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Sequence-Independent DNA Binding Activity of DnaA Protein, the Initiator of Chromosomal DNA Replication in *Escherichia coli*[1]

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The DnaA protein specifically binds to the origin of chromosomal DNA replication and initiates DNA synthesis. In addition to this sequence-specific DNA binding, DnaA protein binds to DNA in a sequence-independent manner. We here compared the two DNA binding activities. Binding of ATP and ADP to DnaA inhibited the sequence-independent DNA binding, but not sequence-specific binding. Sequence-independent DNA binding, but not sequence-specific binding, required incubation at high temperatures. Mutations in the C-terminal domain affected the sequence-independent DNA binding activity less drastically than they did the sequence-specific binding. On the other hand, the mutant DnaA433, which has mutations in a membrane-binding domain (K327 to I344) was inert for sequence-independent binding, but could bind specifically to DNA. These results suggest that the two DNA binding activities involve different domains and perform different functions from each other in *Escherichia coli* cells.

Key words: adenine nucleotides, *Escherichia coli*, membrane-binding, mutational analysis.

The DnaA protein is the initiator of chromosomal DNA replication in *Escherichia coli* (*1*). It specifically binds to the DNA sequence at the origin of DNA replication (*oriC*) and opens up the duplex DNA to enable other replicative proteins to enter (*2, 3*). This activity is regulated by adenine nucleotides bound to DnaA. DnaA has a high affinity for both ATP and ADP. The ATP-DnaA complex is active for duplex opening, but the ADP-DnaA complex and nucleotide-free DnaA are inactive (*4*). The membrane-binding activity of DnaA regulates initiation of DNA replication through modulating the adenine nucleotide–binding capacity of the protein. Acidic phospholipids decrease the affinity of DnaA for adenine nucleotides and activate ADP·DnaA to ATP·DnaA in the presence of high concentrations of ATP, by stimulating an exchange reaction (*5–8*).

In addition to the sequence (*oriC*)-specific DNA binding activity, DnaA also binds to DNA in a sequence-independent manner (*9*). This binding activity has been studied less than the sequence-specific binding activity. For example, the functional domain responsible for sequence-specific binding has been identified (*10, 11*), whereas that for the sequence-independent binding remains unknown. The effects of adenine nucleotides have been reported on sequence-specific DNA binding alone. Adenine nucleotides do not affect the sequence-specific DNA binding activity of DnaA as measured by a filter-binding assay (*4*). It was recently reported that there are ATP·DnaA-specific binding sequences on oriC, based on DNase I footprinting analysis (*12*).

The role of sequence-independent binding of DnaA also remains unclear. We have proposed that it is involved in the regulation of DNA supercoiling in cells. We have previously shown that DnaA protein alters DNA topology through sequence-independent binding *in vitro* (*9*) and regulates DNA supercoiling in cells (*13, 14*). Mutations in the *dnaA* gene affected the expression of a number of genes whose promoters have no DnaA box [a consensus sequence TTAT(A/C)CA(A/C)A recognized by DnaA protein), suggesting that the protein's activity on DNA topology could regulate gene expression (*14-17*)]. In this study, we have characterized the sequence-independent DNA binding activity of DnaA. Binding of ATP and ADP to DnaA inhibited sequence-independent binding. DnaA433, with mutations in a membrane-binding domain (amphipathic helix from K327 to I344), did not bind DNA in a sequence-independent manner, suggesting that the domain is involved in the sequence-independent DNA binding.

MATERIALS AND METHODS

Materials—[α-^{32}P]ATP (3,000 Ci/mmol), [γ-^{32}P]ATP (6,000 Ci/mmol), and φX174 DNA were from Amersham Pharmacia Biotech. T4 polynucleotide kinase was from Takara Biochemicals.

Purification of DnaA Protein—The mutant and the wild-type DnaA proteins were purified as previously described (*18, 19*), except for DnaAΔE. DnaAΔE, produced in *E. coli* KA450 strain [*ΔoriC1071*::Tn10, *rnhA199*(Am), *dnaA17*-(Am), *trpE9828*(Am), *tyrA*(Am), *thr*, *ilv*, and *thyA*], was not

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recovered in soluble fractions after cell lysis (data not shown), and we therefore purified it from precipitates, as for DnaA K178I (*20*). We previously reported that the wild-type DnaA purified from precipitates has similar activities (ATP-binding and DNA replication) to the protein purified from supernatants (*20*), suggesting that we could compare the activity of DnaAΔE to that of the wild-type protein and other mutant DnaA.

