BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **232,** 130–135 (1997)
ARTICLE NO. RC976244

Conformational Transition of DnaA Protein by ATP: Structural Analysis of DnaA Protein, the Initiator of *Escherichia coli* Chromosome Replication[1]

Toshio Kubota, Tsutomu Katayama, Yuji Ito, Tohru Mizushima, and Kazuhisa Sekimizu[2]
Department of Microbiology, Kyushu University Faculty of Pharmaceutical Sciences, Higashi-ku, Fukuoka 812-82, Japan

Received January 20, 1997

DnaA protein binds to the chromosomal origin (*oriC*) to initiate DNA replication. We developed an efficient system for purification of DnaA protein which will facilitate physicochemical analysis of the protein. The yield of DnaA protein was increased at least 6-fold compared to an available method being used, and over 22 mg of the protein were obtained from only 100 g of cells. DnaA protein purified by this procedure showed an indistinguishable affinity for ATP, and activity for *in vitro* replication of *oriC* plasmid. The process of denaturation of DnaA protein, which was blocked by ATP, was monitored by intrinsic fluorescence and circular dichroism. Analysis of circular dichroism revealed that DnaA protein is rich in α-helices, and that ATP-binding leads to a significant transition of protein conformation in that the content of α-helices is decreased. This is the first evidence indicating that ATP-binding profoundly affects conformation of DnaA protein.

DnaA protein is a key factor for initiation of chromosome replication in *Escherichia coli* (1,2), a reaction which requires an unique origin on the chromosome, *oriC,* and the so-called initiator, DnaA protein. This protein binds to the *oriC* site (245 bp), forms a multimeric (20–40 mer) complex, causes local strand-separation in a AT-rich region, and loads DnaB helicase on the generated single-stranded region, a location where complementary DNA is subsequently synthesized.

DnaA protein tightly binds ATP and ADP with a K_D of 30 and 100 nM, respectively (3). The ATP-bound form is active for local unwinding and for initiation, whereas the ADP-bound form is inactive for such processes. Interaction of DnaA protein with acidic phospholipids results in a decrease in the affinity of DnaA protein for adenine nucleotide, and thus may contribute toward regulation of the nucleotide binding of the protein (4–6). A protein factor (IdaA) that is included in a soluble cell extract specifically recognizes DnaA protein to inactivate for initiation at the *oriC* site, and thus is implicated in the control of initiation *in vivo,* by down-regulating DnaA activity (7, 8).

In contrast to detailed characterizations of the functions, structural analysis of this protein remains largely unexplored, mainly because of difficulty in acquiring a large-scale supply of DnaA protein in the active form. The overexpression system of DnaA protein in particular has been inefficient. We developed a new procedure to obtain adequate supplies of DnaA protein, and the method of purification of this protein was improved. The large scale acquisition of the protein made a structural study feasible. Measurements of intrinsic fluorescence indicated that ATP stabilizes the conformation of the protein against heat, while circular dichroism measurement revealed an alteration of the secondary structure of DnaA protein by nucleotide binding.

MATERIALS AND METHODS

Buffers. Buffer C contained: 50 mM Hepes-KOH (pH7.6), 1 mM EDTA, 20% sucrose, and 2 mM dithiothreitol. Buffer D contained: 50 mM Hepes-KOH (pH7.6), 0.1 mM EDTA, 10 mM magnesium acetate, 20% sucrose, 0.2 M ammonium sulfate, and 2 mM dithiothreitol. Buffer K is the same as buffer D except that it contained 0.2 M potassium sulfate instead of 0.2 M ammonium sulfate.

Construction of an overproducing plasmid of DnaA protein. The DnaA protein-overproducing plasmid used here, pKA234, is derived from the DnaAcos protein-overproducing plasmid, pKA233-5 (7). DnaAcos protein is a mutated DnaA protein with base-substitution mutations located within the 1.7 kb *Eco*RI fragment (9), including most of the coding region. This 1.7 kb *Eco*RI fragment of pKA233-5 was replaced with the corresponding part of a plasmid bearing the wild-type *dnaA* gene (T. Katayama, unpublished). The resultant plasmid is pKA234 (Fig. 1).

[1] The first two authors contributed equally to this work.
[2] To whom correpondence should be addressed. Fax: 81(Japan)-92-632-6648. E-mail: sekimizu@bisei.phar.kyushu-u.ac.jp.

