**The Box VII Motif of *Escherichia coli* DnaA Protein Is Required for DnaA Oligomerization at the *E. coli* Replication Origin**

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*Escherichia coli* DnaA protein initiates DNA replication from the chromosomal origin, oriC, and regulates the frequency of this process. Structure-function studies indicate that the replication initiator comprises four domains. Based on the structural similarity of *Aquifex aeolicus* DnaA to other AAA+ proteins that are oligomeric, it was proposed that Domain III functions in oligomerization at oriC (Erzberger, J. P., Pirruccello, M. M., and Berger, J. M. (2002) *EMBO* J. 21, 4763–4773). Because the Box VII motif within Domain III is conserved among DnaA homologues and may function in oligomerization, we substituted conserved Box VII amino acids of *E. coli* DnaA with alanine by site-directed mutagenesis to examine the role of this motif. All mutant proteins are inactive in initiation from oriC in *vitro* and in *vivo*, but they support RK2 plasmid DNA replication in *vitro*. Thus, RK2 requires only a subset of DnaA functions for plasmid DNA replication. Biochemical studies on a mutant DnaA carrying an alanine substitution at arginine 281 (R281A) in Box VII show that it is inactive in *vitro* replication of an oriC plasmid, but this defect is not from the failure to bind to ATP, DnaB in the DnaB-DnaC complex, or oriC. Because the mutant DnaA is also active in the strand opening of oriC, whereas DnaB fails to bind to this unwound region, the open structure is insufficient by itself to load DnaB helicase. Our results show that the mutant fails to form a stable oligomeric DnaA-oriC complex, which is required for the loading of DnaB.

Chromosomal DNA replication is initiated by DNA-binding proteins, which first recognize replication origins and then assemble the enzymatic machinery that functions at each replication fork (reviewed in Refs. 1–3). In *Escherichia coli*, DnaA mediates replication fork assembly for bidirectional fork movement by first binding to each of the five DnaA boxes of oriC (4, 5). DnaA complexed to ATP then opens the duplex DNA near the left oriC boundary (6). However, nucleotide hydrolysis is not required for unwinding because DnaA bound to the nonhydrolyzable analogue ATPγS is also active. DnaA next recruits two DnaB-DnaC complexes (one for each replication fork) to the unwound region of oriC to form an intermediate named the prepriming complex (7–9). After the release of DnaC from the complex, the helicase activity of DnaB enlarges the unwound region of oriC to position the helicase at the apex of each replication fork (10, 11). During the movement of DnaB in this initial phase and subsequently, primase interacts transiently with the helicase to synthesize primers at multiple sites on the parental DNA (12). A dimeric DNA polymerase III holoenzyme at the fork extends these primers to synthesize the leading and lagging strands concurrently in duplication of the bacterial chromosome (13).

Remarkably, DnaA is structurally comparable with archaeal Cdc6/Orc1 (14), supporting the concept that the initiation of DNA replication in all organisms is mechanistically similar. As added support of the similarity, the origin recognition complex and Cdc6p in *Saccharomyces cerevisiae* are functionally equivalent to *E. coli* DnaA, respectively recognizing yeast replication origins and recruiting the minichromosome maintenance complex, the putative replicative helicase, to form the pre-initiation complex (reviewed in Ref. 15).

Because DnaA protein regulates chromosomal DNA replication at the initiation stage, its role in initiation has been the subject of numerous studies (reviewed in Refs. 1–3). On the basis of the comparison of the primary sequence of 67 eubacterial DnaAs, the replication initiator comprises four domains (see Fig. 1; reviewed in Ref. 14). Domain I, proximal to the N terminus, is moderately conserved, and distinct subdomains function in oligomerization and in the recruitment of DnaB from the DnaB-DnaC complex to oriC (see Fig. 1) (16, 17). Domains I and III are separated by Domain II of the nonconserved sequence. Domain III is highly conserved and contains the Walker A and B box motifs, which function in ATP binding, and also contains Sensor I, Sensor II (Box VIII), and Box VII motifs shared by the AAA+ family of ATPases (14, 18). Within Box VII, a conserved arginine at position 281 of *E. coli* DnaA may enable the protein to sense whether ATP or ADP, formed by nucleotide hydrolysis, remains bound to coordinate ATP hydrolysis to a conformational change. In comparison, many oligomeric AAA+ proteins, such as Hs1U and N-ethylmaleimide-sensitive factor, bind ATP within a bipartite binding site formed between adjacent protomers (reviewed in Ref. 19). Hence, DnaA may bind ATP via a similar structure. Domain IV near the C terminus is moderately conserved, and distinct subdomains function in oligomerization and in the recruitment of DnaB from the DnaB-DnaC complex to oriC (14). Domain IV carries a winged helix DNA binding motif in which amino acids in a loop preceding an α-helix make specific contacts with nucleotides in the DnaA box sequence (14, 23).

