Fate of the DnaA Initiator Protein in Replication at the Origin of the
Escherichia coli Chromosome in Vitro*

(Received for publication, September 11, 1989)

Benjamin Yat Ming Yung‡, Elliott Crooke§, and Arthur Kornberg
From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

The dnaA initiator protein binds specific sequences in the 245-base pair Escherichia coli origin (oriC) to form a series of complexes which eventually are opened enough to admit dnaB helicase into a prepriming complex (Bramhill, D., and Kornberg, A. (1988) Cell 52, 743–755). ATP bound to a high-affinity site on dnaA protein is the preferred form for one or more of the early stages, but an elevated level of ATP is needed for a later stage; further evidence for a low-affinity site has now been obtained. We find that at limiting levels of dnaA protein only the ATP form produces an active initial complex; neither the ADP nor the non-nucleotide forms are effective. Augmentation of the activity of a limiting level of the ATP form of dnaA protein by the otherwise inert ADP form implies that at some stage of initiation both forms are active. The dnaA protein is essential up to the stage of forming the prepriming complex; upon salt dissociation from an oriC complex, the protein can be recycled to function at a fresh origin. Distinctive conformational states of the ATP form are implied by interactions with oriC DNA, by the influence of phospholipids on accelerating nucleotide exchange, and by the susceptibility to proteolytic cleavage.

DnaA protein plays the key role in the initiation of a cycle of replication at the origin, oriC, of the Escherichia coli chromosome (1, 2). A cluster of 20 or more dnaA protein molecules binds to the 245-bp oriC sequence and proceeds to open a region of three 13-mers for entry of dnaB helicase into a prepriming complex (3, 4). Further duplex opening generates the sites for priming of DNA synthesis and the forks of bidirectional replication.

At limiting dnaA protein levels, binding of ATP to a high-affinity site is essential for its function; binding of ADP at the same site renders the protein inert (5). The very slow exchange of the bound nucleotide is greatly enhanced by acidic phospholipids residing in vesicles in fluid phase (6, 7). The significance of the phospholipid interaction is strengthened by the demonstration in vivo that initiation of chromosome replication is dependent on membrane fluidity (8).

The importance of dnaA protein and the complexity of its functions focus attention on the numerous extant questions about its structure and behavior. How early among the several stages of initiation is the dependence on ATP manifested? Can the ADP form substitute at any stage? At what stage does the protein become dispensable and can it be recycled to another oriC plasmid? What information regarding conformational changes in the protein can be gleaned from its susceptibility to proteolytic cleavage? Information about these and related questions forms the substance of this report.

MATERIALS AND METHODS

Reagents—Sources were: ATP, deoxynucleotide triphosphates, and HEPES, Pharmacia LKB Biotechnology Inc.; GTP, CTP, UTP; Tricine, N-tosyl-L-phenylalanyl chloromethyl ketone-treated trypsin, and soybean trypsin inhibitor, Sigma; 32P[ATP (410 Ci/mmol), 32P[ATP (600 Ci/mmol), and 32P-adenosyl]methylthioninium (80 Ci/mmol), Amersham Corp.; and [35S]H,S04 (688 Ci/mmol), ICN Radiochemicals. Total E. coli phospholipids were prepared as described previously (6) from E. coli strain W3110 grown with aerobically in Luria broth supplemented with thymine (20 mg/liter) and 0.5% glucose. Buffer A is 50 mM HEPES-KOH (pH 7.6 at 1 M), 2.5 mM magnesium acetate, 0.3 mM EDTA, 20% (v/v) glycerol, 0.006% Triton X-100, and 8 mM dithiothreitol; Buffer B is 50 mM Tricine-KOH (pH 8.25 at 1 M), 2.5 mM magnesium acetate, 0.3 mM EDTA, 20% (v/v) glycerol, 0.007% Triton X-100, and 7 mM dithiothreitol.