0006-291X/97 $25.00

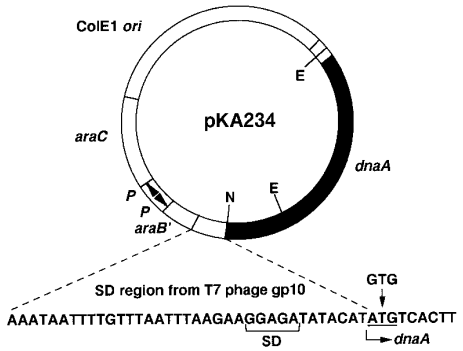

FIG. 1. Structure of pKA234. This plasmid contains the entire coding region of the *dnaA* gene downstream of the T7 phage gp10 Shine-Dalgarno (SD) sequence. Transcriptional promoters are indicated by arrowheads. Restriction sites are: *E, EcoRI; N,NdeI.* Transcription from the *araB* promoter, which is repressed by the *araC* product, is induced by arabinose. The initial GTG codon of *dnaA* gene was replaced with ATG.

In vitro complementation assay of DnaA activity for minichromosome replication. Replication of minichromosome, M13mpRE85 (10), was performed *in vitro,* according to Fuller *et al.* (11). Briefly, a crude protein fraction was prepared from WM433 [*dnaA204*], by a freeze-thaw lysis, isolation of the soluble extract, and precipitation of proteins in 0.28 g/ml of ammonium sulfate. Reaction (25 μl) for minichromosome replication contained: 25 mM Hepes-KOH (pH7.6); 40 mM phosphocreatine; 100 μg/ml creatine kinase; 10 mM magnesium acetate; 7% (w/v) polyvinyl alcohol (MW 30000–70000); 2 mM ATP; 0.5 mM each of GTP, CTP, and UTP; 100 μM each of dATP, dGTP, dCTP and [α-^{32}P] dTTP (70–150 cpm/pmol); 200 μg of the crude protein extract from WM433; and 200 ng (600 pmol as nucleotide) of M13mpRE85 Replicative from I DNA. M13mpRE85 consisted of the minimal *oriC* sequence and M13mp8. Various amounts of DnaA protein were added to the reaction and the preparation was incubated at 30°C for 20 min. Nucleotides incorporated into acid-insoluble materials were retained on GF/C filters, and measured by liquid scintillation counting. One unit of the activity promotes incorporation of 1 pmol of nucleotide for 1 min at 30°C. Protein concentration was determined by the method of Bradford using bovine serum albumin as a standard (12).

In vitro minichromosome replication reconstituted with purified proteins. Replication of minichromosome, pBS*oriC* (pTB101) (13), was performed basically as described (14–16). The reaction (25 μl) contained: 20 mM Tris-HCl (pH 7.4); 0.1 mg/ml bovine serum albumin; 8 mM dithiothreitol; 0.01% Briji 58; 8 mM magnesium acetate; 125 mM potassium glutamate; 2 mM ATP; 0.5 mM each of GTP, CTP, and UTP; 200 ng (600 pmol as nucleotide) pBS*oriC,* 400 ng SSB; 10 ng HU, 180 ng A-subunit of gyrase; 360 ng of B-subunit of gyrase; 150 ng DnaB helicase; 90 ng DnaC protein; 10 ng DnaG primase; 450 ng DNA polymerase III*; 40 ng β-subunit of DNA polymerase III holoenzyme; 100 μM each of dATP, dGTP, dCTP and [α-^{32}P] dTTP (70–150 cpm/pmol); and the indicated amounts of DnaA protein. The mixture was assembled at 0°C, and incubated at 30°C for 30 min.

Assay for ATP binding to DnaA protein. Assay was performed by a filter-retention method, as described by Sekimizu *et al.* (3). Briefly, DnaA protein (1.9 pmol) was incubated at 0°C for 15 min in 25 μl of buffer N (50 mM Hepes-KOH at pH7.6, 2.5 mM magnesium acetate, 0.3 mM EDTA, 20% glycerol, 0.007% Triton X-100, and 7 mM dithiothreitol) containing various concentrations of [α-^{32}P] ATP (0.5–1.5 $\times$ 10^4 cpm/pmol). The mixture was passed through a nitrocellulose filter (Millipore), and the filter was washed with 5 ml of cold buffer containing 50 mM Hepes-KOH (pH 7.6), 0.5 mM magnesium acetate, 0.3 mM EDTA, 17% glycerol, 0.005% Triton X-100, 5 mM dithiothreitol, and 10 mM ammonium sulfate. Radioactivity retained on the filter was measured by liquid scintillation counting.