As described above, a model was proposed whereby arginine 281 within the Box VII motif sensed the state of the bound adenine nucleotide via its interaction with the γ-PO4 of ATP (14). This model leads to two predictions. First, mutant pro-
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FIG. 1. Domains of E. coli DnaA. Secondary structures of E. coli DnaA are derived from the predicted structure of E. coli DnaA and a homology model comparing E. coli DnaA with the structure of the C-terminal two-thirds of A. aeolicus DnaA (14). The Walker A (WA) and B (WB) boxes, Sensor I (SI), Sensor II (SII, Box VIII), and Box VII motifs conserved among AAA+ proteins, and the portion lacking in DnaA220–294 are shown as black bars. Functional domains involved in DnaA oligomerization (21, 46), in retention of DnaB in the oriC prepriming complex (16, 47), in interaction with DnaB (16, 47), in membrane binding (residues 372–381) (48), and in DNA binding (20, 27) are described in the cited references. The scale near the bottom indicates length in amino acid residues.

peptides of the Box VII motif should retain the ability to bind ATP and thus be able to unwind oriC. Second, because the unwound region of oriC is speculated to be sufficient for the binding of two hexamers of DnaB from the DnaB-DnaC complex (24), mutant proteins should be active in DNA replication.

To test these predictions, we substituted each of the strictly conserved amino acids in Box VII with alanine by site-directed mutagenesis (see “Experimental Procedures” and Fig. 1). We show that the arginine 281 substitution (R281A) retains wild-type activity in ATP binding and in unwinding oriC, confirming the first prediction. In contrast, R281A and other mutant Box VII proteins are inactive in initiation at oriC in vivo and in vitro, refuting the second expectation and demonstrating that Box VII residues are essential for initiation. Further biochemical characterization of R281A revealed that its inactivity in initiation is not a result of defects in binding to DnaA box sequences in oriC or oriV of the plasmid RK2 or to DnaB in the DnaB-DnaC complex. Instead, we found that its failure to form a stable DnaA-oriC complex causes its inactivity in initiation at oriC. We conclude that strand opening of oriC is insufficient to provide a structure with which the DnaB-DnaC complex can stably interact. The results additionally suggest that the Box VII motif functions in DnaA oligomerization.

DnaA protein also functions in plasmid DNA replication. In E. coli, the broad host range plasmid, RK2, appears to require the cooperative binding of DnaA protein to the DnaA boxes within the RK2 replication origin (oriV) (25). We show that the Box VII function is apparently unnecessary for initiation at the replication origin of RK2, supporting the notion that a subset of Box VII motif functions in DnaA oligomerization.

EXPERIMENTAL PROCEDURES

Replication Proteins—Monomeric DnaA220–294, an in-frame deletion of amino acids 220–294 (Fig. 1), was purified as described (26) from E. coli HBS174 (DE3) recA hsdR strains (Novagen) carrying pKKDNA220–294 (27) and grown in LB medium at 37 °C with appropriate antibiotics to maintain the dnaA plasmid. Wild-type DnaA protein that was fused at its N terminus to a His tag (Novagen) used in this study was isolated from E. coli BL21 (DE3) pLyS8 strains carrying pKCS97, which was constructed by inserting a 1.7-kb NdeI-BamHI fragment encoding the wild-type dnaA gene and 289 base pairs of the downstream dnaN gene into the multiple cloning site of pET16b (Novagen) by its cleavage with NdeI and BamHI.

In the construction, the dnaA gene and the vector, have been described (7, 28).

