Biochemical analysis of DnaA protein with mutations in both Arg³²⁸ and Lys³⁷²

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The DnaA protein is the initiator of chromosomal DNA replication in *Escherichia coli*. Acidic phospholipids decrease its affinity for adenine nucleotides, and re-activate the ADP-bound form to the ATP-bound form. We have previously reported that two mutant forms, DnaAR328E and DnaAK372E, have decreased affinity for cardiolipin (CL). In the present study, we constructed a mutant DnaA protein, DnaA435, with both R328E and K372E, and compared its biochemical characteristics with those of DnaAR328E and DnaAK372E. DnaA435 could bind to *oriC* DNA, but did not bind ATP or ADP. In DnaA435,

compared with DnaAR328E and DnaAK372E, CL caused less inhibition of *oriC* DNA binding, suggesting that amino acids R328 and K372 are involved in the interaction of DnaA with acidic phospholipids. DnaA435 could initiate DNA synthesis on *oriC* both *in vivo* and *in vitro*. Based on these results, we propose that ATP activates DnaA protein by changing its higher order structure around R328 and K372.

Key words: acidic phospholipids, ATP binding, *Escherichia coli*, mutational analysis.

INTRODUCTION

The DnaA protein is the initiator of chromosomal DNA replication in *Escherichia coli* [1,2]. Its interaction with membrane acidic phospholipids has received much attention, because it provides biochemical evidence for a replicon model in which Jacob et al. [3] hypothesized that DNA replication initiates at membranes, and that replication proteins are regulated by membrane components. Acidic phospholipids, in particular cardiolipin (CL), decrease DnaA protein's affinity for adenine nucleotides [4–7]. DnaA protein has a high affinity for both ATP and ADP, and the ATP–DnaA complex is active, whereas the ADP–DnaA complex is inactive in DNA replication [8–10]. In the presence of high concentrations of ATP, acidic phospholipids can activate the ADP–DnaA complex by stimulating an exchange reaction with ATP [4–7]. Increasing genetic evidence strongly suggests that acidic phospholipids are important regulators of DNA replication in cells. For example, the growth arrest phenotype of *E. coli*, defective in acidic phospholipid synthesis, was suppressed by mutating the gene encoding RNase H, which enables *E. coli* to replicate DNA independently of DnaA [11]. Since bypassing DnaA restores growth, this suggests that acidic phospholipids could regulate DNA replication through their interaction with DnaA [11]. Furthermore, the growth can also be restored by overproduction of certain mutant DnaA proteins [12]. Identification of the amino-acid residues which interact with acidic phospholipids is important in order to identify the precise molecular mechanism of this activation.

We reported that DnaA433 (DnaAR328E, R334E, R342E) and DnaA431 (DnaAR360E, R364E, K372E), which have mutations in potential amphipathic helices K327 to I344 and D357 to V374 respectively, showed decreased interaction with CL, suggesting these amphipathic helices are membrane-binding domains [13,14]. The amphipathic helices (D357 to V374) were overlapping with the domain which was previously suggested to be involved in membrane binding [15–17]. R328 and K372 were the most important of the mutated amino-acid residues in DnaA433 and

DnaA431 respectively [18,19]. R334, located between R328 and K372, is important for the binding of adenine nucleotides to DnaA protein and its intrinsic ATPase activity [19,20]. Based on these results, we proposed that CL binding to both R328 and K372 causes a conformational change, which interferes with the ability of R334 to interact with adenine nucleotides, thus decreasing DnaA protein's affinity for them [19]. To evaluate this model, biochemical analysis of DnaA protein with both R328E and K372E is important. In the present study, we constructed the mutant protein (DnaA435) and examined its interaction with CL. The results suggested that both R328 and K372 interact with acidic phospholipids, supporting the model described above. We also found that DnaA435 had decreased affinity for ATP but supported *in vitro* and *in vivo* *oriC* DNA replication, suggesting that the region including these two amino-acid residues is involved in the regulation of DnaA protein's activity by adenine nucleotides.

EXPERIMENTAL

Materials

A crude extract for an *oriC* complementation assay was prepared from the WM433 strain of *E. coli* as previously described [2,21]. CL was purchased from Sigma. [α -³²P]ATP (3000 Ci/mmol), [α -³²P]dCTP (6000 Ci/mmol) and [³H]ADP (40 Ci/mmol) were from Amersham Pharmacia Biotech or DuPont. The mutant and wild-type DnaA proteins were purified as previously described [22,23].