Fluorescence measurement. Fluorescence of DnaA protein (0.03 mg/ml; 0.57 μM) was measured using a Fluorescence Spectrophotometer F-2000 (Hitachi). The Trp residue of DnaA protein was excited using a UV light at 290 nm and intensity of the emission was scanned over a range of 300–500 nm. A quartz cell with 10 mm pathlength was used.

Thermal stability of DnaA protein. Purified DnaA protein in buffer K was dialyzed at 0°C against buffer containing 5 mM Tris-HCl (pH7.5), 0.1 mM EDTA, 20% glycerol, 2.5 mM magnesium chloride and 0.2 M potassium sulfate. This dialyzed sample (3.2 μM DnaA protein) was incubated with or without 10 μM ATP at 0°C for 15 min, and was then further incubated at 0°C or 37°C for various durations. Residual activities were assayed in the minichromosome replication system reconstituted with purified proteins.

Circular dichroism (CD) spectroscopy. The DnaA sample (3.2 μM DnaA protein) dialyzed as above was used for measurement of CD spectra with a JASCO J-720 spectropolarimeter. The spectra were recorded using UV from 195 nm to 250 nm with a cell pathlength of 1 mm. Each spectrum was the mean of three scans obtained with a time constant of 1 sec and a scan speed of 50 nm/min. The background dichroic absorbance of the buffer used was automatically subtracted, using JASCO software. Estimation of the secondary structure content was obtained using the computer program SSE-338 (JASCO), based on the method of Yang *et al.* (17).

RESULTS AND DISCUSSION

Overproduction of DnaA Protein with Our Newly Constructed Expression Vector

DnaA protein was expressed with an arabinose-inducible *araB* promoter on pKA234 (Fig. 1). This promoter is under the control of the AraC repressor and expression is induced with arabinose (18). pKA234 bears an efficient Shine-Dalgarno sequence derived from T7 phage gp10 and the ATG start codon instead of the GTG start codon in the native *dnaA* gene (7). Without these two modifications, efficient overproduction did not occur (T. Katayama, unpublished data). It thus seems apparent that in the native structure *dnaA* gene, translation initiation was a limiting factor for overexpression.

DnaA protein was overproduced in MC1061 transformed with pKA234. Expression was induced with 1% arabinose, and during 1 h incubation at 37°C, DnaA protein accumulated to about 15% of the entire cell protein (data not shown). Prolonged induction did not increase the product. The accumulation of the protein was similar when the transformant was cultured in the presence of 0.1% arabinose for 3 h at 37°C.

Purification of DnaA Protein

About 50% of the overproduced DnaA protein was recovered in the supernatant after high-speed spin of a cell lysate. DnaA protein was purified according to a reported method, with the following modifications (Table 1, Figs. 2 and 3):

TABLE 1

Purification of DnaA Protein

Fraction	Protein (mg)	Concentration (mg/ml)	Specific activity ($U \times 10^{-6}$/mg)	Activity ($U \times 10^{-6}$)	Yield (%)
I (lysate)	3857	27	0.093	359	[100]
II (ammonium sulfate precipitation, backwash)	582	60	0.51	297	83
III (hydroxyapatite)	292	0.66	0.71	207	58
IV[a] (dialysis precipitation)	81	6.3	(0.058)[b]	(4.7)	(1.3)
V (guanidine treatment)	58	7.2	0.95	55	15
VI (superose 12)	22	0.12	1.2	26	7.2

[a] One sixteenth of fraction IV was used for preparation of fraction V. Thus, the values of fraction V and VI were corrected by a factor of 16.

[b] Activity was underestimated, because the proteins in this fraction aggregated.

