A Common Mechanism for the ATP-DnaA-dependent Formation of Open Complexes at the Replication Origin

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Initiation of chromosomal replication and its cell cycle-coordinated regulation bear crucial and fundamental mechanisms in most cellular organisms. Escherichia coli DnaA protein forms a homomultimeric complex with the replication origin (oriC). ATP-DnaA multimers unwind the duplex within the oriC unwinding element (DUE). In this study, structural analyses suggested that several residues exposed in the central pore of the putative structure of DnaA multimers could be important for unwinding. Using mutation analyses, we found that, of these candidate residues, DnaA Val-211 and Arg-245 are prerequisites for initiation in vivo and in vitro. Whereas DnaA V211A and R245A proteins retained normal affinities for ATP/ADP and DNA and activity for the ATP-specific conformational change of the initiation complex in vitro, oriC complexes of these mutant proteins were inactive in DUE unwinding and in binding to the single-stranded DUE. Unlike oriC complexes including ADP-DnaA or the mutant DnaA, ATP-DnaA-oriC complexes specifically bound the upper strand of single-stranded DUE. Specific T-rich sequences within the strand were required for binding. The corresponding conserved residues of the DnaA ortholog in Thermotoga maritima, an ancient eubacterium, were also required for DUE unwinding, consistent with the idea that the mechanism and regulation for DUE unwinding can be evolutionarily conserved. These findings provide novel insights into mechanisms for pore-mediated origin unwinding. ATP/ADP-dependent regulation, and helicase loading of the initiation complex.

Initiation of chromosomal replication and its cell cycle-coordinated regulation bear crucial and fundamental mechanisms in most cellular organisms. In Escherichia coli, DnaA forms a stable complex with ATP or ADP and binds to 9-mer sequences called DnaA boxes within the replication origin oriC, resulting in the formation of homomultimeric complexes (1–4). A DnaA-binding protein, DiaA, directly stimulates formation of ATP-DnaA multimers on oriC (5, 6). ATP-DnaA multimers, but not ADP-DnaA multimers, promote specific inter-DnaA interactions on oriC, resulting in the adoption of an activated conformation as the initiation complexes, which interact with ATP-DnaA-specific low affinity sites within oriC (7–9). This conformational change triggers duplex unwinding of the AT-rich 13-mer repeats (DNA unwinding element (DUE)) within oriC with the aid of the superhelicity of DNA and heat energy, creating open complexes (10, 11). The mechanisms and functional structures within DnaA directly responsible for the ATP-DnaA-specific duplex unwinding remain unexplored.

Open complex formation is a critical regulatory point for determining whether replication initiation will occur during the cell cycle (1, 2). DnaB helicase is loaded onto the single-stranded (ss) region in open complexes in a manner depending on a DnaA-DnaB interaction and the DnaC helicase loader. The loaded helicase expands the ssDNA region, which leads to the assembly of replication machineries, including DnaG primase and DNA polymerase III holoenzyme, thereby initiating DNA synthesis (12). After the initiation of replication, ATP-DnaA is converted to ADP-DnaA by the promotion of DnaA-ATP hydrolysis in a manner depending on Hda protein and the DNA-loaded form of the clamp subunit of DNA polymerase III holoenzyme (13–16). This DnaA-inactivating system, termed RIDA (regulatory inactivation of DnaA) is required for repressing extra initiation events.

The DnaA protein consists of four functional domains (1, 2). Domain I is required for DnaA self-oligomerization and functional interactions with other proteins such as DnaB helicase and DiaA (5, 17–19). Domain II is a flexible linker (17). Domain III has specific ATP recognition motifs that are characteristic of the AAA+ superfamily, of which DnaA is a member (20, 21). Domain IV is a DNA-binding region that contains a helix-turn-helix motif for specific recognition of the DnaA box (22, 23).
The AAA⁺ superfamily includes various proteins that can induce ATP binding/hydrolysis-dependent conformational changes (21, 24, 25). Of the AAA⁺-specific ATP-interacting motifs, the DnaA sensor 1 Asp-269 residue supports exceptionally tight affinity for ATP/ADP (26). The DnaA sensor 2 Arg-334 is specifically required for RIDA-dependent ATP hydrolysis (27, 28). The DnaA Box VII arginines Arg-281 and Arg-285 most likely interact in different manners with an adjacent DnaA protomer in DnaA multimers assembled on oriC. Arg-281 stabilizes the DnaA multimers (29). Notably Arg-285 plays a crucial role in the ATP-specific conformational change of the initiation complex, which is required for open complex formation (7). This residue is specifically required for binding of ATP-DnaA protomers to the specific low affinity sites within oriC. On the basis of common features of AAA⁺ proteins, it has been suggested that Arg-285 interacts with ATP bound to an adjacent DnaA protomer in the complex, which leads to conformational activation of the complex.