Site-directed mutagenesis and plasmid construction

Site-specific mutagenesis was performed using the method described by Kunkel [24]. Briefly, uracil-containing single-stranded DNA of M13 phage, containing the coding region of the *dnaA*

Abbreviation used: CL, cardiolipin.

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Figure 1 Mutant DnaA proteins

α -Helices (K327 to I344 and D357 to V374) predicted by amino-acid sequence [31].

gene, was hybridized with oligonucleotide primers representing each mutation (Figure 1). The complementary DNA strand was synthesized *in vitro* and the resultant double-stranded DNA was introduced into *E. coli* JM109. The mutation was confirmed by direct DNA sequencing, and double-stranded DNA containing the mutation was prepared.

To construct plasmids for overexpression, the *Eco*RI–*Hind*III region of each *dnaA* gene was ligated into pMZ001 [10], which contains the arabinose promoter. To construct plasmids for complementation analysis, we ligated the coding regions of the mutant *dnaA* gene (*Bam*HI–*Hind*III fragment) into pKP2204 [25], which contains the wild-type promoter of the *dnaA* gene.

ATP or ADP binding to DnaA protein

This was determined by a filter-binding assay [8]. DnaA protein was incubated with 1 μ M [α -³²P]ATP or [³H]ADP at 4 °C for 10 min in 40 μ l of buffer G [50 mM Hepes/KOH (pH 7.6), 0.5 mM magnesium acetate, 0.3 mM EDTA, 5 mM dithiothreitol, 17% (v/v) glycerol, 10 mM ammonium sulphate and 0.005% Triton X-100]. Samples were filtered on to nitrocellulose membranes (Millipore HA, 0.45 μ m) and washed with ice-cold buffer G. The radioactivity remaining on the filter was counted with a liquid scintillation counter.

DNA binding to DnaA protein

This was determined by a filter-binding assay [8]. The pBSoriC plasmid [26,27], containing *oriC*, was digested with *Sca*I and its 3' end was deleted by T4 DNA polymerase. The resultant DNA fragment was radiolabelled by the large fragment of DNA polymerase I and [α -³²P]dCTP. The specific activity of the DNA probe was approx. 23 000 c.p.m./fmol DNA. DnaA protein was incubated with the radiolabelled DNA fragments (21 fmol) at 37 °C for 4 min in 40 μ l of buffer G. Samples were filtered on to nitrocellulose membranes and counted as described above.

DNA replication in a crude extract

Replication of mini-chromosomes was assayed as described previously [2,21] using a crude extract. Template DNA (M13E10; 200 ng, containing 600 pmol of nucleotides), 200 μ g of Fraction II from *E. coli* WM433 (*dnaA204*) and DnaA protein were incubated in reaction cocktails at 30 °C for 20 min. The reaction was terminated by chilling on ice and adding 10% trichloroacetic acid (final concentration). Samples were filtered on to Whatman GF/C glass-fibre filters. The amount of radioactivity on the filter was measured with a liquid scintillation counter, and the amount of synthesized DNA (pmol of nucleotides) was then calculated.

Plasmid complementation analysis

Temperature-sensitive *dnaA* mutant cells were transformed with pKP2204 (a derivative from mini-R plasmid) [25] containing either the wild-type *dnaA* or each mutant *dnaA* gene. Cultures were diluted appropriately and spread on Luria–Bertani agar plates containing 20 μ g/ml chloramphenicol. Plates were incubated at 42 or 30 °C for 12 and 24 h respectively, the number of colonies counted and the colony-forming efficiency determined.

RESULTS

Purification of mutant DnaA proteins

DnaAR328E, DnaAK372E and DnaA435 (see Figure 1) were purified from *E. coli* cells, which overexpressed them. To avoid contamination of purified mutant DnaA protein with wild-type DnaA protein, the KA450 strain [*ΔoriC1071::Tn10*, *rnhA199* (Am), *dnaA17* (Am), *trpE9828* (Am), *tyrA* (Am), *thr*, *ilv* and *thyA*] of *E. coli* was used. Viability is not dependent on the function of DnaA protein in this strain. The addition of 1% arabinose caused significant overproduction of each mutant DnaA protein (results not shown). DnaA protein was purified by ammonium sulphate precipitation, precipitation by dialysis and gel-filtration column chromatography, as described previously [22,23]. All mutant DnaA proteins were purified with approxi-

Figure 2 Purification of mutant DnaA proteins

Active Superose 12 chromatography fractions of each mutant DnaA protein were pooled; 0.2 μ g of each protein was subjected to SDS/PAGE (10%) and stained with Coomassie Brilliant Blue R-250.