Note. MC1061(pKA234) was grown at 37°C in 200 liters of LB medium containing 50 μg/ml of ampicillin. When the A_{595} of the culture reached 0.23, arabinose (0.1%) was added, and the culture was further incubated for 3 h. Cells were harvested by centrifugation at 4°C, resuspended to the A_{595} of 480 in cold buffer containing 50 mM Tris-HCl (pH 7.9), and 10% sucrose, frozen in liquid nitrogen, and stored at −80°C. Frozen cell paste (100 g) was thawed on ice, and resuspended in 100 ml of buffer C containing 250 mM potassium chloride. Lysate was prepared by incubation at 37°C for 4 min in the presence of 20 mM spermidine-HCl and 200 μg/ml of lysozyme, as described (19). All following procedures were done at 4°C or below. The supernatant (fraction I, 143 ml) was obtained by centrifugation in a Beckman type 50.2 Ti rotor at 40000 rpm for 20 min. To this supernatant, 40 g of ammonium sulfate (0.28 g/fraction I 1 ml) was slowly added with stirring. After additional stirring for 30 min, precipitates were collected by centrifugation in a Beckman JA20 rotor at 18000 rpm for 40 min. For backwash, precipitates were resuspended in buffer C (29 ml; 1/5 volume of reaction I) containing 0.28 g/ml of ammonium sulfate with a glass homogenizer, and centrifuged similarly as above. This same procedure was repeated three more times, except for use of buffer C (12 ml; 1/12 volume of fraction I) containing 0.24 g/ml of ammonium sulfate for the first time, use of buffer C (12 ml) containing 0.20 g/ml of ammonium sulfate for the second time, and use of buffer C (12 ml) containing 0.18 g/ml of ammonium sulfate for the third time. Then, the resultant precipitate was dissolved in buffer C (fraction II, 9.7 ml). After being diluted in buffer C to a conductivity equivalent to 0.1 M ammonium sulfate, the sample was further adjusted to a protein concentration of 1.0 mg/ml with buffer C containing 0.1 M ammonium sulfate, and loaded on a hydroxyapatite column (264 ml, 4.0 × 21 cm) equilibrated with buffer the same as above at a flow rate of 200 ml/h. The column was washed with two column volumes of the same buffer, and activity was eluted with a linear gradient (2300 ml) from 0.1 to 1.0 M ammonium sulfate in buffer C. Active fractions were collected (fraction III, 442 ml), and dialyzed for 36 h against 3 liters of buffer C, with three replacements. The samples were centrifuged in a JA20 rotor at 1800 rpm for 30 min, and one eighth of the resulting precipitates was resuspended with brief sonication in buffer C containing 0.6 M ammonium sulfate (fraction IV, 12.8 ml). The sample was further centrifuged in a Beckman TLA100.3 rotor at 100000 rpm for 30 min, and one sixteenth of the precipitates was similarly resuspended in buffer the same as above. After this procedure was repeated, the precipitates were dissolved with brief sonication in buffer C (0.55 ml) containing 4 M guanidine-HCl, 10 mM magnesium acetate, and 0.6 M ammonium sulfate. After a similar centrifugation, the supernatant (fraction V, 0.5 ml) was applied on a superose-12 HR16/50 column (Pharmacia fast protein liquid chromatography) equilibrated with buffer K at a flow rate of 1.0 ml/min. Active fractions were pooled (fraction VI, 11.5 ml).

(i) the backwash step with ammonium sulfate solution; DnaA protein in the soluble cell lysate was precipitated with 0.28 g/ml of ammonium sulfate, and washed with a diluted solution of ammonium sulfate (backwash). The final step of backwash used previously was done with 0.20 g/ml of ammonium sulfate (19). Following to this step, backwash with 0.18 g/ml of ammonium sulfate was added (Table 1), an approach which significantly improved the efficiency of purification, without loss of activity. While the previous backwash procedure yielded only 1.8-fold increase in specific activity compared to that of proteins precipitated with 0.28 g/ml of ammonium sulfate, our new procedure yielded a 2.7-fold increase (data not shown).

(ii) the column step; previously, the backwashed sample was applied on a Bio-Rex70 cation-exchange column, and DnaA activity was eluted with a linear gradient of ammonium sulfate (19). However, the broad peak of the activity appears, and this makes for poor efficiency of purification plus a large loss of activity. With our new procedure, the backwashed sample was applied on a hydroxyapatite column and was eluted with a linear gradient of ammounium sulfate. DnaA activity eluted as a sharp peak with a high yield, and separated well from contaminant proteins (Fig. 2).

Gel filtration was used to separate monomeric from aggregated forms, and monomeric DnaA protein fractions were pooled. Purity of the final fraction (fraction VI) exceeded 95%, as determined by scanning densitometry of an SDS-polyacrylamide gel electrophoresis (Fig. 3).

Replication Activity of the Purified DnaA Protein

Specific activity of the final fraction (fraction VI) in an *in vitro* complementation test for minichromosome replication was much the same as the value previously reported (Table 1).