In well characterized AAA⁺ proteins, a typical functional conformation is ring- or spiral-shaped oligomers (consisting of 5–7 protomers) with a central cavity or pore (24). Certain residues within the pore are crucial for specific functions of proteases, chaperones, SV40 T-Ag helicase, and DNA polymerase III clamp-loader subassembly (30–32). By analogy to these structures, we hypothesized that DnaA molecules oligomerize to form a central pore on oriC and that specific residues on the pore surface play a crucial role in duplex unwinding. However, DnaA does not carry residues in its primary sequence that directly correspond to the important residues within the pores of the AAA⁺ proteins previously characterized. DnaA is structurally classified into a subgroup different from the proteases, chaperones, helicases, and clamp-loaders in the AAA⁺ superfamily (20). Furthermore, unlike the well characterized AAA⁺ proteins, DNA-free DnaA molecules are monomers.

In this study, we first constructed homology models of the DnaA oligomer that forms a pore using a crystal structure of the AAA⁺ domain of the hyperthermophilic eubacterium Thermotoga maritima DnaA ortholog (tmaDnaA). Next, we used this model to select candidates for the crucial residues on the pore surface of DnaA and analyzed the corresponding mutant proteins of tmaDnaA and E. coli DnaA. The candidate residues were highly conserved in DnaA orthologs. The results of our in vitro analyses are in good agreement with this structure. We found that E. coli DnaA V211A and R245A proteins are inactive in in vivo initiation. Moreover, we revealed using in vitro reconstituted systems that these mutant proteins are specifically inactive in origin unwinding, whereas these retain activities for binding to ATP and DnaA boxes and for the formation of the ATP-DnaA-specific multimeric conformation on oriC.

Furthermore, using a newly constructed system of an electrophoretic mobility shift assay (EMSA), we revealed that the ATP form of these mutant DnaA multimers on oriC, unlike that of wild-type DnaA, are inactive in direct binding to ssDUE. Thus, we have revealed specific roles for the previously unexpected residues in open complex formation. These residues reside on the putative pore of the DnaA multimer on oriC and play crucial and specific roles in oriC unwinding. Thus we propose a novel mechanism for ATP-dependent regulation of open complex formation.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Oligonucleotides—**E. coli strains KHS402-1 (wild type), KA450 (ΔoriC1071::Tn10 rnhA199(Am) dnaA17(Am)), KA451 (dnaA::Tn10 rnhA::cat), and KA413 (dnaA46) were previously described (26, 33). pKA234, M13KEW101, pTHMA-1, pOZ14, and pOZ18 were also previously described (26, 34).

For construction of the derivatives of pKA234 and pTHMA-1, mutations were introduced in the dnaA region using a QuickChange site-directed mutagenesis kit (Stratagene) and mutagenic primers. For the sequences of these primers, see supplemental Table S1. The derivatives of pOZ18 were constructed as we previously described (26).

**Assays for DnaA Activities—**Binding activities of DnaA for ATP and DnaA boxes were assessed by a filter-retention assay, surface plasmon resonance analysis, or EMSA as we described previously (7, 26, 34). The P1 nuclease assay using E. coli DnaA was performed essentially as previously described (6, 11, 17, 26). The resultant DNA samples were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. The intensities of DNA bands were quantified by densitometric scanning. When tmaDnaA was used, unwinding reactions were incubated at 48 °C in the presence of pOZ14 (400 ng) instead of M13KEW101 (34). The minichromosome replication and ABC prismsome systems were reconstituted in vitro with purified proteins as previously described (17, 26, 35, 36).

**DNase I Footprint Analysis—**This analysis was essentially performed as described previously (6, 7). DnaA was incubated at 30 °C for 10 min in buffer (10 μl) containing 25 mM Hepes-KOH (pH 7.6), 5 mM calcium acetate, 2.8 mM magnesium acetate, 45 mM ammonium sulfate, 4 mM dithiothreitol, 10% (v/v) glycerol, 0.2% Triton X-100, 0.5 mg/ml bovine serum albumin, 14 μg/ml poly(dA-dT)-(dA-dT), 14 μg/ml poly(dL-dC)-(dL-dC), 3 mM ATP/ADP, and a 419-bp 32P-end-labeled oriC fragment (5.5 ng), followed by incubation for 4 min at the same temperature in the presence of DNase I (0.63 unit/milliliter). After the reaction was stopped by addition of 0.5% SDS, DNA was extracted with phenol/chloroform, precipitated with ethanol, and analyzed by 5% sequencing gel electrophoresis.