Enzymes and DNA—Purified DNA replication proteins (9), dnaA protein (10), 12-mer labeled plasmid DNA (11), and supercoiled plasmid DNAs (12) were prepared as described previously. Plasmid pCM859 (13), a gift from M. Meijer (University of Amsterdam, Netherlands), is a minichromosome consisting solely of E. coli DNA encompassing oriC (base pairs +677 to +3335). Plasmid pBSoriC contains a 678-bp HindIII-PstI fragment spanning oriC (base pairs +189 to +489) cloned into the PstI site of M13mp8. 32P-Labeled DNA Protein—The dnaA overproducer (N4930) with pHF1059 (10) was grown in M9-medium in the absence of methionine and cysteine at 30 °C. At an optical density (A595nm) of 0.5, 5.0 μCi of [35S]H,S04 (688 Ci/mmol) was added. The temperature was shifted to 39 °C and cells were harvested after 2 h. 32S-DnaA protein was purified (10) to greater than 90% homogeneity with a final specific activity of 20,000 cpm/μg protein.

Assays—DnaA protein (150 ng) was incubated with 1 μM ATP and asayed for DNA replication activity in a staged reconstitution assay (20 μl) as described previously (6); non-staged assays (5) were at 30 °C for 20 min using pBSoriC (50 fmol as circles) as template. Assays of dnaA protein for DNA binding and ATP (ADP) binding activities, digestion of dnaA protein with trypsin, SDS-polyacrylamide gel electrophoresis, and gel staining have been described previously (11).

Gel Filtration—Bio-Gel A5m (Bio-Rad) in buffer A was placed in a 1.5-ml plastic centrifugation tube with a pin hole in the bottom plugged with glass wool. The gel was packed by centrifugation (2000 rpm, 2–3 min) in a swinging bucket centrifuge. The sample was applied to the gel (1 ml packed bed volume) and centrifuged 3 min

* This work was supported in part by National Institutes of Health Grant GM 07581 and National Science Foundation Grant DMB-87-107945. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Present address: Chang Gung Medical College, 5 Fu-Hsing St., Kwei-San, Tao-Yuan, Taiwan, R.O.C.
§ Supported by Damon Runyon-Walter Winchell Cancer Fund Fellowship DRG-983.

The abbreviations used are: bp, base pairs; HEPES, 4-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; SDS, sodium dodecyl sulfate.

1 The abbreviations used are: bp, base pairs; HEPES, 4-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; SDS, sodium dodecyl sulfate.

2 T. Baker and A. Kornberg, unpublished data.
into a microcentrifuge tube. Repeatedly, an equal volume of column buffer (200 µl) was applied and the gel was centrifuged again for each successive fraction.

RESULTS

Only the ATP Form of DnaA Protein Produces an Active Initial Complex with oriC—Although dnaA protein in any form (i.e. ATP, ADP, or non-nucleotide) produces a complex with oriC DNA at 0 °C, the isolation of a stable, replication-active complex required that the ATP form be maintained at an elevated temperature (e.g. 37 °C) (15). By replacing the sucrose gradient technique, which consumes several hours, with gel filtration, which takes only a few minutes, an active replication complex could be separated even at 0 °C. The yield of active complex was near 80% with the ATP form (Fig. 1), but less than 10% with either the ADP or non-nucleotide forms (data not shown).

DnaA Protein Is Required for Formation of the Open and Prepriming Complexes But Not Beyond—The open complex of dnaA protein with oriC (4, 15), formed with 35S-labeled protein in the presence of HU protein and 5 mM ATP at 38 °C, was isolated by rapid gel filtration. Virtually none of the complex remained after exposure to salt (e.g. 200 mM K+-glutamate) (Fig. 2). Free 35S-dnaA protein was not detected in the elution profile. Possibly, due to the low level of protein employed, the dissociated dnaA protein was nonspecifically absorbed by the gel matrix. Whereas salt treatment also

DnaA Protein in Replication

DISCUSSION

DnaA Protein Can Be Recycled in oriC Replication—The ability of dnaA protein to function again after prepriming complex formation with pCM959 was demonstrated by challenge with another oriC template (i.e. RE85). Because these plasmids differ in size, their replication products were readily separated by electrophoresis. Prepriming complexes formed with pCM959 were isolated by gel filtration. Upon addition of replication enzymes (but no dnaA protein) and the challenge template (RE85) to the prepriming complex, the replication products analyzed by agarose gel electrophoresis included both the challenge and initial templates (Fig. 4). Thus, the dnaA protein, dispensable for stages of replication beyond

FIG. 1. Isolation of an initial complex of ATP-dnaA protein with oriC DNA. DnaA protein (30 pmol) was incubated in buffer A (200 µl) with 1 mM ATP and pCM959 (1.5 pmol as circles) at 0 °C for 10 min. The initial complex or the control (ATP-dnaA protein without oriC DNA) was then isolated by gel filtration on Bio-Gel A-5m agarose at 0 °C; 20-µl samples were assayed for DNA replication (see "Materials and Methods").

