Effect on DNA Topology by DnaA Protein, the Initiation Factor of Chromosomal DNA Replication in *Escherichia coli*†

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ABSTRACT: We examined effects on supercoiled DNA topology of DnaA protein, the initiator protein of chromosomal DNA replication in *Escherichia coli*. The activity was identified in an analysis of plasmid DNA incubated with DnaA protein and DNA topoisomerase I. In Superose 12 gel filtration chromatography, the activity coeluted with DnaA protein. Incubation of DnaA protein with DNA at temperatures over 24 °C was required for this activity, which was observed with either *oriC* plasmid or the replicative form I of ϕX174 with no DnaA box. As binding of ATP or ADP to DnaA protein prevented the activity of DnaA protein on DNA topology, binding of the adenine nucleotide may regulate the activity.

DnaA protein, the initiation factor of chromosomal DNA replication in *Escherichia coli* (Hirota et al., 1970; Fuller & Kornberg, 1983), specifically binds to DnaA boxes in the *oriC* region, the initiation site of DNA replication (Fuller et al., 1984). DnaA protein is required for the formation of the initial complex on the *oriC* region (Crooke et al., 1993; Funnell et al., 1987), and it guides the entrance of DnaB and DnaC protein to form the prepriming complex (Sekimizu et al., 1988a). Binding of DnaA protein to DNA in a sequence-independent manner has been reported (Fuller et al., 1984). The biological significance of this nonspecific DNA binding has remained to be clarified.

Generally, chromosomal and plasmid DNA in cells is negatively supercoiled. Changes in the extent of DNA supercoiling profoundly affect DNA transactions, such as replication, transcription, and recombination (Drlica, 1984; Wang, 1985, 1987; Kim & Wang, 1989). In *Escherichia coli*, DNA topoisomerase I and DNA gyrase are major DNA topoisomerases regulating the extent of DNA supercoiling (Drlica, 1984; Wang, 1985). In addition to these enzymes, a number of nonspecific DNA binding proteins, such as HU protein, also contribute to maintenance and regulation of the extent of DNA supercoiling (Broyles & Pettijohn, 1986; Yang & Ames, 1990; Tupper et al., 1994). When we examined the influence of DnaA protein on the supercoiling of plasmid DNA, we found that this protein had an effect on supercoiled DNA topology and that this activity was affected by adenine nucleotide binding to DnaA protein.

MATERIALS AND METHODS

Materials. DnaA protein was purified by the method described in a foregoing paper (Sekimizu et al., 1988b) except that a newly constructed overproducer was used (T. Katayama, unpublished results) (Mizushima et al., 1996). The specific activity was 0.7×10^6 units/mg. The purity of the fraction exceeded 90%, as determined by sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis. *Escherichia coli* DNA topoisomerase I was purified by documented methods (Mizushima et al., 1992). The purity of the fraction exceeded 80%, as estimated by SDS−polyacrylamide gel electrophoresis. [α-^{32}P]ATP, [α-^{32}P]dATP, [α-^{32}P]dTTP, [α-^{32}P]dGTP, and [α-^{32}P]dCTP (5 mCi/mmol) were obtained from Amersham Co.

Filter Binding Assay for DNA Binding Activity of DnaA Protein. The replicative form I of ϕX174 DNA was digested with *Ava*I, and the 3′-ends of the restriction fragments were radiolabeled with [α-^{32}P]dTTP, [α-^{32}P]dCTP, [α-^{32}P]dATP, and [α-^{32}P]dGTP by the large fragment of DNA polymerase I (Sambrook et al., 1989). To remove unincorporated nucleotides, the radiolabeled restriction fragments were filtered through a spin column with Sephadex G-50 (Pharmacia). Supercoiled DNA (pUC118) was radiolabeled as follows: Exponentially growing W3110 cells carrying pUC118 plasmid were continuously labeled with [^{3}H]-thymidine. pUC118 plasmid was extracted and purified using alkaline methods (Sambrook et al., 1989). Agarose gel electrophoresis analysis of the fraction revealed that it contained little contamination of chromosomal DNA. Binding of DnaA protein to the radiolabeled DNA was measured by the filter-retention assay, as described (Sekimizu et al., 1987). DnaA protein and DNA were incubated at 37 °C for 4 min in buffer G [50 mM HEPES−KOH (pH 8.0 at 1 M), 0.5 mM magnesium acetate, 0.3 mM EDTA, 5 mM dithiothreitol, 10 mM ammonium sulfate, 17% glycerol, and 0.005% TritonX-100]. Samples were passed through membranes (Millipore, HAWP), and the retained radioactivity was counted in a liquid scintillation counter (Beckman).