Site-Directed Mutagenesis and Plasmid Construction—Site-specific mutagenesis was performed using the method described by Kunkel (*21*). Briefly, uracil-containing single-stranded DNA of M13 phage, containing the coding region of the *dnaA* gene, was hybridized with oligonucleotide primers representing each mutation. The complementary DNA strand was synthesized *in vitro* and the resultant double-stranded DNA was introduced into *E. coli* JM109 cells. The mutation was confirmed by direct DNA sequencing. To create the C-terminal deletion mutant (DnaAΔE), the DNA fragment corresponding to amino acid residues 454–467 was removed by restriction enzyme digestion. To construct a plasmid for over-expression, the *Eco*RI–*Hin*dIII region of each *dnaA* gene was ligated into pMZ001 (*20*), which contains the arabinose promoter.

ATP Binding Activity of DnaA Protein—This was determined by a filter-binding assay (*4*). DnaA protein (1 pmol) was incubated with various concentrations of [α-^{32}P]ATP at 4°C for 10 min in 40 μl of buffer G [50 mM HEPES-KOH (pH 7.6), 0.5 mM magnesium acetate, 0.3 mM EDTA, 5 mM DTT, 17% (v/v) glycerol, 10 mM ammonium sulfate, and 0.005% Triton X-100]. Samples were filtered onto nitrocellulose membranes (Millipore HA, 0.45 μm) and washed with ice-cold buffer G. The radioactivity remaining on the filter was counted with a liquid scintillation counter. Data were analyzed by use of Scatchard plots.

Sequence-Specific and Sequence-Independent DNA Binding Activities of DnaA Protein—These activities were measured by a filter-binding assay using radio-labeled DNA fragments (*4*). Specific 294 bp DNA fragments containing oriC were synthesized by PCR using a template (pBS*oriC*) (*22*) and primers ACCTGGGATCCTTGGGTATTA and ATGTGGATAACTCTGTCAGG. Non-specific 348 bp DNA fragments were synthesized by PCR using a template