FIG. 2. Persistence of dnaA protein is required to form the open complex. 35S-DnaA protein (12 pmol) was incubated in buffer A (200 µl) with ATP (5 mM), pCM959 (1.5 pmol as circles) and HU protein (80 ng), dnaH (0.6 µg), and dnaC (0.2 µg) at 38 °C for 1 h. The prepriming complex (200 µl) was isolated by gel filtration at 0 °C in the presence of potassium glutamate at 0, 75, or 200 mM. Samples were assayed as in Fig. 1. A, complex formation; B, replication activity of the prepriming complex was measured in a subsequent reaction containing SSB, gyrase, primase, and pol II at 16 °C for 30 min (see "Materials and Methods").

FIG. 3. DnaA protein is dispensable after formation of prepriming complex. 35S-DnaA protein (12 pmol) was incubated in buffer A (200 µl) with ATP (5 mM), pCM959 (1.5 pmol as circles), HU protein (80 ng), dnaH (0.6 µg), and dnaC (0.2 µg) at 38 °C for 1 h. The prepriming complex (200 µl) was isolated by gel filtration at 0 °C in the presence of potassium glutamate at 0, 75, or 200 mM. Samples were assayed as in Fig. 2. A, complex formation; B, replication activity of the prepriming complex was measured in a subsequent reaction containing SSB, gyrase, primase, and pol II at 16 °C for 30 min (see "Materials and Methods").

FIG. 4. DnaA protein can be recycled in oriC replication. The prepriming complex with pCM959 was formed and isolated in the absence of glutamate as in Fig. 3. To a 20-µl sample, a challenge with another oriC DNA (RE85, 0.15 pmol as circles) (lane 2) or a second addition of pCM959 (0.15 pmol as circles) (lane 4) was made along with a standard amount (lane 1) or 2-fold amount (lanes 2–4) of replication enzymes (dnaA protein being omitted). DNA synthesis was at 30 °C for 30 min. Product DNAs, labeled by [α-32P]dTTP, were analyzed by electrophoresis on an alkaline agarose gel (0.7%) followed by autoradiography. The sizes of the unit-length products of RE85 (7866 bp) and pCM959 (4012 bp) are indicated.
the prepriming complex, can be reused for initiation of another oriC template.

**Influence of ATP on Tryptic Proteolysis of DnaA Protein in the Early Complexes**—Digestion with trypsin at 37 °C of ATP-dnaA protein complexed with oriC DNA resulted in a prompt loss of replication activity (Fig. 5) along with the disappearance of the 65-kDa band (11) of the protein on SDS-polyacrylamide gel electrophoresis (data not shown). However, the oriC DNA bound to the protein was largely retained. Under these digestion conditions, over 80% of the ATP-dnaA protein in the complex was converted to an NH2-terminal 30-kDa fragment which resisted further digestion for over 2 h (data not shown). By comparison, the 30-kDa fragment from an ADP-dnaA protein complex was digested to smaller fragments within 1 h (data not shown). A strikingly different digestion pattern was observed with the non-nucleotide dnaA protein complex; the 30-kDa as well as the 55-kDa peptide band disappeared within 5 min.

Even with much milder proteolysis (100-fold less trypsin), the nucleotide forms of dnaA protein complexed with oriC DNA were readily converted to the 30-kDa fragment, whereas the nucleotide-free protein (not complexed with oriC DNA) remained undigested (data not shown). We may infer that conformational changes attendant upon binding of ATP-dnaA or ADP-dnaA protein to oriC DNA expose the COOH-terminal region of the protein to trypsinic cleavage.