Replication *in vitro* of minichromosome has been con-

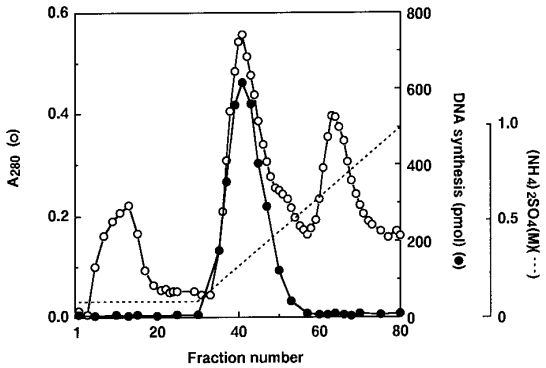

FIG. 2. Hydroxyapatite column chromatography. Fraction II proteins (7.2 mg, 1.5 × 10^6 units) were loaded on a hydroxyapatite column (3 ml, 1.3 × 2.3 cm) equilibrated with buffer C containing 0.1M ammonium sullfate. Activity was eluted in the same buffer by a linear gradient (30 ml) from 0.1 to 1.0 M ammonium sulfate. The flow rate was 3.5 ml/min. A 0.5 ml fraction was collected. Portions (0.13 μl) were assayed for replication activity in *in vitro* DNA synthesis complementation reactions.

structed with the purified proteins, DnaA, DnaB helicase, DnaC, DnaG Primase, SSB (*E. coli* single strand binding protein), HU, Gyrase, and DNA polymerase III holoenzyme (14–16). DnaA samples prepared by the new and previous methods exhibited the same specific activity (Fig. 4).

Nucleotide Binding of Purified DnaA Protein

DnaA protein binds ATP and ADP with a high affinity (3). Our DnaA sample obtained by the previous method showed a Kd of 59 nM for ATP, and the stoichiometric ratio of 0.25 (Fig. 5), values consistent with reported ones (3). Binding of adenine nucleotide is important for functions of DnaA protein; the ATP, but not the ADP, -bound form of the protein is active for initiation of replication of minichromosomes *in vitro.*

K_D and the stoichiometric ratio for ATP of the DnaA sample prepared by the new method were 96 nM, and

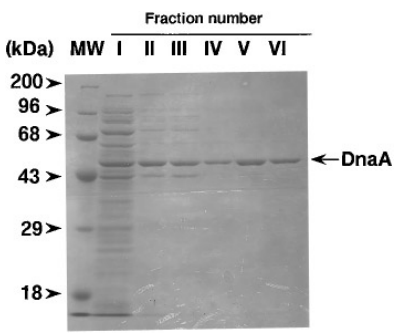

FIG. 3. SDS-polyacrylamide gel electrophoresis of DnaA-containing fractions. Protein fractions (500 units, except for fraction IV) were separated by SDS-polyacrylamide gel (10%) electrophoresis and stained with Coomassie brilliant blue.

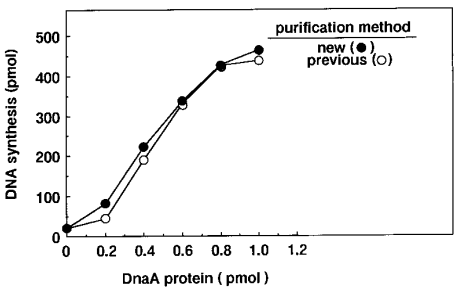

FIG. 4. Minichromosomal replication in the replication system reconstituted with pure proteins. Indicated amounts of purified DnaA protein were added to an *oriC* plasmid replication system reconstituted with purified proteins and incubated for 30 min at 30°C. DNA synthesis using pBS*oriC* as a template DNA (600 pmol, as nucleotide) was measured. DnaA protein purified by a previously reported method (19), was used as a control.

0.42 ATP molecules per DnaA molecule, respectively (Fig. 5). Therefore, affinities for ATP of the DnaA samples purified by the new and previous methods were much the same. Similar results were obtained with ADP (data not shown).

ATP Stabilizes the Conformation of DnaA Protein

The availability of a large quantity of pure DnaA protein facilitated examination of the protein, using a physicochemical strategy. We investigated the correlation between thermal and conformational stabilities of DnaA protein, the ATP-bound or nucleotide-free form. Activity of the ATP-bound form was reported to be more stable than that of the nucleotide-free form (3). Here, we determined the denaturation time-course of DnaA activity, under conditions used for structural analysis, using a replication system reconstituted with purified proteins (Fig. 6, A and B).