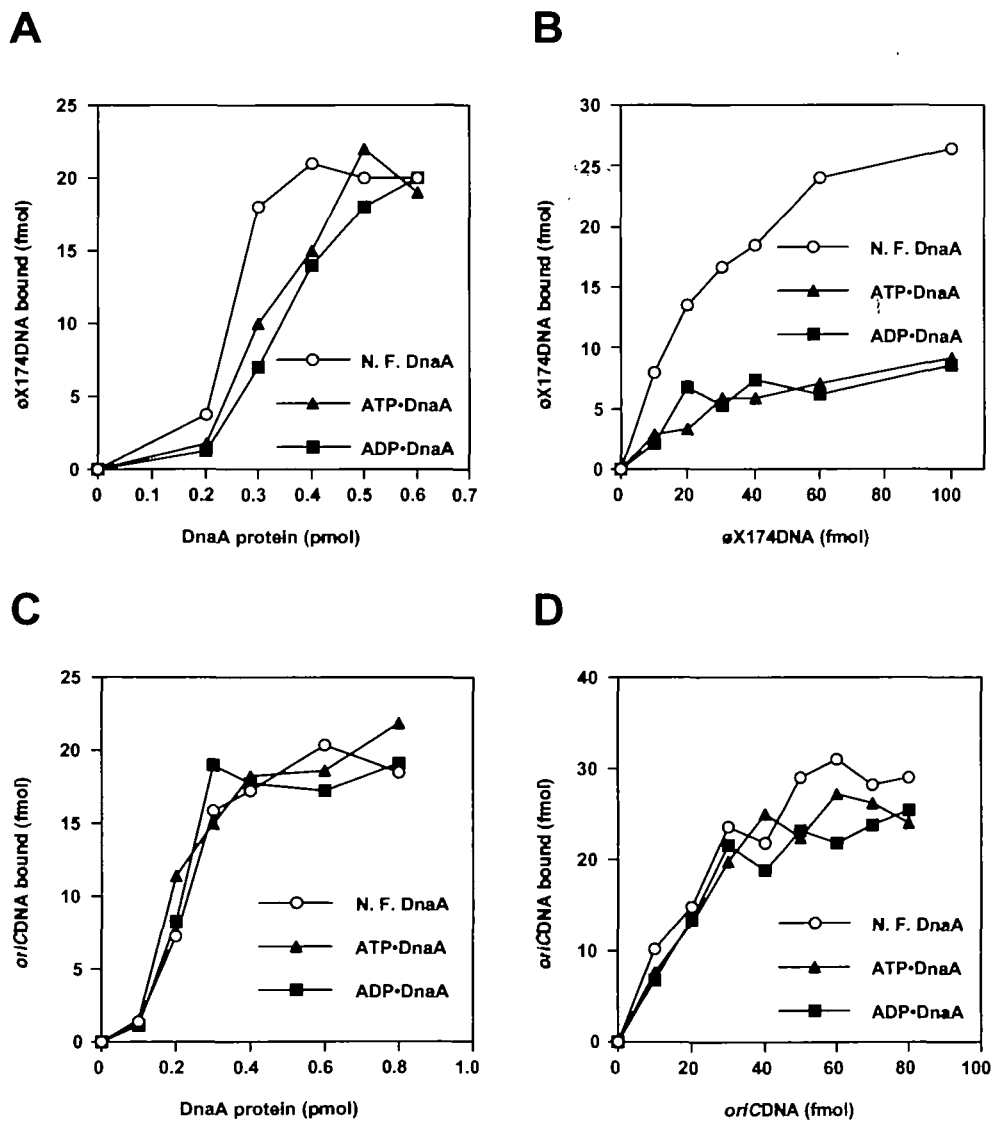

Fig. 1. **Effects of adenine nucleotides on both the sequence-independent and the *oriC* sequence-specific DNA binding of DnaA.** DnaA protein was pre-incubated with ATP or ADP (1 μM) at 4°C for 10 min to form protein-nucleotide complexes. The indicated amounts (A, C) or 0.3 pmol (B, D) of each form of DnaA were further incubated with either 25 fmol (A) or the indicated amounts (B) of radio-labeled φX174 DNA fragments or 20 fmol (C) or indicated amounts (D) of radio-labeled *oriC* DNA fragments in 40 μl of buffer G at 37°C for 4 min. Bound DNA was determined by a filter-binding assay. N.F. DnaA: nucleotide-free DnaA. Data are representative of three similar experiments.

(ϕx174) and primers CCTCCAAATCTTGGAGGCTT and GCATTGTGCCAATTAATCCA. DNA fragments were radio-labeled by T4 polynucleotide kinase and [γ-^{32}P]ATP. The specific activity of each probe was 1,000–7,000 cpm/fmol DNA.

DnaA protein was incubated with radio-labeled DNA at 37°C for 4 min in 40 μl of buffer G. Samples were filtered onto nitrocellulose membranes and radioactivity was counted as described above.

RESULTS

Effects of Adenine Nucleotides on the Sequence-Specific and Sequence-Independent DNA Binding Activities of DnaA—To quantify the DNA-binding activity, we designed a filter-binding assay using radio-labeled DNA fragments. A minimal *oriC* region (*23*) was amplified by PCR and used to detect the *oriC*-specific binding. Since ϕX174 DNA contains no DnaA box sequences (*24*), we used it as a template to prepare DNA fragments for measuring sequence-independent DNA binding.

