Involvement of Arg-328, Arg-334 and Arg-342 of DnaA protein in the functional interaction with acidic phospholipids

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We reported previously that three basic amino acids (Arg-360, Arg-364 and Lys-372) of DnaA protein are essential for its functional interaction with cardiolipin. In this study, we examined the effect of mutation of some basic amino acids in a potential amphipathic helix (from Lys-327 to Ile-345) of DnaA protein on this interaction. ATP binding to the mutant DnaA protein, in which Arg-328, Arg-334 and Arg-342 were changed to acidic amino acids, was less inhibited by cardiolipin than that of the wild-type protein, as was the case for mutant DnaA protein with

mutations of Arg-360, Arg-364 and Lys-372. A mutant DnaA protein with mutations of all six basic amino acids showed the most resistance to the inhibition of ATP binding by cardiolipin. These results suggest that Arg-328, Arg-334 and Arg-342, like Arg-360, Arg-364 and Lys-372, are also involved in the functional interaction between DnaA protein and acidic phospholipids.

Key words: basic amino acid, *Escherichia coli*, initiator protein, membrane binding, site-directed mutagenesis.

INTRODUCTION

The activity of the initiator protein of chromosomal DNA replication must be regulated strictly in order to couple the initiation of DNA replication with cell division. Thus the initiator protein should be activated for the initiation of DNA replication and should be inactivated soon after the initiation of DNA replication. DnaA protein, the initiator of DNA replication in *Escherichia coli* [1–4], has a high affinity for ATP and ADP and the ATP-binding form is the active form for DNA replication [5]. Recent genetic and biochemical studies suggest strongly that adenine nucleotide binding to DnaA protein regulates the initiation of chromosomal DNA replication in *E. coli* cells [6–10]. In particular, DNA-replication-dependent activation of the intrinsic ATPase activity of DnaA protein by its stimulation factors seems to play a major role in the inactivation of DnaA protein soon after the initiation of DNA replication in cells [11–15].

As for the activation of DnaA protein for the initiation of DNA replication, acidic phospholipids in membranes have been proposed to be involved in the process. Acidic phospholipids, in particular cardiolipin (CL), decrease the affinity of the DnaA protein for adenine nucleotides and activate the DnaA protein to change from the ADP-binding form to the ATP-binding form [16–19]. Several lines of genetic evidence support the notion that acidic phospholipids are involved in the regulation of DNA replication in cells [20–23]. In order to better understand how DnaA protein interacts with acidic phospholipids and how it is involved in the regulation of DNA replication, identification of functional domains of DnaA that are involved in membrane binding is important. It has been suggested that a potential amphipathic helix (from Asp-357 to Val-374 of DnaA) is one of the membrane-binding domains [1,24,25]. We reported recently that conserved basic amino acids located in the region of Arg-360, Arg-364 and Lys-372 are essential for the functional

interaction between DnaA protein and CL [26], suggesting that the functional interaction between DnaA protein and acidic phospholipids is mediated by an ionic interaction. In this paper, we focused on another potential amphipathic helix (from Lys-327 to Ile-345) and found that basic amino acids in this amphipathic helix are also involved in the functional interaction between DnaA protein and CL.

MATERIALS AND METHODS

Materials

A crude extract for an *oriC* complementation assay was prepared from the WM433 strain of *E. coli* as described previously [27,28]. CL was from Sigma and [α -³²P]ATP (10 Ci/mmol) and [³H]ADP (40 Ci/mmol) were from Amersham Pharmacia Biotech and DuPont, respectively. The wild-type and DnaA431 (with mutations of Arg-360, Arg-364 and Lys-372 [26]) proteins were purified by gel-filtration column chromatography [26].

Site-directed mutagenesis and plasmid construction

Site-specific mutagenesis was performed using the method of Kunkel [29]. In brief, uracil-containing single-stranded DNA of M13 phage, which contains the coding region of the *dnaA* gene [13], was hybridized with each of two oligonucleotide primers, one of which contained a mismatch sequence for replacement of Arg-328, Arg-334 and Arg-342 each with Glu and the other of which contained a mismatch sequence for replacement of Arg-328, Arg-334, Arg-342, Arg-360, Arg-364 and Lys-372 each with Glu. The complementary DNA strand was synthesized *in vitro* and the resultant double-stranded DNA was introduced into JM109 cells. The mutations were confirmed by DNA sequencing,

Abbreviation used: CL, cardiolipin.

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and double-stranded DNA containing the mutations, plasmids pMZ000-33 and pMZ000-34, was prepared as follows.

For overproduction of the mutant DnaA protein, we used the pMZ001 plasmid [13], which contains the arabinose promoter. The *EcoRI*–*HindIII* region of pMZ000-33 or pMZ000-34 was ligated with pMZ001. The resultant plasmids, named pMZ001-33 and pMZ001-34, respectively, were used for overproduction of the mutant DnaA proteins.