**Elevated Levels of the ADP Form of DnaA Protein Exhibit Replication Activity**—Based on filter retention experiments (2), 20–40 dnaA protein molecules bind per origin sequence; a similar ratio is required for optimal P1 nuclease linearization of an oriC-containing plasmid (4). The dnaA protein-oriC complex observed in the electron microscope (3, 16) also suggests 20–40 dnaA protein monomers enveloped by DNA. As levels of dnaA protein approaching the stoichiometry, only the ATP form of the protein had replication activity, while the ADP form was inert (Fig. 6). However, at a 3-fold greater level, the ADP form was active for replication indicating a clear preference for the ATP form for one or more of the early stages in the initiation of replication.

**Low Levels of the ADP Form of DnaA Protein Augment the Replication Activity of the ATP Form**—When the ADP form was mixed with the ATP form, it neither competed nor inhibited but augmented the activity of a limiting level of the ATP form (Fig. 7). Similar augmentation was observed when the nucleotide forms of dnaA protein were formed in the presence of 100 μM ATP and ADP (data not shown). No replication activity was observed when comparable quantities of ADP-dnaA protein were mixed with ATP (1 or 100 μM) alone (data not shown). Thus, among the several stages at which dnaA protein functions, the ATP form is more efficient for some, while both forms are active at others.

**The Low Affinity Site for Nucleotides on DnaA Protein**—Beyond the very tight binding of a nucleotide (ATP or ADP) with a Kp of 0.1 μM or less (5), dnaA protein requires high levels of ATP (e.g. near 5 mM) to form the open oriC complex (4). Further insight into nucleotide binding at this implied low affinity site is provided by the action of high levels of ADP. When the ATP form of dnaA protein was exposed to 4 mM ADP for 10 min at 0 °C, the protein was severely inhibited in responding to the high level of ATP to form the open

---

**Fig. 5.** Effect of trypsic digestion of dnaA protein on retention of oriC DNA and replication. The complex with ATP-dnaA protein (60 pmol) and [3H]pBSoriC (1.5 pmol as circles) was formed by incubation in buffer A (200 μl) at 0 °C for 10 min. Digestion of the protein in the complex (200 μl) was with trypsin (1.2 μg) at 37 °C. Samples (20 μl) were treated at specified times with 2 μg of soybean trypsin inhibitor at 0 °C. Replication and oriC DNA retention were measured as described under "Materials and Methods."

**Fig. 6.** The ADP form of dnaA protein is active at elevated levels. DnaA protein was incubated in buffer B (16 μl) with 1 μM ATP or ADP at 0 °C for 15 min. The remaining replication components were added and replication activities were assayed (25 μl, non-staged) using 60 fmol of pBSoriC as template (see "Materials and Methods").

**Fig. 7.** Replication activity upon mixing the ATP and ADP forms of dnaA protein. DnaA protein was incubated in buffer B with 1 μM ADP at 0 °C for 15 min. Samples containing ADP-dnaA protein (8 μl) were mixed with 0.6 pmol of ATP-dnaA protein (8 μl, buffer B containing 1 μM ATP). Replication activities were measured as in Fig. 6.

**Fig. 8.** ADP at a high level inactivates the ATP form of dnaA protein. ADP (4 mM) was added to the ATP-dnaA protein complex (3 pmol) at 0 °C, either 10 min before the addition or, as a control, along with the addition of pBSoriC and other replication components. The replication reaction for the indicated times was at 30 °C (see "Materials and Methods").
complex assayed by replication (Fig. 8). Presumably, this ADP occupancy of the low affinity ATP site is responsible for the inactivation.

DISCUSSION

In the course of opening the duplex of the *E. coli* chromosome at its unique origin, oriC, the dnaA protein undergoes a number of conformational changes including aggregation into a sphere of 20 or so molecules (3). Binding of ATP to a high affinity site on the protein (5), we now find, is the most effective form for achieving the earliest active stage that succeeds the initial binding of dnaA protein to its four 9-mer recognition sequences in oriC (Fig. 1). Despite the apparent similarity of the oriC DNA footprints of the ATP, ADP, and non-nucleotide forms of dnaA protein, the capacity to form an active initial complex requires ATP.