DnaA protein can alter DNA topology through its sequence-independent DNA-binding activity (*9*). Interestingly, this activity is regulated by adenine nucleotides (*9*). The nucleotide-free form of DnaA was active, but the ATP- and ADP-bound forms were inactive (*9*), suggesting that ATP or ADP might inhibit sequence-independent binding to DNA. To test this idea, we used a filter-binding assay to examine effects of adenine nucleotides on the sequence-independent DNA binding. DnaA protein was pre-incubated with 1 μM ATP or ADP to form complexes, which were then incubated with radio-labeled DNA fragments. In dose-response experiments for both DnaA protein and DNA, the nucleotide-free form of DnaA bound to non-specific DNA fragments more efficiently than did the ATP- or the ADP-bound forms (Fig. 1, A and B). However, at concentrations higher than 0.5 pmol of DnaA, no difference in the sequence-independent DNA binding of DnaA was observed between the ATP- or ADP-bound form and the nucleotide-free form (Fig. 1A). The K_d values of ATP-bound, ADP-bound, and nucleotide-free DnaA for ϕX174 DNA were 0.74, 0.85, and 6.9 nM, respectively, based on the Scatchard plot analysis. The number of binding sites for ϕX174 DNA per ATP-bound, ADP-bound, and nucleotide-free DnaA was 0.04, 0.04, and 0.7, respectively. ATP did not affect the binding of a mutant DnaA (DnaAΔE) with a defect in its ATP-binding (Fig. 6A). Thus, the results in Fig. 1 suggest that the binding of ATP or ADP to DnaA protein inhibits its sequence-independent DNA-binding activity.

We also examined effects of adenine nucleotides on the *oriC*-specific DNA-binding activity. Figure 1, C and D, shows that the three forms of DnaA protein (ATP-bound, ADP-bound, and nucleotide-free) bound almost equally to the specific DNA, confirming previous results (*4*). The K_d values of ATP-bound, ADP-bound, and nucleotide-free DnaA for *oriC* DNA were 3.9, 4.2, and 12 nM, respectively, based on the Scatchard plot analysis. The number of binding sites for *oriC* DNA per ATP-bound, ADP-bound, and nucleotide-free DnaA was 0.44, 0.42, and 1.2, respectively. When the experiments shown in Fig. 1, C and D, were performed in the presence of non-specific competitor DNA (poly dI/poly dC), results were basically the same (data not shown), suggesting that all forms of DnaA bound to *oriC* only in a sequence-specific manner in Fig. 1, C and D. To further confirm that the binding was sequence-specific, we

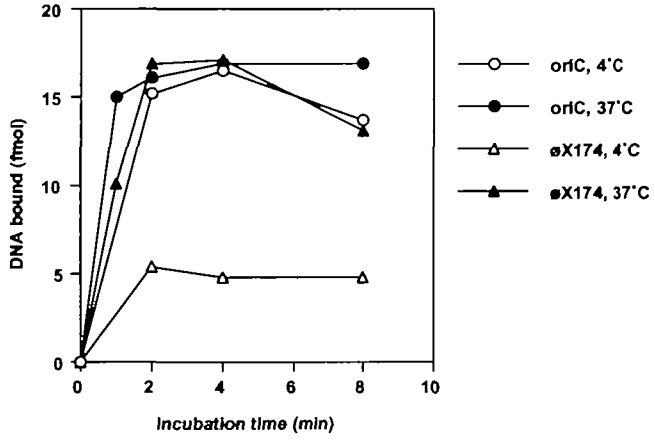

Fig. 3. **Effect of incubation temperature on the sequence-independent DNA binding of DnaA.** DnaA protein (0.3 pmol) was incubated with radio-labeled *oriC* or ϕX174 DNA fragments (40 fmol) in 40 μl of buffer G at 37°C or 4°C for indicated periods. Bound DNA was determined by a filter-binding assay. Data are representative of three similar experiments.

