characterized biochemically in heterogeneous nuclear ribonucleoprotein K (hnRNPK), a major pre-mRNA-binding protein K, and has since been detected in a number of RNA-binding proteins (40, 41). PCBP2 contains several KH domains that specifically and directly interact with telomere poly(dC)ssDNA (39, 42). Therefore, we used NMR to investigate whether DnaA domain I interacts with ssDNA.

A 28-mer ssDNA bearing the oriC 13-mer sequence caused chemical shift changes in the $^1$H-$^1$N HSQC spectra of the $^{15}$N-labeled DnaA N terminus (residues 1–108; Fig. 4A). As the ssDNA concentration increased to a molar ratio greater than 1.6, several signals were shifted. From the chemical shift changes of these peaks, we estimated the $K_d$ to be ~1 mM (in concentration of the DNA fragment) (Fig. 4B). These results suggest that DnaA domain I has weak affinity for ssDNA carrying the 13-mer sequence. We also plotted the chemical shift changes as a function of the residue number of DnaA at 0.6 mM ssDNA (Fig. 4C). The residues causing chemical shift changes of more than 0.02 ppm are localized in the N-terminal half of the $\alpha$3-helix and the loop between $\alpha$1 and $\alpha$2, a region including...
FIGURE 2. Solution structure of the DnaA N terminus. A, stereo view of superposition of 20 simulated annealing structures of the DnaA N terminus (residues Met-1—Thr-80, PDB code 2E0G). B, ribbon model of the DnaA N terminus represented as three α-helices (α1 (residues 5–16), α2 (21–25), and α3 (47–62)) and three β-sheets (β1 (31–34), β2 (38–41), and β3 (73–78)). C, the hydrophobic residues are represented in green on the ribbon model of the DnaA N terminus. D, superposition of DnaA N terminus (type II KH domain, colored blue) and PCBP2 (PDB code: 2AXY; type I KH domain, colored red). Superposition was performed using the Insight II package (Accelrys Inc.). The regions used in r.m.s.d. minimization were β1, β2, α3, and β3 in the DnaA N terminus and β1, α1, α2, β2, and β3 in PCBP2, respectively. The root mean square deviation is 3.5 Å.
Glu-21 (Fig. 4, D and E). Unlike the oriC 13-mer ssDNA, poly(dT) ssDNA did not cause any chemical shift changes (Fig. 4B and supplemental Fig. 3), suggesting that the affinity of domain I for ssDNA is associated with at least some sequence specificity.

Construction and Solubility of Novel DnaA Domain I Mutants—Based on the solution structure and sequence homology of domain I, we selected sites within domain I to mutate for a functional analysis. First, we individually replaced Arg-12, Gln-14, Asp-15, Glu-16, Glu-21, Asn-44, Arg-45, Phe-46, and Lys-54 with alanine using pKA234, an arabinose-inducible DnaA-overproducing plasmid (12, 13). These residues are exposed on the protein surface in solution (Fig. 5; supplemental Fig. 1), and some are structurally affected by ssDNA (Fig. 4D). Next, we replaced with alanine the residues that are highly conserved in DnaA orthologues of eubacterial species bearing Hda orthologues (supplemental Fig. 2). Trp-6, Leu-13, and Leu-17 were individually mutated. Residues 25–27, 28–30, and 40–43 were replaced with the corresponding number of alanine residues.

Each DnaA protein was overexpressed at 37 °C, and soluble lysates were prepared (12, 13, 43). All of the DnaA proteins bearing substitutions of surface residues were obtained from soluble fractions at a level similar to the wild-type DnaA, except that the solubility of DnaA N44A and K54A was slightly reduced (supplemental Fig. 4A). In contrast, all of the other mutant proteins (DnaA W6A, L13A, L17A, 25–27A, 28–30A, and 40–43A) were poorly soluble (supplemental Fig. 4B). Substitution of these evolutionarily conserved residues most likely affected the whole structure of domain I, leading to denatured aggregates. Indeed, considerable parts of the side chains of Trp-6, Leu-13, Leu-17, Trp-25, Ile-26, Leu-29, and Leu-40 are located inside of the domain I structure (Fig. 2C; supplemental Fig. 1). Consistent with this, DnaA L38A, L40A, and I59A proteins are reported to rapidly degrade in cells (17). The side chains of Leu-38 and Ile-59 are also located inside of the solution structure determined in this study (Fig. 2C; supplemental Fig. 1). Thus, the solution structure can reasonably explain all of these observations.