**ssDUE Binding Analysis by EMSA—**DnaA was incubated at 0 °C for 15 min in the presence of 3 mM ATP or ADP as we previously described (26). ATP-DnaA and ADP-DnaA were incubated at 30 °C for 10 min in 10 μl of buffer G containing 13 mM (130 fmol) oriCΔLMR DNA and 2 nM (20 fmol) 32P-end-labeled M28 ssDNA (5’-GATCCTGTCATGATCTTCTTATTAG), its complementary M28-rev ssDNA, or its derivatives. Buffer G contains 20 mM Hepes-KOH (pH 7.6), 1 mM EDTA (pH 8.0), 4 mM dithiothreitol, 5 mM magnesium acetate, 10% (v/v) glycerol, 0.1 mg/ml bovine serum albumin, and 1 mM ATP or ADP. The mixtures were then separated by 4% polyacrylamide gel electrophoresis at room temperature. DNA was visualized using GelStar (Cambrex) staining, and ssDNA was detected using a BAS-2500 Bio-imaging analyzer. The oriCΔLMR fragment was prepared by PCR using M13KEW101 primer pairs.
and primers ori-1 (5'-ATGCAGTGGGCGCCCG) and ori-2 (5'-ATCGCACGCTCCGCTGG). ssDUE Binding Analysis Using Pull-down System—DnaA and 
32P-end-labeled M28 ssDNA were incubated under the same conditions described for EMSA except that 5'-biotinylated oriC/ALMR (bio-oriC/ALMR) was used instead of unmodified oriC/ALMR. The reaction was further incubated for 10 min at room temperature with gentle rotation in the presence of streptavidin-beads (Promega) equilibrated in the buffer G (10 μl). The beads and bound materials were collected and washed in the same buffer (20 μl). M28 ssDNA complexed with bio-oriC/ALMR and DnaA was eluted in buffer (10 μl) containing 10 mM Tris-HCl (pH 7.5 at 1 x), 1 mM EDTA and 0.1% SDS, spotted on the 3MM paper (Whatman) and analyzed using a BAS-2500 Bio-imaging analyzer. The bio-oriC/ALMR fragment was prepared by PCR using primers ori-1 and 5'-biotinylated ori-2.

RESULTS

Structural Models of the Initiation Complex—By analogy to some AAA+ protein multimers, we hypothesized that DnaA multimers on oriC have a central pore that plays a crucial role in DUE unwinding. To predict which residues comprise the putative pore, we first identified the crystal structure of tmaDnaA AAA+ domain (Fig. 1A, Table 1, and supplemental "Experimental Procedures") and then constructed homology models of the multimeric structure using this crystal structure (Fig. 1, C and D). The crystal structure of tmaDnaA AAA+ domain bound to ADP was solved at 3.05 Å and was basically identical to that of Aquifex aeolicus DnaA AAA+ domain (Fig. 1B) (22).

Using the model of a homobexamer ring (Fig. 1C), we searched for the residues that are exposed on the pore surface. We particularly focused on residues bearing hydrophobic or basic side chains that would interact favorably with DNA. tmaDnaA Val-176, Met-179, Lys-180, and Lys-209 residues were found to be strong candidates (Fig. 1E). Identical or chemically similar residues corresponding to these are highly conserved among eubacterial DnaA homologs (Fig. 1F). During the course of this study, a multimeric crystal structure was reported for the A. aeolicus DnaA AAA+ domain (37). Although this complex does not include the cognate oriC DNA, the multimers form a spiral helix in crystals. We therefore used this structure as a second model (Fig. 1D). The residues indicated above are also exposed on the pore of this spiral model (Fig. 1E).

Analyses of tmaDnaA Mutant Proteins—Using tmaDnaA and the predicted oriC (tma-oriC) of T. maritima, we previously determined the minimal oriC region and ATP-tmaDnaA-specific DUE in vitro (34). ATP-tmaDnaA-dependent unwinding within tma-oriC DUE can be assessed by P1 nuclease. To elucidate the functions of the selected residues, we analyzed a set of tmaDnaA mutant proteins with single-alanine substitutions at Val-176, Met-179, Lys-180, or Lys-209. All of the mutant tmaDnaA proteins were purified by the method reported previously for wild-type tmaDnaA. The ATP binding activities of the mutant proteins were similar to that of wild-type tmaDnaA (Table 2). In an EMSA, the tmaDnaA box binding activity of each mutant protein was similar to that of wild-type tmaDnaA (data not shown). In contrast to these activities, tma-oriC unwinding was severely inhibited in each tmaDnaA mutant protein (Fig. 1G).

E. coli DnaA Mutants Inactive in in Vivo Initiation—To assess the in vivo significance of these residues, we constructed plasmids encoding E. coli DnaA mutant proteins that have Ala substitutions at the positions corresponding to the tmaDnaA residues indicated above (Fig. 1, E and F). The E. coli DnaA residues Val-211 to Ile-219 reside in a region corresponding to that of tmaDnA Val-176 to Lys-180 or in its flanking region (positions i–iii in Fig. 1F). E. coli DnaA Lys-243 and Arg-245 residues reside in a region corresponding to tmaDnaA Lys-209 and Gly-211 (positions v and vi, respectively, in Fig. 1F). tma- DnaA Gly-211 is exposed on the pore surface (Fig. 1E). Some DnaA orthologs conserve the basic moiety corresponding to E. coli DnaA Arg-245. In addition, we analyzed E. coli DnaA Lys-223 and Arg-224 (Lys-223 corresponds to position iv in Fig. 1F). These residues have basic side chains that are conserved in this region of DnaA orthologs.