Assay of Effect on DNA Topology. DnaA protein and negatively supercoiled DNA were mixed in buffer M [50 mM HEPES−KOH (pH 7.6 at 1 M), 1.2 mM magnesium acetate, 1 mM EDTA, 2 mg/mL BSA, and 17% glycerol] at 4 °C. The mixture was incubated at 37 °C for 5 min. *Escherichia coli* DNA topoisomerase I (500 ng) was added to samples (including control, without DnaA protein), and the mixture was further incubated at 4 °C for 4 h. DNA was extracted with phenol/chloroform, precipitated with

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ethanol, and dissolved in TE buffer. DNA samples were subjected to agarose (1%) gel electrophoresis (40 V, 12 h) in TAE buffer (100 mM Tris/acetate, 2 mM EDTA) and stained with ethidium bromide, and photographs were taken using a Polaroid apparatus.

Assay of ATP Binding to DnaA Protein. DnaA protein (2 pmol) in 40 μL of buffer M was mixed at 4 °C with [α-^{32}P]-ATP (5 mCi/mmol). The final concentration of ATP was 2 μM. The solution was passed through a membrane filter (Millipore HA 0.45 μm pore) presoaked in buffer G. The filter was washed with 6 mL of ice-cold buffer G and dried under an infrared lamp. Retained radioactivity was measured in a liquid scintillation counter.

RESULTS

Effects of DnaA Protein on Supercoiled DNA Topology. In addition to specific binding to DnaA boxes, DnaA protein has the potential to bind to DNA, in a sequence-independent manner (Fuller et al., 1984). A number of DNA binding proteins, such as HU protein, affect DNA supercoiling *in vitro* (Broyles & Pettijohn, 1986), and mutations in genes encoding HU protein alter levels of DNA supercoiling *in vivo* (Hillyard et al., 1990; Hsieh et al., 1991; Yasuzawa et al., 1992). Thus, we asked whether DnaA protein might also have an effect on DNA topology. Such activity was determined by a change in the linking number of closed circular DNA after treatment of the DNA−DnaA complex with *Escherichia coli* DNA topoisomerase I (Broyles & Pettijohn, 1986; Adams & West, 1995). With this assay, binding of protein which has an effect on DNA topology results in a change in the linking number of DNA after reacting with DNA topoisomerase I. Purified DnaA protein and the replicative form I of ϕX174 were preincubated at 37 °C for 5 min followed by incubation at 4 °C for 4 h with *Escherichia coli* DNA topoisomerase I. Under these conditions, the DNA relaxation reaction induced by DNA topoisomerase I reached saturation within 2 h (data not shown). Following incubation, DNA was extracted and analyzed by agarose gel electrophoresis. The addition of DnaA protein resulted in an increase in the migration rate of DNA, thereby indicating that the binding of DnaA protein to ϕX174 DNA affects DNA topology (Figure 1a). On agarose gel electrophoresis in the presence of 0.25 or 0.5 μg/mL chloroquine, DNA preincubated with DnaA protein migrated more slowly than did control DNA preincubated without DnaA protein (Figure 1b). This means that DNAs are negatively supercoiled in the gel containing no chloroquine, and positively supercoiled in the gel containing 0.25 or 0.5 μg/mL chloroquine, under the present conditions of electrophoresis. The results show that DnaA protein decreases the linking number of the reaction product by DNA topoisomerase I. Even in the presence of a higher amount of DNA topoisomerase I (5 μg), DnaA decreases the linking number of the reaction product, to the same extent (data not shown). As DnaA protein is not likely functioning as an inhibitor of DNA topoisomerase I, we consider that DnaA protein has an effect on DNA topology. Similar results were obtained with mouse DNA topoisomerase I (data not shown). When relaxed circular DNA (reaction product of topoisomerase I) was used as a substrate to examine the activity of DnaA protein on DNA topology, the activity was not observed (data not shown), suggesting that negative supercoiling of DNA is required for the activity.