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DnaA E21A Is Inactive in Vivo—To determine the in vivo activity of the soluble DnaA domain I mutant proteins, we performed dnaA complementation experiments using strain KA451, which carries dnaA::Tn10 rnhA::cat double mutations. Lack of the rnhA gene activates alternative replication origins and induces DnaA-independent replication of the chromosome. The doubling time of KA451 cells is at about 200 min at 37 °C; visible colonies do not form on LB-glucose agar plates for more than 20 h. Introduction of plasmid bearing the wild-type dnaA gene into this strain enhances the growth rate and the
colony formation. After transformation with dnaA allele-carrying plasmids, we deduced the numbers of colonies formed during incubation for 21 and 37 h and calculated the ratio (Table 2). This ratio is 0.5–5 for KA451 bearing the wild-type dnaA. We also introduced mutant dnaA alleles. The dnaA E21A allele did not increase the rate of colony formation (Table 2). An immunoblot analysis indicated that the stability of this mutant protein in vivo was similar to that of the

FIGURE 4. ssDNA binding ability of the DnaA N terminus. A, the 1H-15N HSQC spectrum of the DnaA N terminus showing the chemical shift changes in the absence (black) and presence (red) of 0.6 mM 28-mer ssDNA bearing the oriC 13-mer sequences (5’-GATCTGTCTATTGATCTCTTATTAG-3’). An enlargement of the selected region is shown in the inset. B, NMR titration of the ssDNA to the DnaA N terminus. ssDNA binding was assessed by the change in the NMR chemical shift of amide proton peak of Phe-46 in 1H-15N HSQC. The titration curve was analyzed by a nonlinear iterative fitting procedure, and the dissociation constant, K_d, was estimated to be ~1 mM. Open circles, 28-mer ssDNA bearing the oriC 13-mer sequences; closed triangles, 28-mer poly(dT) ssDNA. C, differences in the chemical shift changes between the absence and presence of ssDNA are displayed for the backbone amides as a function of sequence number. All chemical shifts changes in the 1H-15N HSQC spectra were calculated according to the formula \( \Delta \delta = (\Delta \delta(1H))^2 - (\Delta \delta(15N))^2 \) / 2). The red and orange lines represent 0.03 and 0.02 ppm, respectively. D, ribbon models of the DnaA N terminus indicating residues that experience ssDNA-induced chemical shift changes. The residues represented by stick models are those that underwent chemical shift changes more than 0.03 ppm (red) or 0.02 ppm (orange). E, structural comparison between the KH domain and domain I of DnaA. The structures of the PCBP2-ssDNA complex (PDB code: 2AXY; right panel) and domain I of DnaA (left panel) are shown. Each structure is shown as a ribbon model. Single-stranded DNA is shown as gray stick models. Red, blue, and green represent acidic, basic, and hydrophobic amino acids, respectively. The glycines in the GXGX motif of PCBP2 are colored yellow. To show the bulkiness of the side chains in domain I, acidic and basic amino acids are represented by stick models (left panel).