Figure 3 ATP and ADP binding to DnaA435

The indicated amounts of DnaA435 or wild-type protein were incubated with 1 μ M [α - ^{32} P]ATP or [^{3} H]ADP at 4 °C for 10 min. The amount of bound ATP or ADP was determined by filtration.

Figure 4 DNA binding to mutant DnaA proteins

The indicated amounts of DnaAR328E, DnaAK372E, DnaA435 and the wild-type protein were incubated with radiolabelled *oriC* DNA (21 fmol) at 37 °C for 4 min. The amount of bound DNA was determined by filtration.

mately the same recoveries (6–9 %) as for the wild-type protein (6%). The purity of the proteins was more than 80 %, as judged by densitometric scanning of the gel (Figure 2).

Characterization of ATP- or ADP-binding activities of the mutant DnaA proteins

In our previous papers [13,14,18,19], we measured the interaction between DnaA protein and CL by using both CL-dependent release of ATP (or ADP) from DnaA protein, and CL-dependent inhibition of ATP (or ADP) binding to DnaA protein. Therefore, in order to measure the interaction of DnaA435 with CL using the same method, DnaA435 must maintain ATP-

Figure 5 CL inhibition of DNA binding to mutant DnaA proteins

DnaAR328E, DnaAK372E, DnaA435 and the wild-type protein (0.5 pmol) were incubated with 1 μ M ATP at 4 °C for 10 min and further incubated with radiolabelled *oriC* DNA (21 fmol) in the presence of the indicated amounts of CL at 37 °C for 4 min. The amount of bound DNA was determined by filtration. In the absence of CL, the amounts of DNA bound to DnaAR328E, DnaAK372E, DnaA435 and the wild-type proteins are 7.7, 9.6, 2.8 and 6.3 fmol respectively.

or ADP-binding activity. As shown in Figure 3, DnaA435 showed very low affinity for ATP and ADP. DnaAR328E and DnaAK372E could bind to both ATP and ADP to a similar degree as the wild-type protein (results not shown), as described previously [18,19]. The mutations together may change the conformation of DnaA protein around R334, which is important for ATP binding and ATPase activity of DnaA protein [19,20] (see the Discussion section).

Inhibition of DNA-binding activities of mutant DnaA proteins by CL

Since DnaA435 could not interact with adenine nucleotides, we needed a new method to examine its interaction with CL. It has been shown that CL inhibits DnaA binding to *oriC* DNA [28]. We therefore used the extent of *oriC* DNA-binding to examine the interaction between DnaA435 and CL.

The binding of DnaA protein to *oriC* DNA was examined using radiolabelled *oriC* DNA [8]. As shown in Figure 4, DnaA435 could bind to *oriC* DNA to a similar degree as DnaAR328E, DnaAK372E and the wild-type proteins, suggesting that the interaction between DnaA435 and CL could be studied by monitoring CL-dependent inhibition of *oriC* DNA binding to the protein.

We compared the CL inhibition curve for *oriC* DNA binding obtained for DnaA435 with those of DnaAR328E, DnaAK372E and the wild-type protein. As shown in Figure 5, DnaA435 was most resistant to CL inhibition. These results suggest that both R328 and K372 are involved in the interaction between DnaA protein and acidic phospholipids. The interaction of DnaAR328E or DnaAK372E with CL seems to be similar to that of the wild-type protein, based on results in Figure 5. However, we reported previously [18,19] that these two mutant proteins showed decreased interaction with CL, based on examination of CL-dependent release of ATP from DnaA protein. The CL-dependent release of ATP may be a more sensitive assay for the functional interaction between DnaA and CL than the CL-dependent inhibition of *oriC* DNA binding.

Figure 6 Replication activity of the mutant DnaA proteins *in vitro*

The indicated amounts of DnaAR328E, DnaAK372E, DnaA435 and the wild-type protein were incubated with 1 μ M ATP at 4 °C for 10 min. DNA replication in a crude extract from *E. coli* was performed at 30 °C for 20 min, and the amounts of radiolabel incorporated into acid-perceptible material were assessed.