Replication activity of the nucleotide-free form of

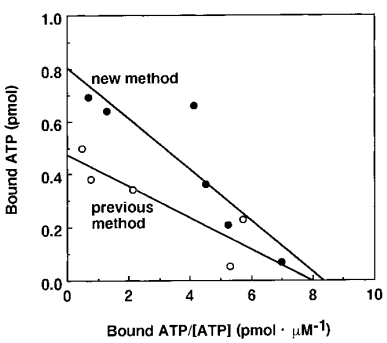

FIG. 5. Affinity of purified DnaA protein for ATP. Each DnaA sample (1.9 pmol of the protein) was incubated with various concentrations of [^{32}P]ATP at 0°C for 15 min and then [^{32}P] ATP bound to DnaA was measured by nitrocellulose filter binding assay (3). DnaA protein, which was purified by a previously reported method (19), was used as a control. Results are shown in the Scatchard plot.

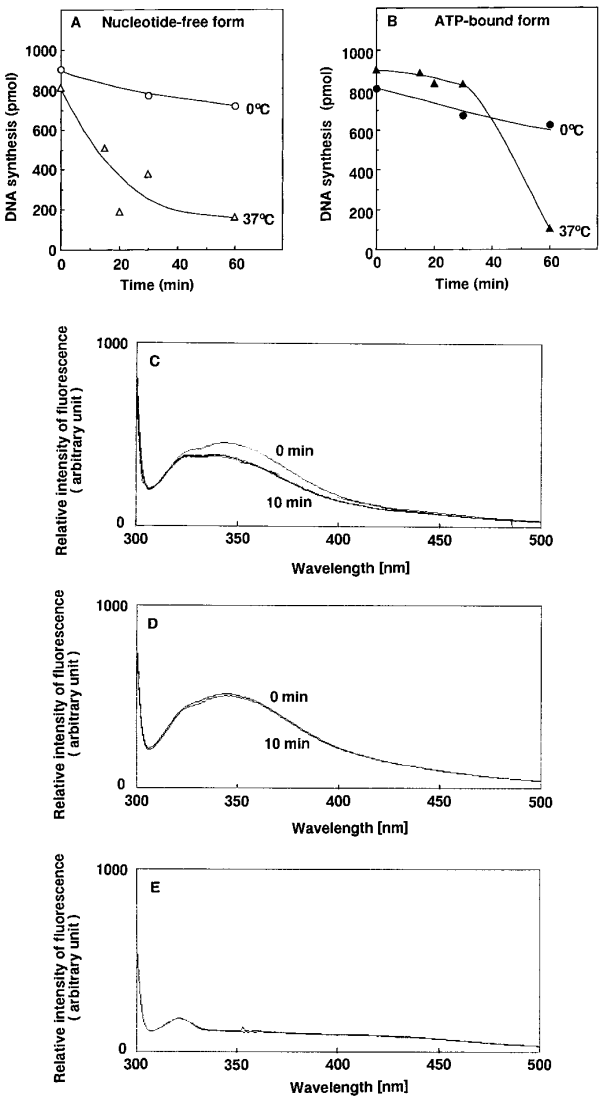

FIG. 6. Thermal stability of DnaA protein analyzed by fluorescence emission spectra. *A* and *B*, DnaA protein were incubated in the (A) absence or (B) presence of 10 μM ATP. Each DnaA protein were incubated at 0°C (circles) or 37°C (triangles) for the indicated time. Residual DNA replication activity of DnaA protein was then measured using an *oriC* plasmid replication system reconstituted with purified proteins. *C*, *D*, and *E*, DnaA protein (0.57 μM) were incubated in the absence (C) or presence (D) of 6.7 μM ATP. Each DnaA protein was then subjected to heat treatment by incubating at 37°C for 10 min. The intrinsic fluorescence of DnaA protein was measured before and after the heat treatment. Background emisson caused by buffer used was also shown (E).

DnaA protein rapidly decreased during preincubation at 37°C, while at 0°C the initial activity remained stable (Fig. 6A). Conversely, activity of the ATP-bound form was stable even at 37°C (Fig. 6B); the residual activity after incubation at 37°C for 30 min was 92% of the initial activity.