FIGURE 5. The surface of the DnaA N terminus and the residues analyzed. The structure of the DnaA N terminus is shown as a space-filling model on which amino acids at sites of mutations are plotted. The color scheme for the mutants is as follows: red, acidic residues; blue, basic residues; green, hydrophobic residues; magenta, polar residues.
proteins might be slightly inhibited. In addition, the initiation activities of DnaA Q14A and E16A mutants was further analyzed. DnaA E21A, Q14A, and E16A proteins were assessed for nucleotide binding affinity for ATP and ADP as the wild type (Table 3).

| Table 2: DnaA activity assay in vivo |
|------------------------------------|
| dnaA allele on plasmid          | No. of transformants/ incubation time | Colony formation ratio |
|----------------------------------|--------------------------------------|------------------------|
| Wild type                        | 21 h  (×10⁴)/μg DNA                  | 21 h/37 h               |
| R12A                             | 5.0                                  | 1.0                    |
| Q14A                             | 1.0                                  | 0.093                  |
| E16A                             | 1.0                                  | 0.32                   |
| E21A                             | <0.01                                | <0.002                 |
| N44A                             | 6.6                                  | 0.5                    |
| F46A                             | 8.3                                  | 0.06                   |
| K54A                             | 8.4                                  | 1.0                    |
| None (vector only)               | 0.18                                 | 0.036                  |

* The colony size was much smaller than that of the strain bearing the wild-type dnaA.

**Table 3: Affinity for ATP/ADP**

Purified wild-type and mutant DnaA proteins were assessed for nucleotide binding activity using a filter retention assay. Means ± S.D. was calculated based on the results of three independent experiments.

| DnaA | ATP ADP | Stoichiometry |
|------|---------|---------------|
|      | kₒ     |               |
| WT   | 0.49 ± 0.08 | 0.35 ± 0.07   |
| Q14A | 0.46 ± 0.02 | 0.32 ± 0.04   |
| E16A | 0.27 ± 0.06 | 0.27 ± 0.15   |
| E21A | 0.29 ± 0.05 | 0.26 ± 0.13   |

**DnaA E21A Is Active in ATP-dependent Open Complex Formation**—To determine the role of Glu-21 in initiation, we assessed open complex formation using the oriC plasmid M13KEW101 and P1 nuclease (12, 13). P1 nuclease breaks DNA specifically at single-stranded sites. If the oriC site of M13KEW101 is specifically broken by P1 nuclease, subsequent digestion with the AlwNI restriction enzyme will produce 3.8- and 4.1-kb fragments (Fig. 7A). The ATP form of the DnaA E21A protein formed open complexes, and the ADP form was inactive similar to the wild-type forms (Fig. 7, A and B), although the maximum level of open complex formation by ATP-DnaA E21A was about half that of the wild-type ATP form across the limited range of DnaA concentrations we examined.

**DnaA E21A Is Defective in DnaB Helicase Loading**—In the initiation process, DnaB helicase loading is the next step of open complex formation. To investigate the role of Glu-21 in this step, we first used an in vitro ssDNA replication system with the ABC primosome (12, 13, 19). The template in this system is ssDNA with a local hairpin structure bearing a DnaA box. Initiation of replication depends on DnaB loading directed by DnaA bound to the hairpin in the presence of DnaC. DnaA E21A had severely limited activity in ABC primosome-dependent replication (Fig. 7, C and D), indicating that this protein has a defect in DnaA-directed DnaB loading.

Next, we assessed DnaB helicase loading onto oriC. When DnaB helicase is loaded onto the open complex of oriC, successive unwinding by the helicase produces a specifically supercoiled form called form I in the presence of DNA gyrase activity (23, 46). Unlike the wild-type DnaA, DnaA E21A was completely unable to produce form I (Fig. 7, E and F). Taken together with the above results, these experiments indicate that the Glu-21 residue of DnaA plays a crucial role in DnaB helicase loading. This is the first result to identify a unique DnaA domain I residue that is specifically required for DnaB helicase loading. We do not exclude a possibility that this role for Glu-21 may be an indirect consequence, because we do not have direct evidence indicating that Glu-21 directly binds to DnaB.

**DnaA E21A Is Active in RIDA**—In the RIDA system, which is required to restrain extra, untimely initiations, DnaA-ATP is hydrolyzed when it interacts with Hda that is complexed with the DNA-loaded form of the clamp (3, 35). DnaA domain I is required for this DnaA-ATP hydrolysis (5). We assessed the role for DnaA Glu-21 in this regulation using a reconstituted, staged RIDA system. The DNA-loaded clamps were isolated and incubated with Hda and ATP-DnaA. The DnaA E21A protein was active in RIDA-specific DnaA-ATP hydrolysis at a level similar to the wild-type DnaA (Fig. 7G). Similar results were obtained for DnaA Q14A and E16A (data not shown). These results highlight the specific role for Glu-21 in helicase loading.