Purification of mutant DnaA proteins

The mutant DnaA proteins were purified, as described previously [30–32], but with some modifications. Strain KA450 [12] transformed with pMZ001-33 or pMZ001-34 was grown in 20 l of Luria–Bertani medium containing 25 µg/ml thymine at 37 °C until the attenuation at 595 nm reached 0.5, and then arabinose was added to a concentration of 1%. After 1 h of incubation at 37 °C, the cells were harvested by centrifugation, resuspended in buffer C [30] containing 250 mM KCl to an attenuation at 595 nm of 220 and stored at –80 °C. The thawed cell suspension was diluted 2-fold with buffer C containing 250 mM KCl, and spermidine/HCl and egg-white lysozyme were added to final concentrations of 20 mM and 400 µg/ml, respectively. After incubation at 0 °C for 30 min, the suspension was centrifuged (140000 g) for 30 min in a Beckman 50.2 Ti rotor. The supernatant was precipitated with 0.22 g/ml ammonium sulphate and re-suspended with buffer C. Samples were dialysed against buffer C and the precipitates that developed were collected by centrifugation at 400000 g in a Beckman TLA100.3 rotor. The precipitates were washed twice with buffer C containing 0.6 M ammonium sulphate and finally resuspended in buffer C containing 4 M guanidine/HCl and 0.6 M ammonium sulphate. Insoluble materials were removed by centrifugation at 400000 g in the TLA100.3 rotor. The supernatant was fractionated by gel filtration on a Superose-12 column (Pharmacia FPLC HR10/30) equilibrated with buffer D [30] at a flow rate of 0.3 ml/min and the active fractions were pooled.

oriC DNA replication in a crude extract

Replication of mini-chromosomes in a crude extract (fraction II) was assayed as described [27,28]. Template DNA (M13E10) (200 ng, 600 pmol as nucleotides), 240 µg of fraction II from WM433 (*dnaA204*, a temperature-sensitive *dnaA* mutant) and DnaA protein were mixed with reaction cocktails [27,28] and incubated for replication at 30 °C for 20 min. The reaction was terminated by chilling on ice and adding 10% trichloroacetic acid. Samples were passed through Whatman GF/C glass-fibre filters. The amount of radioactivity on the filters was measured in a liquid scintillation counter, and the amount of DNA synthesized (pmol of nucleotides) was calculated [27,28].

Filter-binding assay for ATP or ADP binding to DnaA protein

The ATP- and ADP-binding activities of DnaA protein were determined by a filter-binding assay [5]. DnaA protein (2 pmol) was incubated with [α -³²P]ATP or [³H]ADP at 0 °C for 15 min in 40 µl of buffer G [50 mM Tricine/KOH (pH 8.25)/0.5 mM magnesium acetate/0.3 mM EDTA/7 mM dithiothreitol/20% (v/v) glycerol/0.007% Triton X-100]. Samples were passed through nitrocellulose membranes (Millipore HA, 0.45 µm) and washed with ice-cold wash buffer [50 mM Tricine/KOH (pH 8.25)/0.5 mM magnesium acetate/0.3 mM EDTA/5 mM dithiothreitol/17% (v/v) glycerol/10 mM ammonium sulphate/0.005% Triton X-100]. The radioactivity remaining on the filters was counted in a liquid scintillation counter.

Inhibition of ATP-binding to DnaA protein by CL

Liposomes of CL were prepared from dried phospholipids on the bottoms of glass tubes by vigorous vortex mixing in water. The amount of phosphorus in the phospholipid fraction was determined by the method of Chen et al. [33]. Inhibition of ATP-binding to DnaA protein by CL was examined as described [34]. DnaA protein (2 pmol) was pre-incubated with CL at 0 °C for 15 min and further incubated with [α -³²P]ATP at 0 °C for 15 min in 40 µl of buffer G [5]. Bound ATP was determined as described above.

RESULTS AND DISCUSSION

Strategy for site-directed mutation

DnaA protein has five potential amphipathic helices, which are candidates for membrane-binding domains of the protein [1]. Garner and Crooke suggested that the fourth potential amphipathic helix from the N-terminus (from Asp-357 to Val-374) is important for membrane binding based on their result that acidic phospholipids protect Lys-372 from proteolysis by trypsin [24]. Recently, we showed that three basic amino acids (Arg-360, Arg-364 and Lys-372) in the fourth amphipathic helix were essential for the functional interaction between DnaA protein and acidic phospholipids using site-directed mutagenesis [26]. These results suggest that the fourth amphipathic helix is involved in the interaction between DnaA protein and acidic phospholipids. However, the contribution of the other potential amphipathic helices to the interaction has remained unclear. The first and second amphipathic helices are located in the ATP-binding region [1]. The fifth amphipathic helix is in the DNA-binding region [1].