Table 1 Complementation analysis of the temperature sensitivity of *dnaA46* or *dnaA508* mutants with plasmids carrying the mutant *dnaA* genes

Functions of mutant *dnaA* genes *in vivo* were examined by a plasmid complementation analysis. The ratios of colony-forming efficiency at 42 °C compared with that at 30 °C are shown.

Clones	Colony-forming efficiency (42 °C/30 °C)
Using <i>KS1007</i> (<i>dnaA508</i>) [27] cells	
pKP2204 (Vec)	7.3×10^{-9}
pKP2206 (<i>dnaA</i> ⁺)	1.2
pKP2204/ <i>dnaAR328E</i>	1.1
pKP2204/ <i>dnaAK372E</i>	0.4
pKP2204/ <i>dnaA435</i>	1.3
Using <i>KS1003</i> (<i>dnaA46</i>) [27] cells	
pKP2204 (Vec)	6.1×10^{-8}
pKP2206 (<i>dnaA</i> ⁺)	1.3
pKP2204/ <i>dnaAR328E</i>	1.5
pKP2204/ <i>dnaAK372E</i>	0.9
pKP2204/ <i>dnaA435</i>	1.1

Replication activity of mutant DnaA proteins *in vitro*

We measured the abilities of DnaA435, DnaAR328E, DnaAK372E and the wild-type protein to initiate *oriC* DNA replication using an *oriC* complementation assay in a crude extract [2,21]. Surprisingly, DnaA435 supported *oriC* DNA replication *in vitro*, although the specific activity was lower than those of DnaAR328E, DnaAK372E and the wild-type protein (Figure 6). The specific activities of DnaA435, DnaAR328E, DnaAK372E and the wild-type proteins were 0.17, 0.46, 0.53 and 0.48×10^6 units/mg of protein respectively (1 unit of enzyme activity incorporates 1 pmol of nucleotides/min at 30 °C). The

specific activities of DnaAR328E, DnaAK372E and the wild-type protein were similar to those reported previously [18,19]. Since DnaA435 had low affinity for ATP (Figure 3), we consider that DnaA435 can initiate DNA replication without binding to ATP, as was the case for DnaA46, DnaA5, DnaAA184V, DnaA219 and DnaAcos [29–33]. DnaA219 (A184V, H252Y and R342C) was found to be a lethal over-initiation phenotype, as was the case for DnaAcos [31,33]. This phenotype can be explained by the fact that they are active without binding to ATP [31,33], because ATP hydrolysis is involved in DnaA inactivation to suppress the over-initiation [34,35]. However, DnaA435 was not a lethal over-initiation phenotype (Table 1), although this protein was active without binding to ATP (Figures 3 and 6). This may be because DnaA435 is partially, but not fully, active for DNA replication (Figure 6). It is also possible that DnaA435 can bind to ATP under the conditions of *in vitro* DNA replication (in the presence of 5 mM ATP).

Replication activity of mutant DnaA proteins *in vivo*

We used a plasmid complementation analysis with *E. coli* strains bearing temperature-sensitive *dnaA* mutants to examine the DNA replication activities of these mutant DnaA proteins *in vivo*. The coding regions of these mutant and the wild-type *dnaA* genes were conjugated with the wild-type *dnaA* promoter in pKP2204 [25]. Each resultant plasmid was introduced into *E. coli* KS1003, in which DnaA protein has the temperature-sensitive *dnaA46* mutations in its ATP-binding site [36]. Incubations were performed at 42 or 30 °C. Table 1 shows the ratio of colony-forming efficiency at 42 °C compared with that at 30 °C. The ratio with *dnaA435* was approx. 1, as with the wild-type gene. We then performed the same experiments using *dnaA508* (KS1007) [36], which has a temperature-sensitive mutation in DnaA protein's N-terminal region, instead of *dnaA46* (KS1003). The results were similar (Table 1). The colony sizes and growth rates obtained with *dnaA435* were indistinguishable from those obtained with the wild-type gene (pKP2206) [25]. Thus, DnaA435, which shows a decreased interaction with CL and with ATP *in vitro*, is able to initiate DNA replication *in vivo*.