To determine the importance of these residues in vivo, we performed plasmid complementation tests (Table 3). First, we used as a host a dnaA46 mutant strain, KA413, that is defective in colony formation at 42 °C. The initiation activity of the DnaA46 protein is temperature-sensitive, and the protein is labile at 42 °C, with a shortened half-life in vivo (33, 38). The first series of DnaA-producing plasmids bearing wild-type or mutant dnaA alleles was introduced into KA413 cells, and the transformant cells were incubated overnight at 30 °C or 42 °C (Table 3, experiment A). At 30 °C, colonies formed with a similar efficiency among the plasmids, whereas the plasmids bearing the dnaA V211A allele (pKW44-1) and dnaA K223A allele (pKW47-1) did not support colony formation at 42 °C. In these experiments, the inducer arabinose was not included in the medium, thereby allowing only leaky expression. Immunoblot analysis indicated that DnaA proteins were expressed from the plasmids at a similar level even at 42 °C (supplemental Fig. S1).

Next, we used as a host a dnaA-disrupted strain, KA451 (rnhA::cat dnaA::Tn10) (Table 3, experiment B). In KA451, the lack of the rnhA gene activates an alternative oriC-independent initiation system and allows dnaA-independent cell growth (39). Plasmid pOZ18 is a low copy mini-R derivative bearing wild-type rnhA and dnaA genes (26). When KA451 and a wild-type strain, KH5402-1, were transformed with pOZ18, transformants were obtained at 30 °C with the same efficiency. In KA451 cells bearing pOZ18, the plasmid-encoded rnhA gene product represses the oriC-independent replication system, and instead the plasmid-encoded dnaA gene product activates the oriC initiation system, which results in colony formation. When pOZ18-derivatives (pOZ20 and pOZ21) bearing dnaA K243A and dnaA R245A were used for transformation, colony formation was sustained by KH5402-1 but not KA451 (Table 3, experiment B). Immunoblot analysis indicated that the expression levels of both mutant DnaA proteins were similar to that of wild-type DnaA (supplemental Fig. S1). These results indicate that DnaA K243A and DnaA R245A are inactive for initiation at oriC in vivo at 30 °C. DnaA N216A was also unable to support the colony formation of KA451 (Table 3) but was found to degrade rapidly in vivo (supplemental Fig. S1); moreover, purified DnaA N216A was active in in vitro minichromosomal replication (data...
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A

B

C

D

E

Ring

Spiral

K209 (v) G211 (vi)

K180 (iii) M179 (ii) V176 (i)

F

| Organism | α3 | α4 | α5 | α6 |
|----------|----|----|----|----|
| Ecoli    | MHSERVQDMVKALQNNAIPEEHRYR-SVDALLYDIPANKERSQEEFFHTFNALLSEG |   |    |    |
| Thema    | ITSEKFNLDVMKLEKNEFSKYRKKVDDLDDVQFLIGTXTVQTELHFHTFNEHDSEG |   |    |    |
| Aquae    | SSADDFAQAMVEHLPKGETHEFENMY-KSVDDLDDVQFLSGKERTIQIEFFHIPNTLYLLE |   |    |    |
| Helph    | VTESDFLTEFLKLDNKMDQAQK-RCDFLQDAQFLQKPLLGEFFHTFNEHLANS |   |    |    |
| Bacsu    | LSEKFTNEFINSIRDNKAVDFNRYR-ZVDVLLDDVQFLGKEQTEIEFFHTFNTLHEES |   |    |    |
| Myctu    | VSTTEFNDPINSRDDRKVAPESYR-DRVDDVDDIQIECGEQIEFFHTFNTLHNN |   |    |    |
| Chlmu    | VSSLFTEHLSIISKSEGQMRFAYR-NVEALFIEDIEVLSGKATQIEFFHTFNSLHTEG |   |    |    |

G

Open complex (%) vs. tmaDnaA (pmol)

WT (O)

V176A (△)

M179A (▲)

180A (○)

209A (●)

0 1 2 3 4

tmaDnaA (pmol)
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not shown). These results taken together demonstrate that the E. coli DnaA Val-211, Lys-223, Lys-243, and Arg-245 residues correspond to positions i, iv, v, and vi (Fig. 1, E and F).

ATP/DNA Binding Activities of the DnaA Mutant Proteins—
To reveal the functions of these residues, we overproduced and purified the mutant DnaA proteins according to a method described previously for wild-type DnaA (7, 26, 27). A filter binding assay demonstrated that all of the mutant DnaA proteins sustained affinities for ATP at levels similar to that of wild-type DnaA (Table 2). Also, surface plasmon resonance analysis and EMSAs showed that the affinity of each mutant DnaA protein for DnaA box R1 was similar to that of wild-type DnaA (Fig. 2).

The DnaA Mutant Proteins Are Inactive in Origin Unwinding—
We used a P1 nuclease assay to examine whether the mutant DnaA proteins sustain oriC unwinding activity (6, 11, 17, 26). Unlike the results with wild-type ATP-DnaA, oriC-specific digestion was barely detectable for any of the mutant ATP-DnaA proteins (Fig. 3), which indicates that these residues are crucial for oriC unwinding.