We then measured intrinsic fluorescence of the protein which monitors changes in environments of Trp residues of proteins. The patterns of fluorescence emission by the ATP-bound form or nucleotide-free form of DnaA protein were compared before and after incubation for 10 min at 37°C (Fig. 6, C and D).

With this heat treatment, the emission for the nucleotide-free form was significantly altered to decrease quantum yield, with a slight, blue shift of the emission maximum (Fig. 6C). Therefore, tryptophan residues exposed to the surface shifted into an internal location. In contrast, the fluorescence emission for the ATP-bound form was unchanged by heat treatment (Fig. 6D). This evidence suggests that ATP confers conformational stability on the protein.

CD Assessment of the Secondary Structure of DnaA Protein

To analyze the secondary structure, we next measured circular dichroism of the nucleotide-free and ATP-bound forms of DnaA protein, under non-denaturing conditions (Fig. 7, A and B).

The nucleotide-free form of DnaA protein showed the spectrum of a typical, α-helix rich protein (Fig. 7A). The contents of α-helix, β-structure, turn, and random coil, as deduced according to the method of Yang *et al* (17) using a computer program, were 62, 2, 12, and 24%, respectively.

The spectrum of the ATP-bound form was similar to that of the nucleotide-free form, but there was a significant reduction in content of the α-helix (Fig. 7B). The contents of α-helix, β-structure, turn, and random coil, calculated as described above, were 51, 12, 16, and 22%, respectively.

The decrease in helical content was small but significant, thereby suggesting that the interaction of ATP slightly decreased the population of the helical structure of DnaA protein. There are putative α-helices in the region that has an ATP-binding motif of DnaA protein (20, 21). In addition, we have data that γ-phos-

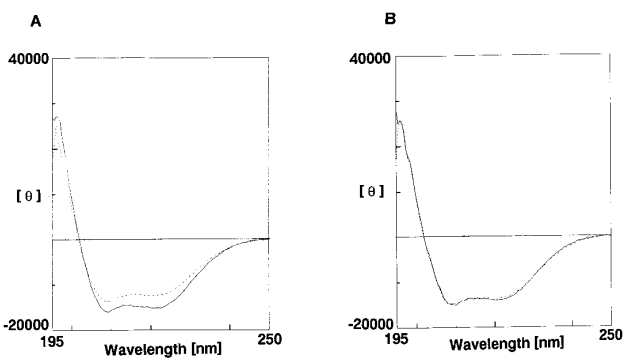

FIG. 7. CD spectra of DnaA protein. Circular dichroism spectra of the nucleotide-free (A) and ATP-bound (B) forms of DnaA protein were recorded at 4°C (solid line). The samples were then incubated at 37°C for 20 min, and circular dichroism spectra were again recorded (broken line). [θ]; molecular ellipticity (deg·cm^2/dmol).

phate of ATP bound to DnaA protein is close to Lys-415 located in a putative α-helix in the C-terminal region (Katayama, T., Kubota, T., Ito, K., Tagaya, M., Mizushima, T., and Sekimizu, K. *submitted*).

Content of the β-structure of DnaA protein seems to increase by interaction with ATP, an event perhaps caused by a structural transition of the P-loop structure (the ATP-binding motif A) that is present in DnaA protein (3, 20). The P-loop structure interacts with one or more phosphate residues of the bound nucleotide, and seems to retain a changeable conformation (20, 22, 23). An ATP-binding peptide derived from the β-subunit of mitochondrial F_1 ATPase changes its structure, depending on ATP-binding, to increase the β-structure content; the P-loop structure in the peptide may well be responsible for this change (23).

To compare heat stability of the two forms of DnaA protein, we incubated the samples at 37°C for 20 min, and monitored circular dichroism (Fig. 7, A and B). As expected, no significant change was seen in the ATP-bound form, whereas the nucleotide-free form was clearly denatured, as determined by the CD profile. Thus, the secondary structure of DnaA protein was considerably stabilized against heat by interaction with ATP, an observation consistent with results of the stability of replication activity (Fig. 6, A and B) and measurement of intrinsic fluorescence (Fig. 6, C and D). Taken together, binding of ATP apparently leads to a conformational change required for the replication activity of DnaA protein, and stability of the structure is thus enhanced.