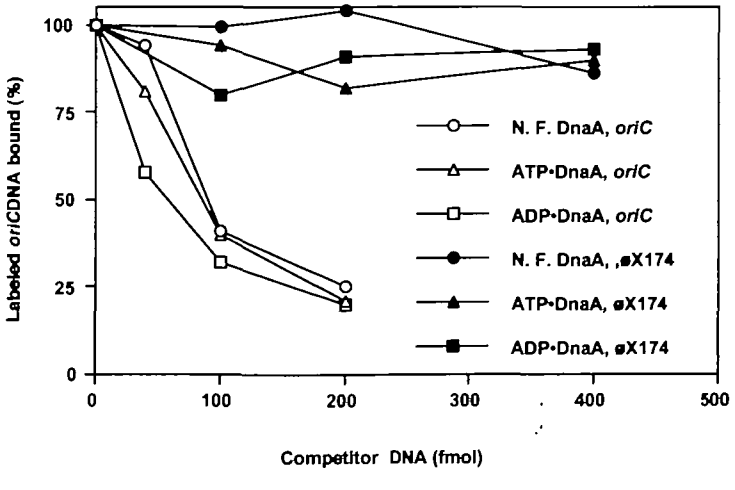

Fig. 2. **Competition analysis to demonstrate that *oriC*-DnaA binding is sequence-specific.** ATP·DnaA and ADP·DnaA complexes (0.3 pmol) prepared as in Fig. 1 were incubated with radio-labeled *oriC* DNA fragments (20 fmol) and various amounts of either unlabeled *oriC* fragments or unlabeled ϕX174 fragments in 40 μl of buffer G at 37°C for 4 min. Bound radio-labeled *oriC* DNA was determined by a filter-binding assay. N.F. DnaA: nucleotide-free DnaA. In the absence of competitor DNA, 18.0, 17.4, and 19.5 fmol of *oriC* DNA was bound to N.F. DnaA, ATP·DnaA, and ADP·DnaA, respectively. Data are representative of two similar experiments.

performed competition analysis. Each form of DnaA protein was incubated with radio-labeled *oriC* DNA fragments in the presence of various concentrations of non-labeled *oriC* or ϕX174 DNA fragments. The binding of radio-labeled *oriC* DNA fragments was inhibited by excess non-labeled *oriC* DNA, but not by excess ϕX174 DNA (Fig. 2). This result suggests that not only ATP- and ADP-bound forms, but also nucleotide-free DnaA protein binds to *oriC* only in a sequence-specific manner. Thus the binding of ATP or ADP to DnaA protein inhibits sequence-independent DNA binding, but not its *oriC*-specific binding.

Effect of Incubation Temperatures on Sequence-Specific and Sequence-Independent DNA Binding Activities—The effect of DnaA on DNA topology, which may be mediated by sequence-independent DNA binding, required incubation at relatively high temperature (37°C) (*9*). The experiments on the sequence-independent DNA binding of DnaA protein (Fig. 1) were routinely done at 37°C. We therefore examined the effects of incubation temperature on sequence-independent DNA binding activity. As shown in Fig. 3, the sequence-independent binding of the nucleotide-free form of DnaA required incubation at 37°C, but sequence-specific DNA binding did not require incubation at such high temperature. Thus, non-specific binding requires higher incubation temperature than sequence-specific binding, as was the case with the activity of DnaA protein on DNA topology (*9*).

The report that the binding of ATP or ADP to DnaA reduces the tendency of the protein to aggregate (*25*) raises the possibility that the aggregated form of DnaA is responsible for the sequence-independent DNA binding of DnaA, even though we used the monomer form of DnaA in all experiments. However, the aggregated form of DnaA which was separated from the monomer form on gel-filtration column chromatography (*18, 19*) did not bind to DNA in either a sequence-specific or a sequence-independent manner (data not shown), suggesting that the aggregated form of DnaA is not responsible for the sequence-independent DNA binding of DnaA.

Mutational Analysis of the Sequence-Independent DNA Binding Activity of DnaA Protein—Previous reports located the *oriC*-specific DNA binding in the C-terminal region and indicated the importance of T435 (*10, 11*). To determine whether the sequence-independent DNA binding activity involves the same domain, we used site-directed mutagenesis. We constructed two mutant proteins, DnaAT435M and DnaAΔE (Fig. 4), and purified them from over-producing strains of *E. coli*. Both are reportedly unable to bind specifically to *oriC* DNA (*10, 11*).

We first examined the *oriC*-specific DNA-binding activities of these mutant proteins. To detect specific DNA binding, we added non-specific competitor DNA (poly dI/poly dC) to the reaction mixture. As shown in Fig. 5, DnaAT435M and DnaAΔE did not bind to the *oriC* DNA fragments. In the absence of non-specific competitor DNA, both mutant proteins bound to the *oriC* DNA fragments (data not shown), suggesting that they can bind to DNA non-specifically (see below). The results in Fig. 5 are much the same as reported previously (*10, 11*). We also examined the ATP-binding activity of these two mutant DnaA proteins. ATP-binding to mutant and wild-type DnaA were measured at various concentrations of ATP, and the K_d value and the number of binding sites were determined by Scatchard plot analysis. As shown in Table I, the K_d value and the number of binding sites of DnaAT435M were much the