We also assessed the minichromosome replication activity of the mutant DnaA proteins by using an in vitro system reconstituted with purified proteins. In this system, initiation is promoted by ATP-DnaA but not ADP-DnaA (10, 11, 17, 26). All of the mutant DnaA proteins were inactive in this assay (Fig. 4A), which coincides well with the defects in initiation in vitro and in vivo (Fig. 3 and Table 3).

To assess the activity of the mutant DnaA in DnaB helicase loading, we used the ABC primosome system (17, 26, 35, 36). In this system, DnaA binds to a DnaA box-carrying local hairpin structure within the ssDNA template and interacts with DnaB to load this onto ssDNA, leading to DNA replication. Only slight inhibition in this activity was seen for DnaA V211A, R245A, and K223A (Fig. 4B), indicating that the mutated residues are not important for DnaB helicase loading. Although substantial inhibition was seen for DnaA K243A, considerable residual activity remained. This mutation might indirectly affect the domain III structure. A site in the DnaA domain III N terminus is required for interaction with DnaB (1).

DnaA V211A and R245A Are Active in the ATP-DnaA-Specific Inter-DnaA Interactions on oriC—To assess the conformation of the ATP-DnaA-specific complex on oriC (7, 8), we performed DNase I footprint experiments using various DnaA-oriC complexes (Fig. 5). Similar to wild-type ATP-DnaA, each mutant ATP-DnaA protein bound to DnaA boxes R1 and R4 with high affinity (Fig. 5), consistent with their DnaA box binding activities (Fig. 2). ATP-DnaA V211A and ATP-DnaA R245A proteins also interacted with the ATP-DnaA-specific sites at higher DnaA levels, resulting in footprint patterns indistinguishable from those of wild-type DnaA proteins (Fig. 3), which indicates that these residues are crucial for oriC unwinding.

TABLE 2

| Position | tmaDnaA | E. coli DnaA |
|----------|---------|-------------|
|          | Protein | Stoichiometry | DnaA | Stoichiometry |
| WT       | V176A   | 0.22         | WT   | 0.45         |
| i        | M179A   | 0.24         | V211A| 0.45         |
| ii       | K180A   | 0.23         | i    | 0.45         |
| iii      | K223A   | 0.30         | iv   | 0.30         |
| iv       | K243A   | 0.37         | v    | 0.37         |
| v        | K245A   | 0.27         | vi   | 0.37         |

TABLE 1

Data collection and refinement statistics of tmaDnaA crystals

| Space group | R32 | R2 |
|-------------|-----|----|
| Unit cell   | a = 221.82 Å, c = 55.60 Å, α = 90°, γ = 120° | a = 133.34 Å, b = 221.79 Å, c = 55.60 Å, β = 106.13° |
| Resolution (Å) | 50.0-3.00 | 50.0-3.05 |
| Unique reflections | 10,290 | 27,730 |
| Redundancy | 3.7 | 2.3 |
| Completeness (%) (last shell) | 96.3 (74.6) | 94.1 (68.3) |
| R(Fo−Fc) (%) (last shell) | 13.4 (2.46) | 13.7 (2.31) |
| R(Fo−Fc) (%) | 7.9 (35.1) | 7.0 (29.7) |
| R(Fo−Fc) (%) | 23.4 | 23.2 |
| R(Fo−Fc) (%) | 29.1 | 26.4 |

Stoichiometry

Stoichiometry

Stoichiometry
TABLE 3

Plasmid complementation tests

| Experiment | Host | Plasmid | Allele | Transformation efficiencya |
|------------|------|---------|--------|---------------------------|
| A          | KA413| pKA234  | WT     | 3.4 CFU/µg DNA            |
|            |      | pKW44-1 | V211A  | 9.6 x 10⁵                  |
|            |      | pKW46-1 | K212A  | 5.2 x 10⁶                  |
|            |      | pL214R  | L214R  | 2.4 x 10⁶                  |
|            |      | pQ215A  | Q215A  | 1.6 x 10⁶                  |
|            |      | pN216A  | N216A  | 2.1 x 10⁵                  |
|            |      | pN217A  | N217A  | 2.4 x 10⁶                  |
|            |      | pI219A  | I219A  | 7.7 x 10⁵                  |
|            |      | pKW47-1 | K223A  | 1.0 x 10⁶                  |
|            |      | pKW48-1 | R244A  | 5.2 x 10⁵                  |
|            |      | pING1 (vector) | None | 1.1 x 10⁶                  |
| B          | KH5402-1 | pOZ18 | WT | 3.4 x 10⁶                  |
|            |      | pMNDR-N216A | N216A | 1.1 x 10⁵                  |
|            |      | pMNDR-N217A | N217A | 1.3 x 10⁵                  |
|            |      | pMNDR-I219A | I219A | 1.6 x 10⁵                  |
|            |      | pOZ20 | K243A  | 5.6 x 10⁵                  |
|            |      | pOZ21 | R245A  | 2.6 x 10⁵                  |
|            |      | pI219A | I219A  | 7.5 x 10⁴                  |
|            |      | pOZ20 | K243A  | <25                       |
|            |      | pOZ21 | R245A  | <25                       |

a CFU, colony-forming units; WT, wild-type.