The supply of DnaA protein has heretofore been limited, due to difficulties in overproduction and purification. The overproduced DnaA protein formed an insoluble aggregate in cells, and this solubilization without loss of activity proved unsuccessful in our experiments (data not shown). We constructed several overproducing systems of DnaA protein using various expression vectors and host strains, and tested various conditions for overproduction. Among them, only the system reported herein produced a significant amount of DnaA protein in the soluble fraction, perhaps because of the tight repression of the non-induced *dnaA* gene on the plasmid. In other systems, a significant leaky expression occurred, an event which might lead to aggregation of the DnaA protein (Katayama, T., unpublished observation).

We obtained over 10 mg of DnaA protein using a simple procedure with high reproducibility. This efficient acquisition of the protein will not only pave the way for biochemical studies of functions of the protein, but also make feasible unexplored studies such as structural analysis by X-ray crystallography. In addition, this system will stimulate other aspects of studies such as development of specific antibodies, analysis of structure-function relationship by site-directed mutagenesis, biophysical analysis of the tertiary structure, and physicochemical analysis of DnaA-membrane interaction.

ACKNOWLEDGMENTS

The overproducer of DnaA protein was constructed in the laboratory of Dr. A. Kornberg by the second author, who was supported there in part by the International Human Frontier Science Program. We are grateful to Drs. E. Crooke, D.-S. Hwang, D. Bramhill, and N. E. Dixon for suggestions on overproduction and purification of DnaA protein. We thank Dr. T. Ueda and M. Ohara for suggestions on analysis of CD data and for comments on the manuscript, respectively. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

REFERENCES

1. Kornberg, A., and Baker, T. A. (1992) DNA Replication, 2nd ed., Freeman, New York.
2. Messer, W., and Weigel, C. (1996) *in Escherichia coli* and *Salmonella typhimurium:* Cellular and Molecular Biology, 2nd ed. (Neidhardt, F. C., Curtiss III, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E., Eds.), pp. 1579–1601, Am. Soc. Microbiol., Washington, DC.
3. Sekimizu, K., Bramhill, D., and Kornberg, A. (1987) *Cell* **50,** 259–265.
4. Sekimizu, K., and Kornberg, A. (1988) *J. Biol. Chem.* **263,** 7131–7135.
5. Castuma, C. E., Crooke, E., and Kornberg, A. (1993) *J. Biol. Chem.* **268,** 24665–24668.
6. Mizushima, T., Ishikawa, Y., Obana, E., Hase, M., Kubota, T., Katayama, T., Kunitake, T., Watanabe, E., and Sekimizu, K. (1996) *J. Biol. Chem.* **271,** 3633–3638.
7. Katayama, T. (1994) *J. Biol. Chem.* **269,** 22075–22079.
8. Katayama, T., and Crooke, E. (1995) *J. Biol. Chem.* **270,** 9265–9271.
9. Braun, R. E., O'Day, K., and Wright, A. (1987) *J. Bacteriol.* **169,** 3898–3903.
10. Smith, D. W., Garland, A. M., Herman, G., Enns, R. E., Baker, T. A., and Zyskind, J. W. (1985) *EMBO J.* **4,** 1319–1326.
11. Fuller, R. S., Kaguni, J. M., and Kornberg, A. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7370–7374.
12. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
13. Baker, T. A., Sekimizu, K., Funnell, B. E., and Kornberg, A. (1986) *Cell* **45,** 53–64.
14. Ogawa, T., Baker, T. A., van der Ende, A., and Kornberg, A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 3526–3566.
15. van der Ende, A., Baker, T. A., Ogawa, T., and Kornberg, A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 3954–3958.
16. Crooke, E. (1995) *Methods Enzymol.* **262,** 500–506.
17. Yang, J. T., Wu, C. S., and Martinez, H. M. (1986) *Methods Enzymol.* **130,** 208–269.
18. Johnston, S., Lee, J.-H., and Ray, D. S. (1985) *Gene* **34,** 137–145.
19. Sekimizu, K., Yung, B. Y.-M., and Kornberg, A. (1988) *J. Biol. Chem.* **263,** 7136–7140.
20. Koonin, E. V. (1993) *Nucleic Acids Res.* **21,** 2541–2547.
21. Skarstad, K., and Boye, E. (1994) *Biochim. Biophys. Acta.* **1217,** 111–130.
22. Saraste, M., Sibbald, P. R., and Wittinghofer, A. (1990) *TIBS* **15,** 430–434.
23. Chuang, W.-J., Abeygunawardana, C., Pedersen, P. L., and Mildvan, A. S. (1992) *Biochemistry* **31,** 7915–7921.