DNAs—M13oriC2LB5 (29), pM3595-Cm4 (31), pSP6 (25), pF10 (32), and pH100 (33) have been described in the respective references. pBR322 and pACYC184 were from laboratory stock. DNA fragments carrying oriC or oriV from plasmid RK2 were prepared by cleavage of M13oriC2LB5 with EcoRI or of pSP6 with EcoRI and HindIII endonucleases, respectively. Radioactive labeling of these DNAs was performed by end filling with the large fragment of E. coli DNA polymerase I (New England Biolabs) and [α32P]dATP (PerkinElmer Life Sciences).

RESULTS

ATP Binding and ATPase Activity—Monomeric DnaA protein binds ATP with high affinity (26, 34). In a complex with ATP and bound to oriC, it unwinds a region within oriC, which is then bound by DnaB from the DnaB-DnaC complex to assemble the prepriming complex (6, 8). If the primary function of arginine 281 is to sense whether ATP or ADP is bound to DnaA,
the substitution of arginine 281 by alanine should not affect ATP binding or its hydrolysis. To test these predictions, we purified a mutant DnaA carrying an alanine substitution at arginine 281 (R281A) (see “Experimental Procedures”).

2 ATP binding assays were performed, which measured the amount of radioactive ATP complexed to DnaA by nitrocellulose filter retention. The assays revealed that R281A was comparable with DnaA in both affinity ($K_d$) for ATP and the ratio of ATP bound per DnaA monomer (Fig. 2A), substantiating our expectation.

DnaA is a weak ATPase, which is stimulated by DNA (34). We measured the ATPase activity of R281A and found that the rate of appearance of R281A-ADP was comparable with DnaA or R281A protein as described (35), and 25 fmol of a $^{32}$P-labeled 337-base pair EcoRI DNA fragment carrying $E. coli$ oriC was separated. The mobility shift assays were performed with the indicated amounts of DnaA or R281A as described (35). For simplicity, the His tag modification has been omitted from the protein nomenclature.

The assays showed that R281A is active in unwinding oriC in a supercoiled plasmid unwinds a region of duplex DNA within oriC (6). The open structure is sensitive to P1 nuclease, which cleaves within the unwound region to linearize the plasmid. The amount of linear DNA, separated from the supercoiled DNA by agarose gel electrophoresis, can be quantified by its fluorescence after ethidium bromide staining. In this assay, we found that the mutant protein was comparable with DnaA$^+$ protein at 3–50 ng in

For this study, protein purification of both mutant and wild-type DnaA was facilitated by an N-terminal polyhistidine sequence and metal chelation chromatography. The presence of the N-terminal polyhistidine sequence does not affect the activity of wild type DnaA in DNA replication (see “Experimental Procedures”) (35). For simplicity, the His tag modification has been omitted from the protein nomenclature.

unwinding of oriC (Fig. 4). Consequently, the substitution did not impair this activity. However, R281A was less inhibitory than DnaA$^+$ at levels above 50 ng.

Box VII Mutants Are Inactive in Initiation from oriC—Despite showing that R281A is active in unwinding oriC and in binding to ATP and the DnaA boxes of oriC, we found that the mutant protein was inert in DNA replication of an oriC-containing plasmid in vitro (Fig. 5A). To ascertain whether other conserved Box VII residues are also essential for function, we substituted these residues (Fig. 1) with alanine by site-directed mutagenesis and expressed the mutant DnaAs as well as R281A and wild-type DnaA in an in vitro coupled transcription-translation system (RTS 100, Roche Applied Science). These proteins, and also R281A, were essentially inactive in oriC plasmid replication, whereas DnaA$^+$ protein expressed in vitro was similar in activity to purified DnaA$^+$ protein assayed in parallel as a control (Fig. 5B). Correspondingly, an in vivo assay showed that the respective dnaA alleles, including pBR322 as a negative control, were inactive in initiation (Table 1). The control with the dnaA$^+$ gene showed that it was active. The genetic assay relied on a host strain lacking a functional chromosomal dnaA locus (dnaA850::Tn10). In this strain, we established plasmids encoding the respective dnaA alleles and then introduced an oriC plasmid (pCM959-Cm$^+$). Hence, dnaA function is plasmid-encoded. These results indicate that the Box VII motif is functionally important for initiation from oriC.