For experiment A, KA413 (dnaA46) cells were transformed with plasmid bearing the indicated dnaA allele and incubated on LB agar plates containing thymine (50 µg/ml) and ampicillin (50 µg/ml) at 30 °C for 21 h or at 42 °C for 12 h. Transformation efficiencies and the ratio are shown.

For experiment B, KH5402-1 (wild-type dnaA) cells and KA451 (dnaA::Tn10 rnhA::cat) cells were transformed with mini-R derivative plasmid bearing wild-type rnhA and the indicated dnaA allele, and incubated as above at 30 °C for 20 h.

ATP-DnaA (Fig. 5). These results demonstrate that these mutant proteins do not disrupt the ATP-DnaA-specific inter-DnaA interaction and the overall conformation of the activated initiation complex.

In contrast, the affinities of ATP-DnaA K223A and K243A proteins for ATP-DnaA-specific sites such as the box M and I/τ-sites were decreased, although these proteins associated with R2 and R3 boxes at levels similar to wild-type ATP-DnaA (Fig. 5). This result is consistent with the idea that the DnaA Lys-223 and Lys-243 residues play direct or indirect roles in the inter-DnaA interaction in the initiation complex, and thus the ATP-dependent conformational change of the complex is incomplete in these mutant proteins.

DnaA Assembled onto oriC Associates with ssDUE—We hypothesized that the unwound DUE directly binds to specific residues in the pore of an ATP-DnaA multimer on oriC. We developed a novel method to analyze the interaction of ssDUE with DnaA complexes. Electron micrographic study indicates that DUE does not affect overall DnaA complex formation on oriC (40). ATP-DnaA was incubated in the presence of a DUE-deleted minimal oriC fragment (oriCΔLMR) and a 32P-labeled 28-mer ssDUE, followed by EMSA (Fig. 6, A and B). To monitor DnaA assembly on oriCΔLMR, DNA was detected by an intercalating fluorocin (Fig. 6A) before detection of 32P-labeled ssDUE (Fig. 6B). ssDUE formed discrete bands at positions corresponding to DnaA multimers complexed with oriCΔLMR in an ATP-DnaA-dependent manner. Virtually all input ssDUE molecules were bound to the oriC-DnaA complex when DnaA was present at 120 nM; at this concentration, the molar ratio of DnaA to oriC was 9.2. Mobility shifts of ssDUE depended on the presence of oriCΔLMR. These observations suggest that ssDUE directly and specifically binds to ATP-
DnaA multimers complexed with oriC/H9004 LMR. In the absence of oriC/H9004 LMR, a slight amount of ssDUE remained in the gel well, especially in the presence of ATP, suggesting that DnaA formed irregular aggregates that bound ssDUE. DnaA has a propensity to form irregular aggregates during incubation in the absence of oriC and a high salt concentration. Faint bands seen in the middle of the gel (Fig. 6, A and B) are derived from nonspecific nucleic acids slightly included in a bovine serum albumin sample and ssDUE weakly hybridized with them, respectively.

Unlike the DUE upper strand, the DUE lower strand did not stably bind to ATP-DnaA multimers complexed with oriC/H11002 LMR. In the absence of oriC/H11002 LMR, a slight amount of ssDUE remained in the gel well, especially in the presence of ATP, suggesting that DnaA formed irregular aggregates that bound ssDUE. DnaA has a propensity to form irregular aggregates during incubation in the absence of oriC and a high salt concentration. Faint bands seen in the middle of the gel (Fig. 6, A and B) are derived from nonspecific nucleic acids slightly included in a bovine serum albumin sample and ssDUE weakly hybridized with them, respectively.

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Mechanism and Regulation of the Origin Unwinding

To further elucidate direct binding of ssDUE to ATP-DnaA multimers, we performed a pull-down experiment. DnaA and the $^{32}$P-labeled upper strand DUE were incubated in the presence of bio-oriC-LMR DNA. ssDUE was recovered in an ATP-DnaA-dependent manner (Fig. 6D), indicating that ATP-DnaA complexes with oriC-LMR were associated with ssDUE.