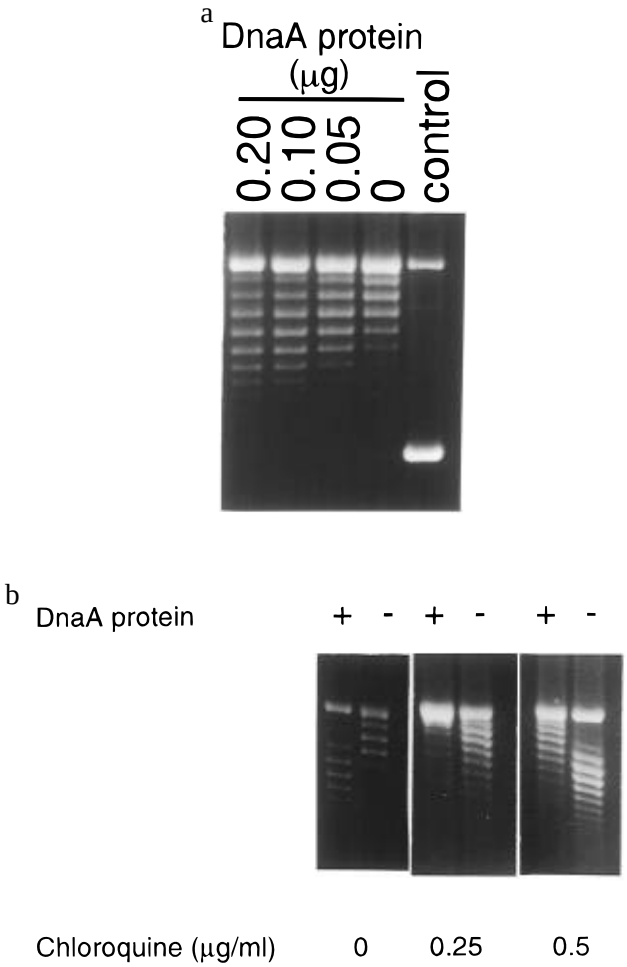

FIGURE 1: Effects on supercoiled DNA topology by DnaA protein. DnaA protein (a, indicated amounts; b, 0.1 μg) and the replicative form I of ϕX174 DNA (0.5 μg) were preincubated at 37 °C for 5 min followed by incubation with *Escherichia coli* DNA topoisomerase I at 4 °C for 4 h. ϕX174 DNA was analyzed by agarose (1%) gel electrophoresis in the absence (a) or presence (b) of chloroquine. DNA incubated with neither DnaA nor topoisomerase I is shown as a control.

Coelution of the Activity on DNA Topology and DnaA Protein on Superose 12 Gel Filtration Chromatography. To gain support for the proposal that DnaA protein is responsible for the activity on DNA topology, we examined whether the activity and DnaA protein would coelute on Superose 12 column chromatography. Fraction V of the DnaA purification procedure (Sekimizu et al., 1988b) (guanidine fraction) was gel-filtered on a Superose 12 column. Recovery of the activity of DnaA protein for DNA replication in this chromatography was about 50%, a value much the same as reported earlier (Sekimizu et al., 1988b). The activity on DNA topology of DnaA protein was quantified by densitometric scanning of DNA topoisomers, as described in the legend of Figure 2. The amount of DnaA protein was determined by SDS−polyacrylamide gel electrophoresis and by measuring replication activity in the mutant complementation assay of *oriC* DNA replication (Fuller et al., 1981). The activity on DNA topology coeluted with the monomer of DnaA protein on this gel filtration chromatography. Fraction 42 in Figure 2 was a peak fraction for both the activity on DNA topology and DnaA protein. The fraction showed a single band with a molecular mass of 50 kDa on SDS−polyacrylamide gel electrophoresis (data not shown). Recovery of the activity of DnaA protein on DNA topology