Arginine 281 Functions in DnaA Oligomerization at oriC—After the unwinding of oriC, DnaA recruits the DnaB-DnaC complex

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**Fig. 3.** DnaA and R281A bind comparably to $E. coli$ oriC. Gel mobility shift assays were performed with the indicated amounts of DnaA or R281A protein as described (35), and 25 fmol of a $^{32}$P-labeled 337-base pair EcoRI DNA fragment carrying $E. coli$ oriC from M13oriC2LB5 DNA, followed by autoradiography of the dried gel.

**Fig. 4.** R281A is active in unwinding oriC. Assays (10 μl) to measure the unwinding of oriC were performed with the indicated amounts of either DnaA or R281A as described (16). After incubation at 38 °C for 15 min, 0.75 unit of P1 nuclease (Roche Diagnostics) in 0.01 M sodium acetate (pH 5.3) was added followed by incubation at 38 °C for 15 s. A, the electrophoretic separation of DNAs was visualized by ethidium bromide staining. The reverse image is shown. The lane labeled ‘M13oriC2LB5’ represents the untreated control, which contains both supercoiled and nicked DNA. The position of this DNA linearized by cleavage with HindIII is also indicated. B, the amount of linear DNA was quantified by densitometric analysis of the stained gel.
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via a direct interaction with DnaB to assemble the prepriming complex (7, 36). Subsequently, the hydrolysis of ATP bound to DnaC releases it from the DnaB-DnaC complex to unmask the helicase activity of DnaB (24, 37, 38). Without concomitant DNA replication, DnaB can further unwind the parental duplex DNA (39). The positive superhelicity that accumulates from DNA unwinding can be removed by DNA gyrase to produce a highly negative supercoiled DNA, Form 1*, which can be separated from the naturally supercoiled oriC plasmid by agarose gel electrophoresis and quantified. Because R281A is active in unwinding oriC (Fig. 4), its inactivity in initiation may be at a following step. R281A was inert when it was examined, whereas DnaA \(^{-}\) protein was active in Form 1* formation (Fig. 6), suggesting that R281A is defectate in the assembly of the prepriming complex.

Because DnaA interacts with DnaB to load the helicase at oriC, we considered the possibility that Box VII residues function in DnaB recruitment. As DNA replication of a single-stranded DNA carrying a DnaA box in a hairpin structure requires an interaction between DnaA and DnaB (7, 40), R281A may be inactive in this system. However, we found that R281A was comparable in replication activity to DnaA \(^{-}\) (Fig. 7). We conclude that Box VII is not required for DnaA to interact with DnaB and that R281A fails to assemble the prepriming complex for another reason.

Several studies indicate that a specific nucleoprotein complex forms at oriC during initiation. In one study, we determined by quantitative immunoblot analysis that 10 DnaA...
monomers and two DnaB hexamers are bound to oriC in the isolated prepriming complex (9). DnaC was not quantified because the reaction conditions should have permitted DnaC-dependent hydrolysis of ATP to release it from the DnaB-DnaC complex (24, 37, 38). In a second study, we showed that an N-terminal domain of DnaA must physically interact with another DnaA monomer bound to oriC to form a DnaA-oriC complex that is functionally active (16, 17). A third study involving molecular modeling of Domains III and IV of a DnaA homologue led to the suggestion that Domain III of one DnaA monomer interacts with an adjacent monomer in a proposed DnaA hexamer (14). If Box VII residues within Domain III participate in DnaA oligomer formation to assemble a specific DnaA-oriC complex, which is then recognized by the DnaB-DnaC complex to assemble the prepriming complex, we expect a decreased stoichiometry of the mutant protein because of its failure to assemble properly at oriC, and background levels of DnaB and DnaC as a consequence. To test this model, we incubated R281A, DnaB, and DnaC with an oriC plasmid under conditions optimal to form the prepriming complex and then separated the plasmid DNA and proteins bound to it from unbound proteins by gel filtration chromatography. Quantitative immunoblot analysis of the proteins associated with the oriC plasmid revealed that the mutant DnaA was present at near background levels (Table II). DnaB representing the DnaB-DnaC complex was also barely detectable. As a control, the prepriming complex assembled with wild-type DnaA contained 14 DnaB monomers, consistent with recent results showing that two DnaB hexamers are bound per oriC plasmid (8, 9). The ratio of about seven DnaA monomers per oriC is lower than the expected ratio of 10 monomers per oriC in the prepriming complex but is generally consistent with previous studies (9). When the stoichiometry of DnaA+ bound to the oriC plasmid in the absence of the DnaB-DnaC complex was determined, the ratio of five DnaA monomers agrees with previous observations that one DnaA monomer is at each of the five DnaA boxes of oriC (9). In summary, these results indicate that the mutant DnaA is impaired in DNA replication from its inability to form a stable DnaA-oriC complex. We suggest that the specific defect of R281A is in self-oligomerization.