DISCUSSION

In this study, we measured the interaction between DnaA protein and acidic phospholipids using a system of CL-dependent inhibition of DNA-binding activity of DnaA protein [28]. This is because DnaA435 cannot bind to ATP and, therefore, the previous method of studying the effect of acidic phospholipids (CL-dependent inhibition of DnaA–ATP binding) [13,14,18,19] could not be used. CL-dependent inhibition of DnaA–DNA binding and CL-dependent inhibition of DnaA–ATP binding are closely related, because we recently found that the inhibition of DnaA–DNA binding by phospholipids required acidic moiety in phospholipids (M. Makise, S. Mima, T. Tsuchiya and T. Mizushima, unpublished work), as was the case for the inhibition of DnaA–ATP binding by phospholipids [13,14,18,19].

We found in the present study that DnaA435, with both R328E and K372E, has a lower affinity for CL than DnaA proteins with either one of the mutations. These results suggest that R328 and K372 are co-operatively involved in the interaction with acidic phospholipids. K372 included the domain which was suggested to be involved in the membrane-binding activity [15–17]. DnaA protein belongs to a family of ATPases associated with a variety of cellular activities [37]. X-ray structure analysis of other proteins belonging to this family, and comparison of amino-acid sequences, suggested that R334 of DnaA protein interacts with the phosphates of adenine

Figure 7 A proposed model for conformation of the wild-type DnaA protein and DnaA435 in the presence of ATP or CL

nucleotides [37–40]. In fact, we showed that both ATP–DnaAR334E and ADP–DnaAR334E complexes were unstable at higher temperatures, compared with the wild-type protein [19]. It was also reported that DnaAR334H has decreased intrinsic ATPase activity, suggesting that this amino-acid residue interacts with phosphates of adenine nucleotides [20]. Since R334 is located between R328 and K372, it is reasonable to hypothesize that CL binds to R328 and K372 and causes a conformational change around R334, which in turn inhibits the interaction of R334 with phosphates of adenine nucleotides, lowering the affinity of DnaA protein for adenine nucleotides [19,20].

Although DnaA435 has low affinity for ATP, it can initiate DNA replication *in vivo* and *in vitro*. Figure 7 shows a model, which can explain all the following phenomena: (1) the wild-type DnaA protein is activated by binding to ATP; (2) DnaA435 has low affinity for ATP; (3) DnaA435 is active; (4) CL interacts with the wild-type DnaA protein to decrease the affinity of the protein for ATP or ADP; and (5) DnaA435 has decreased affinity for CL. In the model, we hypothesize that both ATP binding to the wild-type DnaA protein and mutations in DnaA435 (R328E and K372E) cause a similar conformational change in DnaA protein around two amphipathic helices (K327 to I344 and D357 to V374). This conformational change in DnaA435 may modify the association of ATP with the ATP-binding pocket of DnaA protein, in which K178 (Walker A motif) [10] and R334 are involved. In the wild-type protein, CL may bind to R328 and K372 and block the ATP binding to DnaA protein.