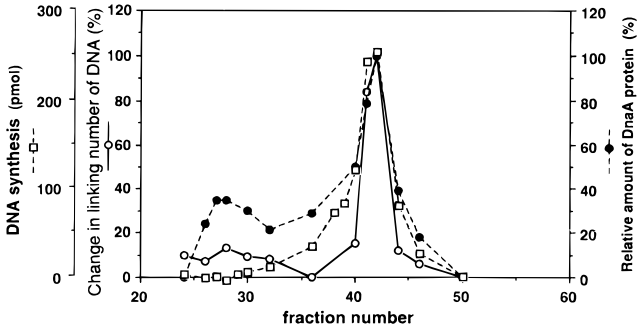

FIGURE 2: Coelution of the activity on DNA topology with DnaA protein on Superose 12 gel filtration chromatography. Superose 12 chromatography of DnaA protein was done as described (Sekimizu et al., 1988b). The activity on DNA topology in each fraction was analyzed as described in the legend of Figure 1a and quantified by determination of the decrease in the linking number of DNA, by methods described elsewhere (Keller, 1975). The relative change of the linking number to that of fraction 42 is shown. Replication activity was determined by the mutant complementation assay, as described (Fuller et al., 1981). The relative amount of DnaA protein was determined by densitometric scanning of the stained band corresponding to DnaA protein on SDS−polyacrylamide gel electrophoresis.

in this chromatography was 55%. Therefore, proteins contaminated in the preparation of DnaA protein apparently did not contribute to the activity.

DNA Specificity for the Activity of DnaA Protein on DNA Topology. As ϕX174 DNA has no DnaA box, the effect on DNA topology of DnaA protein did not require the DnaA box in template DNA (Figure 1). To determine whether DnaA boxes in the duplex DNA would affect the activity, the effect on DNA topology by DnaA protein was analyzed using negatively supercoiled forms of pCM959 (Meijer et al., 1979), M13*oriC26* (Fuller et al., 1981), and pBS*oriC* (pTB107) (Baker & Kornberg, 1988), which contain the *oriC* region, pBR322; it has one DnaA box and the replicative form I of ϕX174, which does not contain the DnaA box (Figure 3, lanes 1, 2). The effect on DNA topology by DnaA protein was observed with all DNA species, and the extent of change in the linking number was indistinguishable among all these species. This means that the DnaA box and the *oriC* sequence do not affect the activity on DNA topology of DnaA protein.

Requirement of High Temperature for the Effect of DnaA Protein on DNA Topology. To examine the effect on DNA topology of DnaA protein described in previous sections, DnaA protein and closed circular duplex DNA were preincubated at 37 °C for 5 min followed by incubation with DNA topoisomerase I at 4 °C. A decrease in the linking number of DNA by DnaA protein was not observed with preincubation at 4 °C (data not shown). Thus, one can separate the reaction of the topological change of DNA by DnaA protein from that of DNA relaxation by topoisomerase I. As shown in Figure 4, the activity on the DNA topology of DnaA protein was stimulated to a greater extent with a higher preincubation temperature. At 42 °C, the activity was less than that seen at 37 °C, perhaps because the DnaA protein had denatured (see next paragraph).

The DNA replication activity of purified DnaA protein is unstable; the protein loses the activity within a few minutes of incubation at 37 °C (Sekimizu et al., 1987). Therefore, only heat-denatured DnaA protein may have the activity on DNA topology of DnaA protein. In other words, the