Cooperative Binding of DnaA to RK2 oriV—Doran et al. (25) proposed that DnaA binds cooperatively to the DnaA boxes in the replication origin of RK2 plasmid. By gel mobility shift analysis, they showed that DnaA forms four discrete complexes on binding to a DNA fragment carrying oriV (Fig. 5A), suggesting that a DnaA monomer binds to each of the four DnaA boxes within oriV. At low DnaA levels, Complex I, which had the greatest electrophoretic mobility, was never seen in the absence of the next retarded complex, Complex II, suggesting that Complex II was formed through a cooperative interaction between DnaA molecules bound to respective DnaA boxes. In support of this interpretation this binding mode was not observed when the rightmost DnaA box in oriV was inverted (25). Instead, DnaA binding appeared to be sequential. We compared R281A with wild-type DnaA and observed similar binding to RK2 oriV by gel mobility shift analysis (Fig. 8B). At lower protein levels in this and other experiments, Complex I was not seen in the absence of Complex II, in agreement with the observations of Doran et al. (25). However, we observed three instead of four complexes and do not know the reason for this difference. Hence, the inability of R281A to form a stable complex at oriC is not apparently from a defect in cooperative binding, suggesting that the functions of self-oligomerization and cooperative binding are distinct. In contrast to DnaA protein, an in-frame deletion lacking amino acids 220–294 (DnaAΔ220–294) did not appear to bind cooperatively to oriV because the appearance of Complexes I and II was sequential (Fig. 8C). Of the AAA+ motifs of DnaA, the Walker B box, Sensor I, and Box VII motifs are absent in DnaA 220–294 (Fig. 1) (14). Because R281A appears to bind normally to the oriV-containing fragment, other AAA+ motifs of Domain III may be responsible for cooperative DNA binding.

We also measured the activity of R281A and other Box VII mutants in RK2 DNA replication in vivo (Table I). In contrast to their inactivity at oriC in vivo, the Box VII mutants maintained the RK2 plasmid about as effectively as wild-type DnaA protein. These results suggest that a subset of DnaA functions is needed at the RK2 plasmid origin and that, if arginine 281

![FIG. 7. R281A is active in DNA replication of a single-stranded DNA carrying a DnaA box in a hairpin structure.](image)

**TABLE II**

Stoichiometry of DnaA and DnaB at oriC

Prepriming complexes were assembled on an oriC plasmid (M13oriC2LB5) and isolated by gel filtration chromatography (Sepharose 4B, Amersham Biosciences) as described (9). Fractions corresponding to the void volume were analyzed for the amount of DNA in ethidium bromide-stained agarose gels and for DnaA and DnaB protein by quantitative immunoblot analysis. Known amounts of DNA or protein were used to prepare standard curves from which the amounts of DnaA and DnaB retained on the oriC plasmid were determined. The results from identical sets of experiments were averaged to calculate the standard deviation for the stoichiometry of DnaA and DnaB protein. The range in the ratios of DnaA and DnaB per oriC plasmid is also indicated. In one set of experiments under conditions optimal to form the prepriming complex (9), the amounts of DnaA+ or R281A and DnaB were determined. DnaC was not quantified because the experimental conditions include ATP, which, when hydrolyzed by DnaC, permits the release of DnaC from the prepriming complex. In the second set of experiments, the amount of wild-type or mutant DnaA bound to the oriC plasmid in the absence of DnaB and DnaC was determined.

| Complex     | Protein  | DnaA No. of experiments | DnaA Monomer/孕期 | Range  | DnaB No. of experiments | DnaB Monomer/孕期 | Range |
|-------------|----------|-------------------------|--------------------|--------|-------------------------|--------------------|--------|
| Prepriming  | DnaA+    | 3                       | 1.2 ± 0.4          | 0.7–1.7| 2                       | 14.1 ± 0.8         | 13.5–14.7|
|             | R281A    | 5                       | 0.55               | 0.5–0.6| 3                       | 2.5 ± 0.6          | 2.1–3.1 |
| DnaA-oriC  | DnaA+    | 4                       | 4.9 ± 1.3          | 4.2–6.3| 2                       | 0.5 ± 0.4          | 0.4–0.6 |
|             | R281A    | 2                       | 0.55               | 0.5–0.6| 3                       | 2.5 ± 0.6          | 2.1–3.1 |
functions to sense the hydrolysis of ATP, it is unnecessary for RK2 DNA replication.