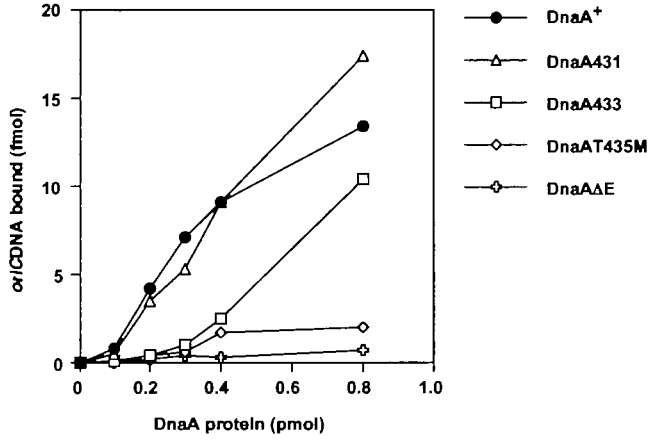

Fig. 5. **The *oriC*-specific DNA-binding activity of mutant DnaA proteins.** Mutant and wild-type DnaA proteins were pre-incubated with ATP (1 μM) at 4°C for 10 min to form ATP·DnaA complexes, and then incubated with radio-labeled *oriC* DNA fragments (20 fmol) in the presence of polydI/polydC competitor (38 ng) in 40 μl of buffer G at 37°C for 4 min. Bound DNA was determined by a filter-binding assay. Data are representative of four similar experiments.

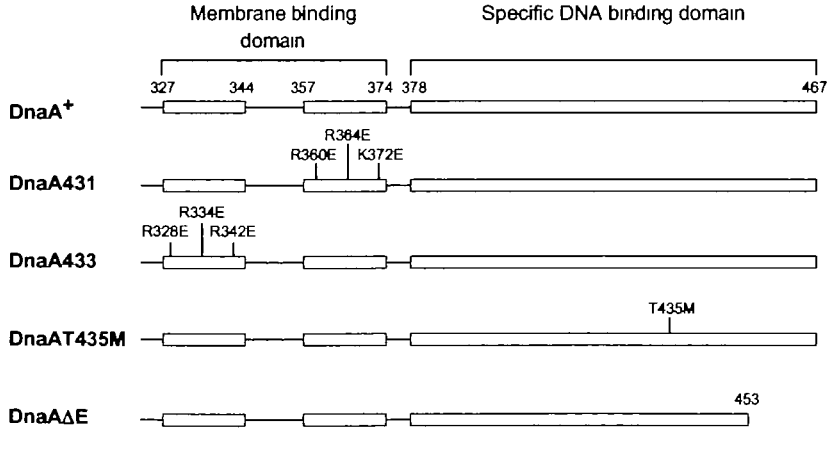

Fig. 4. **Mutant DnaA proteins.** Amino acid residues of DnaA are numbered, and mutations in each mutant DnaA are shown (amino acid residues from 454 to 467 are deleted in DnaAΔE). α-Helixes (K327 to I344 and D357 to V374) suggested in the previous paper (*10*) to be the membrane-binding domain (*26–29*) and specific DNA binding domain are boxed.

same as those of the wild-type protein (*11*); and values for the wild-type protein were nearly the same as reported previously (*4*). On the other hand, DnaAΔE did not bind to ATP (Table I). Since DnaAΔE has intact Walker A and B motifs, we have no clear explanation of why the deletion in DnaAΔE (from 454 to the C-terminal) affects the ATP-binding activity.

We then examined the non-specific DNA binding activity of both mutant proteins in the presence or absence of ATP. As shown in Fig. 6A, the non-specific DNA-binding activity of DnaAΔE was similar to that of the wild-type protein, except that it was not inhibited by ATP. This may be because DnaAΔE has lower affinity for ATP (Table I). The non-specific DNA binding activity of DnaAT435M was lower than that of the wild-type protein, but significant amounts of non-specific DNA fragments were bound to DnaAT435M. Thus, both mutant DnaA proteins were still able to bind to DNA in a sequence-independent manner.