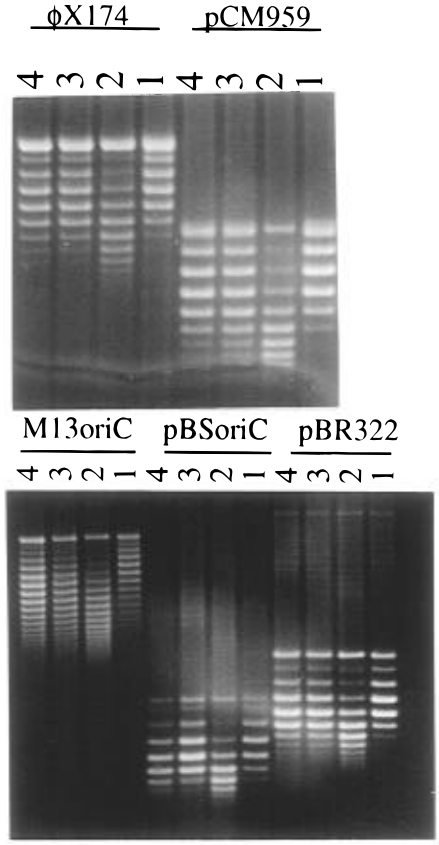

FIGURE 3: DNA specificity for the activity on DNA topology of DnaA protein. DnaA protein (0.1 μg) was preincubated with 1 μM ATP (lane 3) or 1 μM ADP (lane 4) or without nucleotide (lane 2). Lane 1 is a control without DnaA protein. The effect on DNA topology by DnaA protein was examined using negatively supercoiled forms of pCM959, M13oriC (M13*oriC26*), pBSoriC (pTB101), pBR322, or the replicative form I of ϕX174 (0.5 μg). DNAs were extracted and analyzed by agarose gel electrophoresis as in Figure 1a.

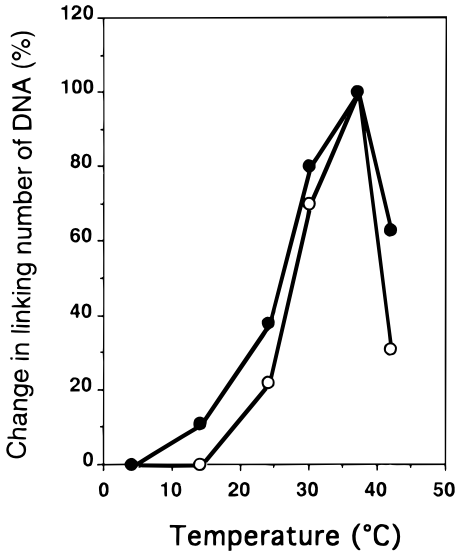

FIGURE 4: Influence of temperature on the activity on DNA topology of DnaA protein. DnaA protein (0.1 μg) and negatively supercoiled forms of DNA (0.5 μg) were preincubated for 5 min at various temperatures. The effect on DNA topology by DnaA protein was quantified, as described in the legend of Figure 2. The relative change of the linking number to that of sample at 37 °C is shown. (○) ϕX174; (●) pBS*oriC*.

requirement of a high temperature at the preincubation step might be because DnaA protein is denatured. To examine

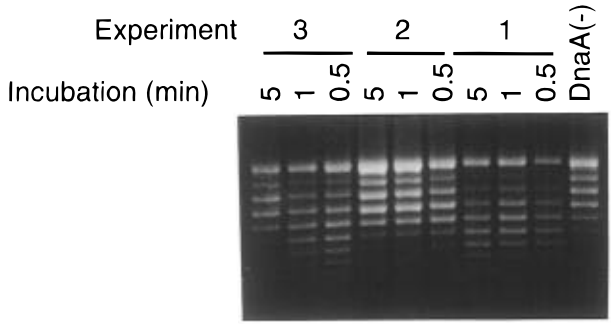

FIGURE 5: Influence of preincubation of DnaA protein on the activity on DNA topology of DnaA protein. Experiment 1: DnaA protein (0.1 μg) and replicative form I of ϕX174 DNA (0.5 μg) were incubated at 37 °C for the indicated time. Experiment 2: DnaA protein (0.1 μg) was incubated at 37 °C for the indicated time followed by addition of the replicative form I of ϕX174 DNA (0.5 μg) at 4 °C. Experiment 3: DnaA protein (0.1 μg) was preincubated at 37 °C for the indicated time in the absence of DNA; then ϕX174 DNA (0.5 μg) was added to the mixture and the preparation was incubated at 37 °C for 5 min. After the DNA relaxation reaction with DNA topoisomerase I, DNA was extracted and analyzed by agarose gel electrophoresis.

this possibility, we investigated whether or not heat-denatured DnaA protein has the activity on DNA topology. Judged by the ATP binding activity, DnaA protein was completely inactivated after incubation for 4 min at 37 °C in the buffer for the assay of the effect on DNA topology by DnaA protein, while most activity was retained after incubation for 1 min at 37 °C (data not shown). The change in DNA topology by DnaA protein reached a saturation level within 1 min at 37 °C (Figure 5, experiment 1). Therefore, it does not seem likely that the effect on DNA topology by DnaA protein is caused by denaturation of DnaA protein. This conclusion is supported by the results of experiment 2 in Figure 5, showing that there is no decrease in the linking number of DNA when the preincubation is done in the absence of DNA.