**DISCUSSION**

**Domain I Dimerization**—In this study, we have described the structure of DnaA domain I. DnaA domain I has close structural similarity to the KH domains, including secondary structure topology, i.e. three β-strands and two or three α-helices (Fig. 2D). A recently published crystal structure of the KH domain in PCB2 revealed that it forms homodimers (39) (PDB...
The hydrophobic surface, which is composed of the β1-strand and α3-helix in PCBP2, contributes to the dimerization. Similar to the PCBP2 dimer, domain I has a hydrophobic patch that is influenced by salt concentration (Fig. 3). Based on PCBP2 dimer structure and our experimental results, we have proposed a structural model of dimerization between domain I monomers (Fig. 3D). The domain I structure contains a hydrophobic surface within the N-terminal α1-helix and the loop between β1 and β2; based on chemical shift changes, we suggest that this hydrophobic surface, which includes Trp-6, plays an important role in DnaA dimerization (Fig. 3). This is consistent with previous reports that a DnaA W6A mutant protein fails to form homooligomers (17, 18). A considerable part of the Trp-6 side chain is inside of the domain I structure, but some of it is exposed on the surface (Figs. 3C and 5A; supplemental Fig. 1). This structure explains the reduced solubility of DnaA W6A and its role in interactions between DnaA molecules. Previous studies have also indicated that DnaA domain I can form homo-oligomers; for example, the C-terminal domain of the λ cI repressor is required for both self-dimerization and repressor activity, and a hybrid λ cI repressor in which the C-terminal domain has been replaced by the DnaA domain I continues to have repressor activity in vivo (16). We were not able to determine the chemical shift of the N-terminal amino acids Met-1 to Leu-3, because these signals disappeared. This is consistent with the model of the domain I dimer structure in that these residues are located on the dimer interface, thereby causing signal broadening (Fig. 3D).

Interaction with DnaB—We found that DnaA domain I Glu-21 plays a crucial and specific role in DnaB helicase loading. DnaA E21A retains the ability to form an open complex, indicating that the interaction between DnaA molecules and DNA binding are sustained in this mutant. In contrast, DnaA E21A has no activity for loading DnaB onto template DNA. DnaB-interacting regions of DnaA were previously identified by deletion analysis and by functional inhibition using a monoclonal antibody (20, 21, 23). These methods can alter regions neighboring the deletion or bound epitopes, which reduces the resolution of the analysis. For the first time, we have identified a residue specifically required for DnaB loading. Based on this finding and the domain I structure, we suggest that the region including Glu-21 and the N terminus of the α2-helix is crucial for DnaB loading (Figs. 2B and 3D). This is consistent with previous analyses obtained using truncated forms of DnaA and monoclonal antibodies as described above.

DnaA Multimerization and DnaB Loading on oriC—The sites required for intermolecular DnaA interactions and DnaB loading (Trp-6 and Glu-21, respectively) are located on opposite surfaces of domain I (Figs. 3D and 5A). This is reasonable in that the DnaA-DnaA and DnaA-DnaB interactions must occur simultaneously in the oriC initiation process. The DnaA com-
plex formed by the DnaA R281A protein is unstable on oriC, which results in failure of DnaB loading onto the open complex, although DnaA R281A is active in DnaB loading onto ssDNA in the ABC primosome system (46). Thus, formation of a stable DnaA multimer on oriC is required for DnaB loading onto the open complex. The Arg-281 residue is located in domain III, which most likely forms head-to-tail homomultimers on oriC as it does in several AAA+ proteins (9, 12).