Analysis of the DnaA-binding Elements within ssDUE—To elucidate the essential sequence elements within ssDUE for binding to ATP-DnaA-oriC complexes, we performed EMSA using a set of mutant ssDUEs (Fig. 7). M28 ssDNA carried 13-mer M and R motifs. Deletions including M or R motif severely decreased the binding activity (Fig. 7, A and B). As T-rich sequences are evolutionarily conserved in DUEs (34), we next focused on these. TTGT sequence located in the M motif and downstream from it was important for the binding activity (M28-3 and M28-10 in Fig. 7, A, C, and D). Base substitutions for TTATT sequence within the R motif decreased the binding activity (Fig. 7, A, C, and E). Simultaneous substitutions for the both sequences completely diminished the binding activity (M28-9 and M28-11 in Fig. 7, A and D). A possible explanation would be that TTGT sequence played a primary role in binding to oriC-DnaA complexes, and TTATT sequence reinforced it. Requirement of T-rich sequences is consistent with the data that ATP-DnaA-oriC complexes specifically bind the upper strand rather than the lower strand (Figs. 6C, 7A, and 7D). These results are also consistent with the previous report that mutations in these sequences reduce in vitro and in vivo initiation activities (41). Other AT-rich sequences than these within the 13-mer might be important in a process of duplex unwinding, a preceding step for ssDUE-DnaA binding.

DnaA V211A and R245A Do Not Associate with ssDUE—To identify the residues required for ssDUE binding, we examined whether the ATP-DnaA mutant proteins could complex with oriC-bound ssDUE. Like wild-type DnaA, the mutant DnaA proteins formed multimeric complexes on oriC (Fig. 8, A–D), and the mutant multimers were comparable to wild-type DnaA multimers in size. However, the ATP forms of the mutant DnaA proteins were evidently inactive in ssDUE binding (Fig. 8, E–H). The defects of the DnaA K223A and K243A proteins in ssDUE binding are consistent with their inability to achieve the activated conformation of ATP-DnaA-oriC complexes (Fig. 5). Unlike these proteins, DnaA V211A and R245A proteins formed ATP-DnaA-specific complexes that were indistinguishable from wild-type DnaA (Fig. 5). By considering these experimental results together with the structural model, it is reasonable to infer that the DnaA Val-211 and Arg-245 residues play direct and specific roles in ssDUE binding of ATP-DnaA-oriC complexes.

Activities of Heteromultimers Containing Wild-type and Mutant DnaA Proteins—To elucidate the ATP-DnaA-dependent dynamics of initiation complexes, we further examined E. coli DnaA V211A, K243A, R245A, and R285A (Fig. 9). We analyzed mixtures of the ATP forms of wild-type and mutant DnaA. Heterocomplexes of DnaA R285A and wild-type DnaA can initiate minichromosomal replication, indicating that only a subgroup of DnaA protomers in an initiation complex require the Arg-285 residue for conformational activation (7). This idea was also supported by the greater ssDUE binding (data not shown) and open complex formation (Fig. 9A) of a mixture of DnaA R285A and wild-type DnaA than would be expected from the amount of wild-type DnaA alone. These are consistent with the previous report that only a subgroup of DnaA protomers
must be the ATP form for formation of active initiation complexes (42).

When mixed with wild-type DnaA, DnaA V211A, but not DnaA R245A, enhanced the open complex formation activity (Fig. 9, A and B). Similar results were seen in the ssDUE binding activity (Fig. 9C). These results suggest that the DnaA Val-211 residue is required only by a subgroup of DnaA protomers within an initiation complex. DnaA R245A did not enhance or inhibit the initiation activities of wild-type DnaA in a 1:1 mixture (Fig. 9, B and C), suggesting distinctive roles for these residues (see “Discussion”).

When a mixture of wild-type DnaA and DnaA K243A was used, the open complex formation activity of wild-type DnaA was inhibited (Fig. 9A), presumably because of the irregular inter-DnaA interactions as indicated by the footprint analysis (Fig. 5). Consistent with this, inhibition for initiation was observed using flow cytometry in KH5402-1 (wild-type dnaA) cells bearing a low copy mini-R vector, which carried dnaA K243A, but not wild-type dnaA or dnaA R245A (supplemental Fig. S2).

**DISCUSSION**

In this study, we revealed *E. coli* DnaA Val-211 and Arg-245 as residues that play specific and crucial roles in oriC duplex unwinding. Moreover, DnaA-oriC complexes interact specifically with ssDUE, which requires these residues. The upper strand of DUE is preferentially bound by the DnaA complexes, which is logical in that DnaB helicase initially loads on the lower strand of ssDUE (43). Formation of ATP-DnaA multimers on oriC is a prerequisite for the ssDUE binding. ATP-DnaA multimers bind several specific sites within oriC, which might enhance the topological torsion stress for oriC DNA, resulting in initial labile unwinding within the DUE in the presence of the superhelicity of template DNA and heat energy. We infer that DnaA Val-211 and Arg-245 bind and stabilize the resultant ssDUE for promoting the subsequent initiation reactions. TTGT and TTATT sequences within ssDUE could bind to different DnaA protomers within a single DnaA-oriC complex for initiation.

Furthermore, the results obtained are consistent with our model that DnaA molecules form a ring- or spiral-shaped multimer with a pore on oriC, that the Val-211 and Arg-245 residues are projected on the pore surface, and that these residues play direct and distinctive roles in open complex formation. These motifs are specific to the DnaA-subclass of the AAA + family (supplemental Fig. S3). DnaA Val-211 resides on a uniquely inserted helix in this subclass. DnaA Arg-245 is basic, whereas crucial residues at the corresponding position within the clamp-loader subunits and FtsH protease are neutral Thr/Ser and acidic Glu, respectively. In addition, those within helicases reside on unique β-sheet structure (30, 32, 44).