The heat stability of the activity on the DNA topology of DnaA protein was also examined. DnaA protein was preincubated at 37 °C for various periods followed by incubation for 5 min with DNA at 37 °C, and this led to a complete loss of the activity (Figure 5, experiment 3). Thus, the activity on DNA topology of DnaA protein is as heat-labile as the ATP binding activity.

Inhibition of the Activity on DNA Topology by Adenine Nucleotide Binding to DnaA Protein. DnaA protein has a high affinity for ATP (K_d = 30 nM) and ADP (K_d = 100 nM) (Sekimizu et al., 1987). The ATP binding form is active in an *oriC* replication system reconstituted with purified proteins, while the ADP-binding form and the nucleotide-free form are inactive (Sekimizu et al., 1987). In the foregoing experiments described in this report, we used the nucleotide-free form of DnaA protein to assay the effect on DNA topology. Next, we examined whether binding of ATP or ADP to DnaA protein would affect the activity on DNA topology of DnaA protein. The ATP or ADP binding form of DnaA protein was prepared by preincubation of DnaA protein with 1 μM ATP or ADP, respectively (Sekimizu et al., 1987). We confirmed the formation of the DnaA−ATP complex, using [α-^{32}P]ATP, under the present conditions (data not shown). The nucleotide-free form of DnaA protein showed saturation at 0.1 μg for the activity on DNA topology, while 0.2 μg of DnaA protein preincubated with

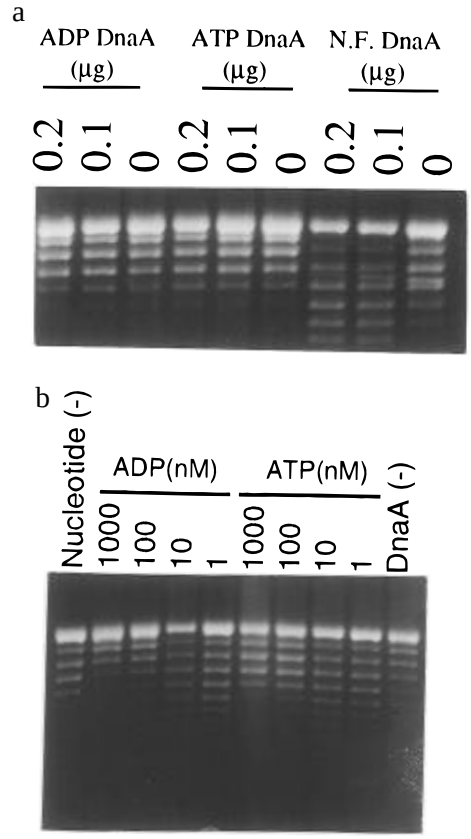

FIGURE 6: Inhibition of the activity on DNA topology by ATP or ADP. DnaA protein (a, indicated amounts; b, 0.1 μg) was preincubated with ATP or with ADP, or with no nucleotide (a, 1 μM; b, indicated concentrations) at 0 °C for 15 min. The effect on DNA topology by DnaA protein was analyzed (see Figure 1a).

ATP or ADP showed no activity (Figure 6a). Concentrations of ATP or ADP required to inhibit the activity were approximately 100 nM (Figure 6b), such being consistent with the K_d value for ATP or ADP binding of DnaA protein (Sekimizu et al., 1987). These results suggest that the inhibitory effects of ATP or ADP on the activity on DNA topology of DnaA protein are caused by formation of the ATP− or ADP−DnaA complex. This conclusion was supported by findings that ATP or ADP did not inhibit the activity, when added after the preincubation step at 37 °C (data not shown). The inhibition was observed even when either *oriC* DNA or pBR322 was used for assay (Figure 3), indicating that the ATP or ADP binding form of DnaA protein has no activity, even for DNA containing DnaA boxes or the *oriC* sequence.