We suggest that domain I forms head-to-head homodimers within the initiation complex through the hydrophobic patch including Trp-6 (Fig. 8). We infer that domain I dimerization is led by rotation of domain I via the domain II flexible linker, which effectively exposes the DnaB interaction site of domain I on the surface of the initiation complex (Fig. 8). DnaB helicase is a homo-hexamer, and at least two DnaB hexamers are loaded onto a single oriC open complex (47, 48). The affinity of DnaA for DnaB is relatively weak; surface plasmon resonance analysis suggests a dissociation constant ($K_d$) of 2 μM between the two (20). In our model, several sites on DnaA homomultimers that interact with DnaB line up on the surface when the two domains interact, which would be a reasonable way to recruit DnaB efficiently (Fig. 8). In addition, the weak affinity of the domain I of certain DnaA molecules in the open complex might support the interaction between unwound oriC DNA and DnaB helicase by holding the single-stranded region in the vicinity of the DnaB entry site (Fig. 8 and see below). DnaA multimerization on oriC would thus be required for effective interaction with DnaB and for leading DnaB to interact precisely with the limited ssDNA region of unwound oriC.

Although Trp-6 in E. coli DnaA is highly conserved over evolution among DnaA orthologues, conservation of Glu-21 is moderate (supplemental Fig. 2). In addition to Glu, Gln is also moderately conserved at...
DnaA Structure and Mechanisms for Interprotein Interactions

**FIGURE 8. Model of the mechanism of DnaA multimerization and interaction with DnaB.** A, schematic presentation of DnaA. DnaA domains I–IV, Glu-21, the hydrophobic patch (HP) including Trp-6, and the second DnaA-binding site on the N terminus of domain III are indicated. B, schematic presentation of the structure of a DnaA multimer on oriC and interaction with DnaB. DnaA domain III forms a head-to-tail multimer. Domain I forms a head-to-head dimers, which causes rotation of the domain to expose Glu-21 on the surface of the DnaA multimer. Thus, a series of DnaB interaction sites form a DnaB entry gate. The unwound ssDNA region may be localized near the DnaB entry site.

this position. The total conservation of Glu, Gln, and Asp is 71% at this position, implying that these residues use hydrogen bonds for DnaA-DnaB or DnaA-ssDNA interactions.

**ssDNA Interaction**—Some KH domains, such as those of heterogeneous nuclear ribonucleoprotein K and NusA, repeat several times within a single peptide, and these repeats increase the binding affinity 100–1000-fold (49, 50). Similarly, multimerization of DnaA would increase the overall affinity of domain I for ssDNA. A subset of DnaA molecules in open complex might use this domain for ssDNA interaction (Figs. 3D and 8), contributing to the efficient unwinding of the DNA duplex (Fig. 7B). Like Glu-21, Phe-46 is suggested to interact with ssDNA (Fig. 4C). This residue is highly conserved among DnaA orthologues (supplemental Fig. 2). DnaA domain I has a rigid structure and is connected to domain III via a flexible linker domain II. This overall structure suggests that the function of domain I is basically independent of the other domains. Thus, we infer that the weak affinity of domain I for ssDNA is associated with the full-length protein.

Several studies of the KH domains using NMR and x-ray structures have indicated that ssDNA interacts with the groove composed of hydrophobic residues and surrounding electrostatic residues (39, 42). Consistent with this finding, the ssDNA-binding surface of domain I contains exposed hydrophobic residues and surrounding electrostatic residues (Fig. 4E). The GXXG motif is a conserved feature in KH domains of various proteins. This motif usually causes a kink in the α-helix, forming a groove on the protein surface (Fig. 4E, right panel). This groove and the compact side chains of the glycines of the GXXG motif are important for interactions with DNA and RNA (39, 40). The affinity of typical KH domains for DNA and RNA is at the micromolar level. A unique feature of the DnaA domain I is that it does not carry the GXXG motif; instead, Tyr-55, a helix breaker, kinks the α3-helix (Figs. 1B and 2, B and D). In addition, the N-terminal half of this helix carries bulky and electrostatic side chains (Fig. 4E, left panel), which interrupt the formation of the groove. Therefore the area of ssDNA-binding site within domain I is smaller that that of other KH domains, which most likely causes the weak affinity of domain I for ssDNA.

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