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REFERENCES

- Hirota, Y., Mordoh, J. and Jacob, F. (1970) On the process of cellular division in *Escherichia coli*. 3. Thermosensitive mutants of *Escherichia coli* altered in the process of DNA initiation. *J. Mol. Biol.* **53**, 369–387
- Fuller, R. S. and Kornberg, A. (1983) Purified dnaA protein in initiation of replication at the *Escherichia coli* chromosomal origin of replication. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5817–5821
- Jacob, F., Brenner, S. and Cuzin, F. (1963) On the regulation of DNA replication in bacteria. *Cold Spring Harbor Symp. Quant. Biol.* **28**, 329–348
- Sekimizu, K. and Kornberg, A. (1988) Cardiolipin activation of DnaA protein, the initiation protein of replication in *Escherichia coli*. *J. Biol. Chem.* **263**, 7131–7135
- Yung, B. Y. and Kornberg, A. (1988) Membrane attachment activates DnaA protein, the initiation protein of chromosome replication in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7202–7205
- Castuma, C. E., Crooke, E. and Kornberg, A. (1993) Fluid membranes with acidic domains activate DnaA, the initiator protein of replication in *Escherichia coli*. *J. Biol. Chem.* **268**, 24665–24668
- Mizushima, T., Ishikawa, Y., Obana, E., Hase, M., Kubota, T., Katayama, T., Kunitake, T., Watanabe, E. and Sekimizu, K. (1996) Influence of cluster formation of acidic phospholipids on decrease in the affinity for ATP of DnaA protein. *J. Biol. Chem.* **271**, 3633–3638
- Sekimizu, K., Bramhill, D. and Kornberg, A. (1987) ATP activates DnaA protein in initiating replication of plasmids bearing the origin of the *E. coli* chromosome. *Cell* **50**, 259–265
- Mizushima, T., Sasaki, S., Ohishi, H., Kobayashi, M., Katayama, T., Miki, T., Maeda, M. and Sekimizu, K. (1996) Molecular design of inhibitors of *in vitro* *oriC* DNA replication based on the potential to block the ATP binding of DnaA protein. *J. Biol. Chem.* **271**, 25178–25183
- Mizushima, T., Takaki, T., Kubota, T., Tsuchiya, T., Miki, T., Katayama, T. and Sekimizu, K. (1998) Site-directed mutational analysis for the ATP binding of DnaA protein. Functions of two conserved amino acids (Lys-178 and Asp-235) located in the ATP-binding domain of DnaA protein *in vitro* and *in vivo*. *J. Biol. Chem.* **273**, 20847–20851
- Xia, W. and Dowhan, W. (1995) *In vivo* evidence for the involvement of anionic phospholipids in initiation of DNA replication in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 783–787
- Zheng, W., Li, Z., Skarstad, K. and Crooke, E. (2001) Mutations in DnaA protein suppress the growth arrest of acidic phospholipid-deficient *Escherichia coli* cells. *EMBO J.* **20**, 1164–1172
- Hase, M., Yoshimi, T., Ishikawa, Y., Ohba, A., Guo, L., Mima, S., Makise, M., Yamaguchi, Y., Tsuchiya, T. and Mizushima, T. (1998) Site-directed mutational analysis for the membrane binding of DnaA protein. Identification of amino acids involved in the functional interaction between DnaA protein and acidic phospholipids. *J. Biol. Chem.* **273**, 28651–28656
- Yamaguchi, Y., Hase, M., Makise, M., Mima, S., Yoshimi, T., Ishikawa, Y., Tsuchiya, T. and Mizushima, T. (1999) Involvement of Arg-328, Arg-334, and Arg-342 of DnaA protein in its functional interaction to acidic phospholipids. *Biochem. J.* **340**, 433–438
- Garner, J. and Crooke, E. (1996) Membrane regulation of the chromosomal replication activity of *E. coli* DnaA requires a discrete site on the protein. *EMBO J.* **15**, 3477–3485
- Garner, J., Durrer, P., Kitchen, J., Brunner, J. and Crooke, E. (1998) Membrane-mediated release of nucleotide from an initiator of chromosomal replication, *Escherichia coli* DnaA, occurs with insertion of a distinct region of the protein into the lipid bilayer. *J. Biol. Chem.* **273**, 5167–5173
- Crooke, E. (2001) *Escherichia coli* DnaA protein–phospholipid interactions: *in vitro* and *in vivo*. *Biochimie* **83**, 19–23
- Makise, M., Mima, S., Tsuchiya, T. and Mizushima, T. (2000) Identification of amino acids involved in the functional interaction between DnaA protein and acidic phospholipids. *J. Biol. Chem.* **275**, 4513–4518
- Makise, M., Mima, S., Tsuchiya, T. and Mizushima, T. (2001) Molecular mechanism for functional interaction between DnaA protein and acidic phospholipids: identification of important amino acids. *J. Biol. Chem.* **276**, 7450–7456
- Su'etsugu, M., Kawakami, H., Kurokawa, K., Kubota, T., Takata, M. and Katayama, T. (2001) DNA replication-coupled inactivation of DnaA protein *in vitro*: a role for DnaA arginine-334 of the AAA⁺ Box VIII motif in ATP hydrolysis. *Mol. Microbiol.* **40**, 376–386
- Fuller, R. S., Kaguni, J. M. and Kornberg, A. (1981) Enzymatic replication of the origin of the *Escherichia coli* chromosome. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7370–7374
- Sekimizu, K., Yung, B. Y. and Kornberg, A. (1988) The dnaA protein of *Escherichia coli*. Abundance, improved purification, and membrane binding. *J. Biol. Chem.* **263**, 7136–7140
- Kubota, T., Katayama, T., Ito, Y., Mizushima, T. and Sekimizu, K. (1997) Conformational transition of DnaA protein by ATP: structural analysis of DnaA protein, the initiator of *Escherichia coli* chromosome replication. *Biochem. Biophys. Res. Commun.* **232**, 130–135
- Kunkel, T. A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 488–492
- Guo, L., Katayama, T., Seyama, Y., Sekimizu, K. and Miki, T. (1999) Isolation and characterization of novel cold-sensitive *dnaA* mutants of *Escherichia coli*. *FEMS Microbiol. Lett.* **176**, 357–366
- Baker, T. A., Sekimizu, K., Funnell, B. E. and Kornberg, A. (1986) Extensive unwinding of the plasmid template during staged enzymatic initiation of DNA replication from the origin of the *Escherichia coli* chromosome. *Cell* **45**, 53–64