Influence of ATP and ADP on the Nonspecific DNA Binding of DnaA Protein. Data presented in foregoing sections suggest that DNA binding of DnaA protein in a sequence-independent manner leads to the effect on DNA topology. With the knowledge that the activity of DnaA protein was inhibited by adenine nucleotide binding to DnaA protein, we examined by filter binding assay the influence of the adenine nucleotide on nonspecific binding of DnaA protein to radiolabeled restriction fragments of the replicative form I of ϕX174 which does not contain the DnaA box. There was no significant difference on the binding to DNA fragments among the ATP binding form, ADP binding form, and the nucleotide-free form (data not shown). Incubation at 37 °C was not required for the nonspecific binding of DnaA protein to DNA (data not shown).

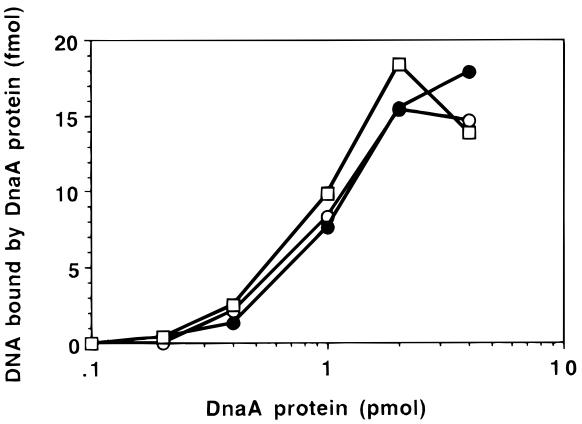

FIGURE 7: Binding of DnaA protein to the supercoiled form of pUC118 DNA. The ATP binding, ADP binding, and nucleotide-free forms of DnaA protein were formed by preincubation with 1 μM ATP or 1 μM ADP, or with no nucleotide in 85 μL of Buffer G at 0 °C for 15 min. The mixture was further incubated for 4 min at 37 °C with the supercoiled form of pUC118 DNA prepared by *in vivo* labeling (20 fmol, 12 000 cpm). Samples were passed through membranes (Millipore, HAWP), and the retained radioactivity was counted. (○) ATP-DnaA; (●) ADP-DnaA; (□) nucleotide-free DnaA.

When we examined the effect of adenine nucleotide on DnaA protein binding to circular and supercoiled DNA (Figure 7), the results were much the same as seen with linear DNA. Thus, inhibition of the activity on DNA topology of DnaA protein by adenine nucleotide cannot be explained by decreases in the affinity of DnaA protein for DNA.

DISCUSSION

We obtained evidence for effects on supercoiled DNA topology by a purified fraction of DnaA protein. The activity comigrated with DnaA protein on Superose 12 gel filtration chromatography. A search of the literature revealed no evidence for the effect on DNA topology by DnaA protein. Previous studies revealed that negative supercoiling of the template is necessary for the initiation of *oriC* replication. For example, relaxed DNA templates are inert for *oriC* DNA replication *in vitro*, as reconstituted from purified proteins (Baker & Kornberg, 1988). It was suggested that transcription-induced DNA supercoiling affects the initiation of chromosomal DNA replication *in vivo* (Asai et al., 1992). Analysis of *oriC* DNA replication *in vitro* also suggests that conformational changes of the *oriC* region resulting from transcription activate the initiation of *oriC* replication (Baker & Kornberg, 1988). The effect of DnaA protein on DNA topology described in this present report may also contribute to the initiation of *oriC* DNA replication.

The activity on DNA topology was inhibited by preincubation of DnaA protein with ATP or ADP, suggesting that the adenine nucleotide binding forms of DnaA protein are inert for the activity on DNA topology. Apparently only the ATP binding form of DnaA protein is active in *oriC* replication (Sekimizu et al., 1987). Therefore, both activities of DnaA protein in cells, strand-opening of the *oriC* region and the effect on DNA topology, may be regulated by adenine nucleotide binding.

There is a sensitive phenotype of *dnaA* mutants to coumarin antibiotics (Filutowicz, 1980). These reagents inhibit DNA gyrase and decrease negative supercoiling of DNA *in vivo*, which suggests that DnaA protein contributes to maintenance of DNA supercoiling in cells. DnaA protein may directly contribute to formation of supercoiled chromosomal DNA, which we described in the present report.

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