Next, we tried to identify the functional domain for the sequence-independent DNA binding. We predicted that the conserved basic amino acid residues (R328, R334, R342, R360, R364, and K372) located in two amphipathic helixes (K327 to I344 and D357 to V374) of the membrane-binding domain of DnaA would be important for its sequence-independent DNA binding. This is because these basic amino acid residues seem to be located on one side only of each helix, which may allow ionic interaction with a DNA molecule. These basic residues interact with the acidic moiety of acidic phospholipids (*26–29*). Therefore, we examined the sequence-independent DNA-binding activity of two more mutants, DnaA431 (R360E, R364E, and K372E) (*26–28*) and DnaA433 (R328E, R334E, and R342E) (*27, 29*), by a filter-binding assay. As shown in Fig. 6B, the sequence-independent DNA binding activities were much lower than those of the wild-type protein. In particular, DnaA433 (0.3 pmol) had no affinity for non-specific DNA fragments under the conditions employed (Fig. 6B). Larger amounts of DnaA433 (up to 0.8 pmol) also showed no affinity for non-specific DNA fragments; less than 2% of input non-specific DNA (25 fmol) bound to DnaA433 (0.8 pmol), whereas more than 95% of the input DNA bound to the wild-type DnaA (0.8 pmol) (data not shown). On the other hand, the *oriC*-specific DNA binding activities were less affected by these mutations (Fig. 5). More than 50% of input *oriC* DNA (20 fmol) bound to DnaA433 (0.8 pmol) (Fig. 5). The K_d values and numbers of binding sites for ATP (Table I) of these mutant proteins were much the same as described previously (*26–29*). All these results suggest that the amphipathic helix (K327 to I344) is involved in sequence-independent DNA binding. Compared to the wild-type DnaA, the *oriC*-specific DNA binding activity of DnaA433 was slightly decreased (Fig. 5). We consider that mutations in DnaA433 affect the higher order structure of DnaA, resulting in not only decreased stability of DnaA433-ATP complex (*29*) but also its decreased affinity for *oriC* DNA.

TABLE I. **ATP-binding to mutant and the wild-type DnaA proteins.** DnaA protein (1 pmol) was incubated with various concentrations of [α-^{32}P]ATP, and bound ATP was determined by a filter-binding assay. Data were analyzed by Scatchard plots to determine K_d and the number of ATP-binding sites. The mutant DnaAΔE did not bind ATP, and thus the K_d value and number of ATP-binding sites could not be determined.

Protein	K_d value (nM)	Binding sites/DnaA molecule
DnaA$^+$	52	0.54
DnaA431	90	0.50
DnaA433	120	0.25
DnaAT435M	51	0.34
DnaAΔE	–	–

DISCUSSION

Compared to the sequence-specific binding of DnaA to *oriC*, its sequence-independent DNA binding activity had not been well studied. In this paper, we characterized this non-specific DNA binding activity and compared it with the sequence-specific binding. The ATP of and ADP-binding to DnaA inhibited the sequence-independent DNA binding but did not affect *oriC*-specific binding. Mutations in the C-terminal region affected non-specific DNA binding activity less severely than the specific binding. On the other hand, DnaA433, a mutant DnaA protein with mutations in an amphipathic helix (K327 to I344) involved in membrane binding (R328E, R334E, and R342E), showed a defect in