As a simple explanation of the regulation of open complex formation, we postulate a model of the complex consisting of 6 DnaA protomers with a central pore (Fig. 10A). A crucial event in our model is the ATP-DnaA-dependent structural change of the pore. This conformational change depends on the interaction between DnaA-bound ATP and the DnaA Arg-285 arginine finger (7). This model contains three pairs of protomers,
each containing an arginine finger “donor” DnaA and a “recipient” ATP-DnaA (Fig. 10A).

Another key to our model is the distinct roles of two residues on the pore: one is the basic residue (B-motif; E. coli DnaA).
Arg-245), and the other is the hydrophobic Val (H-motif; E. coli DnaA Val-211) (Figs. 1F and 10A). The positively charged residue of the B-motif can interact with backbone phosphates of DNA. The hydrophobic residue of the H-motif can interact with the DNA bases. Based on the data shown in Fig. 9, we also infer that the Val-211 residues (the H-motif) of only a subset of the DnaA protomers project into the pore in a manner that depends on DnaA-ATP and the arginine finger. Based on this model, a possible mechanism of open complex formation can be explained as follows. In ATP-DnaA homomultimers, the B-motif and H-motif residues are projected on the pore surface, creating the critical activity of ssDUE binding and an unwinding-competent conformation. In homomultimers of ADP-DnaA or DnaA R285A, the H-motif, B-motif or both are not fully projected into the pore due to the absence of the ATP-Arg-285 interaction, resulting in failure of ssDUE binding (Fig. 10A).

The model can also explain the abilities of heteromultimers of the wild-type and mutant DnaA protomers to form open complexes (Fig. 9). As a simple illustration, we have shown possible heteromultimers containing wild-type and mutant DnaA in a 1:1 ratio (Fig. 10B). When mixed complexes are formed with DnaA protomers of the wild-type and B-motif mutant (DnaA R245A), a decrease of the positive charges within the pore results in complexes with a decreased activity (Fig. 10B, panel i). In contrast, only a subset, but not all, of the ATP-DnaA protomers on oriC must carry the arginine finger and H-motif for open complex formation (Fig. 9, A and C) (7), because a pair of wild-type and mutant (R285A or V211A) DnaA protomers can create the same activated conformation as seen for the wild-type ATP-DnaA complexes (Fig. 10B, panels ii and iii).

Herein, we report also the tmaDnaA residues required for oriC unwinding in vitro (Fig. 1G). Results of further analyses are consistent with the idea that tmaDnaA Val-176 and Lys-209 perform similar roles to E. coli DnaA Val-211 and Arg-245.5 tmaDnaA Val-176 resides at the position corresponding to the E. coli DnaA H-motif Val-211 (position i in Fig. 1F). The B-motif residues reside at position v (tmaDnaA Lys-209) or position vi (E. coli DnaA Arg-245) (Fig. 1F). Positions v and vi are very close in sequence and in protein structure (Fig. 1, E and F). These are consistent with the idea described above. E. coli DnaA Lys-243 at position v was required for constructing ATP-form initiation complexes (Figs. 5 and 9) and might reside on inter-DnaA interface. The tmaDnaA residue corresponding to position vi is Gly (Fig. 1F). These might be species-specific minor differentiations in structure of initiation complexes relating to those in oriC primary structure. T. maritima is speculated to be one of the most evolutionarily ancient organisms (45). The primary structure of DnaA AAA5 and DNA-binding domains and the basic structure of oriC, consisting of AT-rich DUE and DnaA box repeats, are highly conserved among eubacterial species (2, 4, 34, 46). Activity for oriC unwinding in tmaDnaA depended on ATP and the arginine finger motif,5 like

5 S. Ozaki and T. Katayama, unpublished results.
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that in *E. coli* DnaA (7). Taken together, it is conceivable that the oriC-unwinding mechanism that we proposed here can be a common system in eubacteria.

An initiation complex at oriC is thought to include ~20 DnaA molecules (2, 4). As a single round of a putative multimeric DnaA spiral consists of 6–7 protomers, 2–4 rounds of the spiral might be formed in an initiation complex (Fig. 1D) (4, 37). This idea is consistent with the proposed mechanism of open complex formation, as configuration of DnaA multimers can be a ring or a spiral in the model shown in Fig. 10. Some base-substitution mutations within the ATP-DnaA-specific low affinity sites inhibit open complex formation by wild-type ATP-DnaA substitution mutations within the ATP-DnaA-specific low affinity sites inhibit open complex formation by wild-type ATP-DnaA (8). Stable binding of ATP-DnaA molecules to these sites might enhance torsion stress of DNA nearby DUE, enhancing DnaA (8). Taken together, it is conceivable that ATP-DnaA-specific conformational change of an initiation complex as shown in Fig. 10 requires stable binding of ATP-DnaA and the specific sites to anchor a subset of DnaA molecules.

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