To proceed to the open and then to the prepriming complexes, dnaA protein requires HU protein, a high level of ATP (e.g. 5 mM) and an elevated temperature (>25 °C) (4, 11). The stages beyond the prepriming complex proceed effectively despite the removal of virtually all the dnaA protein (Figs. 2 and 3). Upon dissociation of dnaA protein from the prepriming complex, effected by salt, the protein can be recycled to a fresh oriC template (Fig. 4). Whether recycling occurs in vivo is uncertain.

With ATP tightly bound, dnaA protein interacts with and opens a sequence of three 13-mers adjoining the 9-mer region in oriC (4, 11). Conformational changes in dnaA protein, not achieved with ADP binding, are implied by altered susceptibility to proteolytic cleavage. Binding of the ATP form of dnaA protein exposes its COOH-terminal region to tryptic action, conceivably a structural change related to the entry of the dnaB helicase (from the dnaB - dnaC complex) into the prepriming complex.

The ADP form of dnaA protein, though ineffective in forming the initial complex (data not shown) and supporting oriC replication (Fig. 6), augments rather than inhibits the activity of limiting levels of the ATP form (Fig. 7). From this result, we infer that while the ATP form is required for one or more of the several stages of initiation, the ADP form may be substituted at some stage, and at high levels, is effective at all stages (Fig. 6). Consistent with this interpretation is the ability of the ADP form to support the replication of R1 plasmids, the origins of which possess a single dnaA box.4

The numerous molecules of dnaA protein involved in initiation and their several functions dependent on ATP imply complex binding kinetics of ATP to the protein. Beyond occupancy of the high affinity site (Kd = 0.03 μM) for producing the initial complex, the requirement for a high level of ATP (near 5 mM) for proceeding to the open and prepriming complex stages implies a low-affinity site. Direct evidence for this site has been difficult to obtain, but the striking inactivation by a high level of ATP of the protein with its high-affinity site occupied by ATP, is a clear indication of an irreversible occupancy of such a low affinity site (Fig. 8).

REFERENCES

1. Hirota, Y., Mordoh, J., and Jacob, F. (1970) J. Mol. Biol. 53, 369-387
2. Fuller, R. S., and Kornberg, A. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5817-5821
3. Fuller, R. S., Funnell, B. E., and Kornberg, A. (1984) Cell 38, 889-900
4. Bramhill, D., and Kornberg, A. (1988) Cell 52, 743-755
5. Sekimizu, K., Bramhill, D., and Kornberg, A. (1987) Cell 50, 259-265
6. Sekimizu, K., and Kornberg, A. (1988) J. Biol. Chem. 263, 7131-7135
7. Yung, B. Y. M., and Kornberg, A. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7202-7205
8. Fralick, J. A., and Lark, K. G. (1973) J. Mol. Biol. 80, 459-475
9. Kaguni, J. M., and Kornberg, A. (1984) Cell 38, 183-190
10. Sekimizu, K., Yung, B. Y., and Kornberg, A. (1988) J. Biol. Chem. 263, 7126-7140
11. Yung, B. Y., and Kornberg, A. (1989) J. Biol. Chem. 264, 6146-6150
12. Ogawa, T., Baker, T. A., van der Ende, A., and Kornberg, A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3562-3566
13. Meijer, M., Beck, F., Hansen, F. G., Bergman, H. F., Messer, W., von Meyenburg, K., and Schaller, H. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 580-584
14. Smith, D. W., Garland, A. M., Herman, G., Enns, R. E., Baker, T. A., and Zyskind, J. W. (1985) EMBO J. 4, 1319-1326
15. Sekimizu, K., Bramhill, D., and Kornberg, A. (1988) J. Biol. Chem. 263, 7124-7130
16. Funnell, B. E., Baker, T. A., and Kornberg, A. (1987) J. Biol. Chem. 263, 10327-10334

4 H. Masai, personal communication.

---

K. Sekimizu, D. Bramhill, and A. Kornberg, unpublished data.
Fate of the DnaA initiator protein in replication at the origin of the Escherichia coli chromosome in vitro.
B Y Yung, E Crooke and A Kornberg

J. Biol. Chem. 1990, 265:1282-1285.

Access the most updated version of this article at http://www.jbc.org/content/265/3/1282

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/3/1282.full.html#ref-list-1