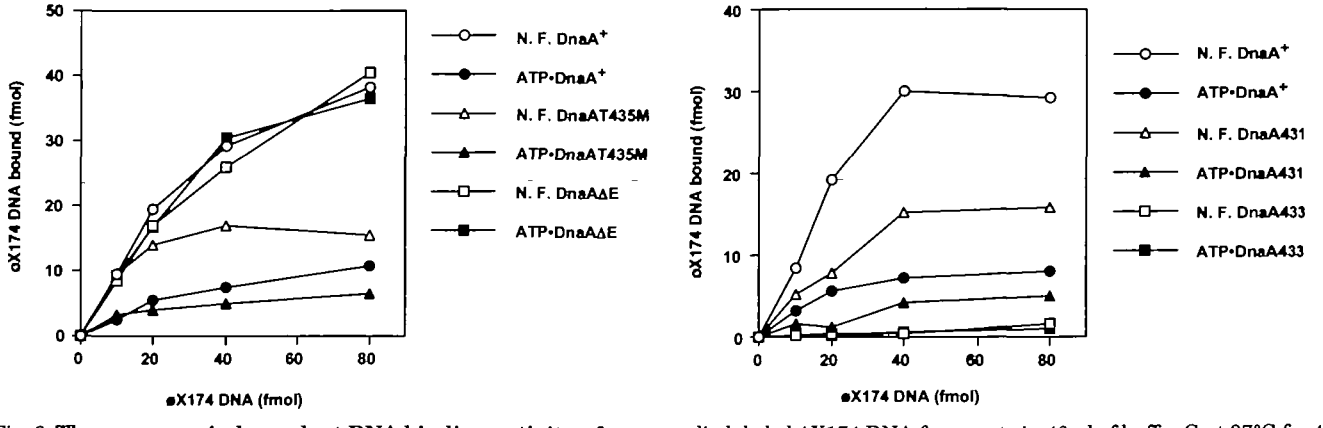

Fig. 6. **The sequence-independent DNA binding activity of mutant DnaA proteins.** Mutant DnaA and wild-type DnaA were preincubated with or without ATP (1 μM) at 4°C for 10 min. Each preparation (0.3 pmol) was further incubated with indicated amounts of radio-labeled φX174 DNA fragments in 40 μl of buffer G at 37°C for 4 min. Bound DNA was determined by a filter-binding assay. N.F. DnaA: nucleotide-free DnaA. Data are representative of four similar experiments.

non-specific DNA binding activity but could specifically bind to *oriC* DNA. The amphipathic helix was previously shown to be dispensable for specific DNA binding activity (*10*). Based on all these results, we consider that the two DNA binding activities of DnaA involve different domains.

Although only 20–30 molecules of DnaA are required for initiation of DNA replication *in vitro* (*2*), the amounts of DnaA protein *in vivo* are estimated at about 2,000 molecules per cell (*18, 30*). There are 308 DnaA box sequences in the *E. coli* chromosome (*31*). Thus, the high abundance of DnaA in cells suggests that this protein could play roles other than initiation of DNA replication, through its sequence-independent DNA-binding activity. However, the physiological roles of the sequence-independent binding activity of DnaA are unclear. Furthermore, because the K_d value of DnaA for ATP is much lower than the concentration of ATP in cells and the sequence-independent DNA binding activity of DnaA is inhibited by ATP and ADP (Fig. 1), the possibility remains that DnaA is unable to bind to DNA in a sequence-independent manner in cells.

DnaA433, which has mutations in the membrane-binding domain, is unable to interact with acidic phospholipids *in vitro* (*27, 29*). Thus, the observation that DnaA433 also lacked sequence-independent DNA binding activity (Fig. 7B) suggests that the two activities share the functional domain. We recently found that acidic phospholipids inhibit the sequence-independent DNA binding activity of DnaA (Makise *et al.*, unpublished). Acidic phospholipids also inhibited the *oriC*-specific DNA binding activity as previously reported (*32*), but the inhibitory effect was weaker than that for the sequence-independent DNA binding activity (Makise *et al.*, unpublished). These results suggest that acidic phospholipids regulate both adenine nucleotide binding and DNA binding.

The binding of ATP and ADP to DnaA selectively inhibited the sequence-independent DNA binding and thus increased the sequence-specificity of DNA binding. As described above, sequence-independent DNA binding involves an amphipathic helix (K327 to I344). We recently reported that R334 is involved in the adenine nucleotide binding of DnaA (*28*). The binding of adenine nucleotides to DnaA may change its conformation and inhibit the access of DNA to this amphipathic helix. We also recently reported that a candidate initiator of chromosomal DNA replication in eukaryotes, Origin Recognition Complex (ORC), can bind to DNA not only in a sequence (origin)-specific manner but also in a sequence-independent manner (*33*). Cdc6p, a key regulator of eukaryotic DNA replication, increases the DNA-binding specificity of ORC by inhibiting its non-specific DNA binding through changing the conformation of ORC (*33*). It is very interesting that bacterial and eukaryotic initiators of DNA replication share common biochemical characters (both specific and sequence-independent DNA-binding activity). It is also interesting that ATP and Cdc6p affect the non-specific DNA binding activity of DnaA and ORC, respectively, in the same way.

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