**DISCUSSION**

**The Box VII Motif**—This study investigates the function of the Box VII motif, testing the idea that Box VII mutants should remain active in ATP binding and thus be able to unwind oriC. Because the unwound region of oriC appears to be where DnaB initially binds before moving as a DNA helicase (8), mutants of this motif should be active in initiation if the unwound region of oriC is sufficient for the binding of DnaB from the DnaB-DnaC complex. Our results show that the mutant DnaA bearing an R281A substitution is active in ATP binding and in unwinding of oriC. However, R281A is inactive in initiation from the failure to form a stable complex at oriC. The inactivity of other Box VII mutants in DNA replication suggests that they share similar defects. Thus, the open region within oriC is insufficient to load DnaB. The single-stranded DNA binding activity of DnaC, thought to enable DnaC to bind to the unwound region of oriC to load the helicase (24), does not suffice either. These and previous results (7) indicate that the loading of DnaB requires direct interaction with DnaA assembled at oriC as an oligomer.

**Oligomerization of DnaA at oriC**—Recent studies show that DnaA self-oligomerizes on binding to the DnaA boxes of oriC and that this activity is required for initiation. Specific N-terminal residues (leucine 5, tryptophan 6, glutamine 8, and cysteine 9 in a predicted α-helix), which function in oligomerization (17), reside within a region of DnaA that can replace the dimerization domain of a bacteriophage λ CI repressor (41). Because this N-terminal domain is separate from the Box VII motif relative to the primary structure of DnaA, two regions of DnaA appear to be involved. As additional support for oligomerization, the spatial arrangement of the individual DnaA boxes of oriC must be preserved to maintain oriC function. Insertion of four or eight base pairs between DnaA boxes R3 and R4 near the right oriC border disrupts oriC function but not when the insertion is 10 base pairs, which is one helical turn (42). Apparently, DnaA bound at these sites must interact on one side of the DNA helix in forming an oligomeric complex to load DnaB. Finally, the unstable binding of R281A to oriC supports the proposal based on modeling studies that Domain III functions in DnaA oligomerization (14).

The quantitative immunoblot experiments (Table II) revealed that R281A fails to bind stoichiometrically to oriC, in contrast to gel mobility shift assays in which the mutant DnaA was comparable with wild-type DnaA in binding to the DnaA boxes of oriC (Fig. 3). Furthermore, a previous study concluded that Domain IV is sufficient for sequence-specific DNA binding (20), which is ostensibly at odds with the observation that the R281A substitution causes an unstable DnaA-oriC complex. Because of the rapid off-rate of DnaA when its binding to the DnaA box sequence was measured by surface plasmon resonance (22) or by competitive DNA binding assays (43), we suggest that R281A dissociates from the oriC plasmid as it is being isolated by gel filtration chromatography because of its failure to oligomerize. In contrast, it is well documented that nucleoprotein complexes, which are labile in solution, are stabilized by the caging effect of the polyacrylamide gel in the gel mobility shift method (44, 45), which provides an explanation for the comparable binding of R281A and DnaA* to oriC in this assay.

**DnaA as an AAA⁺ ATPase**—DnaA is a member of the AAA⁺ family of ATPases (19). By analogy with other AAA⁺ ATPases, arginine 281 is proposed to function as the arginine finger (14) to sense whether ATP has been hydrolyzed in coordinating ATP hydrolysis to a conformational change. In other AAA⁺ proteins, the change in conformation modulates enzyme function (reviewed in Ref. 19). If the role of arginine 281 is to function as the arginine finger, the results suggest that the ability to discriminate ATP from ADP is essential for the formation of an active DnaA-oriC complex and the recruitment of DnaB helicase.

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