- 27 Mizushima, T., Katayama, T. and Sekimizu, K. (1996) Effect on DNA topology by DnaA protein, the initiation factor of chromosomal DNA replication in *Escherichia coli*. *Biochemistry* **35**, 11512–11516
- 28 Crooke, E., Castuma, C. E. and Kornberg, A. (1992) The chromosome origin of *Escherichia coli* stabilizes DnaA protein during rejuvenation by phospholipids. *J. Biol. Chem.* **267**, 16779–16782
- 29 Hwang, D. S. and Kaguni, J. M. (1988) Purification and characterization of the *dnaA46* gene product. *J. Biol. Chem.* **263**, 10625–10632
- 30 Hupp, T. R. and Kaguni, J. M. (1993) DnaA5 protein is thermolabile in initiation of replication from the chromosomal origin of *Escherichia coli*. *J. Biol. Chem.* **268**, 13128–13136
- 31 Katayama, T. and Kornberg, A. (1994) Hyperactive initiation of chromosomal replication *in vivo* and *in vitro* by a mutant initiator protein, DnaAcos, of *Escherichia coli*. *J. Biol. Chem.* **269**, 12698–12703
- 32 Carr, K. M. and Kaguni, J. M. (1996) The A184V missense mutation of the *dnaA5* and *dnaA46* alleles confers a defect in ATP binding and thermolability in initiation of *Escherichia coli* DNA replication. *Mol. Microbiol.* **20**, 1307–1318
- 33 Weigel, C., Schmidt, A., Seitz, H., Tungler, D., Welzeck, M. and Messer, W. (1999) The N-terminus promotes oligomerization of the *Escherichia coli* initiator protein DnaA. *Mol. Microbiol.* **34**, 53–66
- 34 Mizushima, T., Nishida, S., Kurokawa, K., Katayama, T., Miki, T. and Sekimizu, K. (1997) Negative control of DNA replication by hydrolysis of ATP bound to DnaA protein, the initiator of chromosomal DNA replication in *Escherichia coli*. *EMBO J.* **16**, 3724–3730
- 35 Katayama, T., Kubota, T., Kurokawa, K., Crooke, E. and Sekimizu, K. (1998) The initiator function of DnaA protein is negatively regulated by the sliding clamp of the *E. coli* chromosomal replicase. *Cell* **94**, 61–71
- 36 Mizushima, T., Yokoyama, K., Mima, S., Tsuchiya, T. and Sekimizu, K. (1997) Inhibition of thymidine transport in *dnaA* mutants of *Escherichia coli*. *J. Biol. Chem.* **272**, 21195–21200
- 37 Neuwald, A. F., Aravind, L., Spouge, J. L. and Koonin, E. V. (1999) AAA⁺: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res.* **9**, 27–43
- 38 Guenther, B., Onrust, R., Sali, A., O'Donnell, M. and Kuriyan, J. (1997) Crystal structure of the delta' subunit of the clamp-loader complex of *E. coli* DNA polymerase III. *Cell* **91**, 335–345
- 39 Bochtler, M., Hartmann, C., Song, H. K., Bourenkov, G. P., Bartunik, H. D. and Huber, R. (2000) The structures of HslU and the ATP-dependent protease HslU-HslV. *Nature* **403**, 800–805
- 40 Schaper, S. and Messer, W. (1997) Prediction of the structure of the replication initiator protein DnaA. *Proteins* **28**, 1–9

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