In this study, we focused on the third potential amphipathic helix (from Lys-327 to Ile-345), which is located very close to the fourth. A helical wheel projection shows that all five basic amino acids of this amphipathic helix are located on one side of the wheel (Figure 1, top left panel) [26]. Among these five basic amino acids, Arg-328, Arg-334 and Arg-342 are more conserved among DnaA proteins from various species than the other two basic amino acids (Figure 1, top right panel). We considered the possibility that these three amino acids, in addition to Arg-360, Arg-364 and Lys-372 in the fourth amphipathic helix, are involved in the membrane-binding activity of DnaA. Thus we decided to construct mutant DnaA proteins, DnaA433 and DnaA434, in which Arg-328, Arg-334 and Arg-342, and Arg-328, Arg-334, Arg-342, Arg-360, Arg-364 and Lys-372 were each mutated into Glu, respectively (Figure 1, bottom panel), to examine the role of the fourth amphipathic helix of DnaA in its membrane binding.

At first, we predicted the helicity of the mutant proteins, considering the possibility that these mutations (Figure 1, bottom panel) could affect the helicity of the third and fourth amphipathic helices of DnaA. We computed the helicity using SSTRhread [35], a widely used threading (three-dimensional/one-dimensional) program offered by the National Institute of Genetics in Japan on the worldwide web. We calculated the secondary structure of the portion of DnaA protein (from Arg-156 to the C-terminus). Figure 2 shows the simulated α -helix, β -sheet and random-coil portions in the Lys-327 to Val-374 region of the wild-type and mutant DnaA proteins. The two helices of the wild-type DnaA protein predicted by this program match those described elsewhere [1], except for the first six amino acids. These helix-moieties appear on the mutant DnaA431, DnaA433 and DnaA434 proteins at almost the same positions. Thus these

Figure 1 Strategy for site-directed mutagenesis

(Top left panel) helical wheel projection of the third amphipathic helix (from Lys-327 to Ile-345). Outlined characters, basic amino acids; underlined characters, mutated amino acids. (Top right panel) comparison of amino acid sequences of the third amphipathic helix of 14 bacterial species (data from [1]). Outlined characters show conserved basic amino acids. (Bottom panel) conversion of amino acids in the mutant DnaA proteins.

Figure 2 Predicted secondary structures of mutant DnaA proteins

The threading method was applied to the sequences from Arg-156 to the C-terminus of the wild-type (DnaA⁺), DnaA431, DnaA433 and DnaA434 proteins. Underlined sequence in DnaA⁺ indicates the helix predicted by Skarstad and Boye [1]. Closed bars, open bars, and blank portions under each amino acid indicate α -helix, β -sheet and random-coil moieties, respectively. The arrows indicate mutated residues.

Figure 3 ATP and ADP binding to mutant DnaA protein measured by a filter-binding assay

DnaA433, DnaA434, DnaA431 and the wild-type DnaA protein were incubated with 1 μ M [α -³²P]ATP (top panel) or [³H]ADP (bottom panel) for 15 min at 0 °C. The amount of bound ATP or ADP was determined, as described in the Materials and methods section.

mutations do not appear to deform drastically the helicity of DnaA.

Purification of mutant DnaA proteins

We constructed mutant DnaA proteins, DnaA433 and DnaA434, in which Arg-328, Arg-334 and Arg-342, and Arg-328, Arg-334, Arg-342, Arg-360, Arg-364 and Lys-372 were mutated into Glu, respectively (Figure 1, bottom panel). The coding regions of the *dnaA433* and *dnaA434* genes were conjugated with the promoter of the arabinose operon to construct plasmids for overproduction of the mutated DnaA proteins. The KA450 strain [Δ oriC1071::Tn10, *rnhA199*(Am), *dnaA17*(Am), *trpE9828*(Am), *tyrA*(Am), *thr*, *ilv* and *thyA*], in which the *dnaA* gene on chromosomal DNA was deleted [12], was used as the host for overproduction to avoid contamination of the wild-type DnaA protein. Purification of the mutant DnaA protein was done as

Figure 4 Inhibition of ATP binding to the mutant DnaA protein by CL

DnaA433, DnaA434, DnaA431 and the wild-type DnaA protein were pre-incubated with the indicated amounts of CL for 5 min in 40 μ l of buffer G and further incubated with 1 μ M [α -³²P]ATP for 15 min in the same buffer at 4 °C. The amount of bound ATP was determined, as described in the Materials and methods section.

described in the Materials and methods section. At the final step of purification by gel-filtration column chromatography, both monomer and aggregated forms of the wild-type DnaA protein were recovered [30]. However, only the monomer forms of the DnaA433 and DnaA434 proteins were recovered (results not shown), as was the case for DnaA431 [26]. Recovery of the DnaA433 and DnaA434 proteins from the gel-filtration column was not significantly different from the recovery of the wild-type protein. The aggregated form of DnaA protein is considered to be a complex of DnaA with phospholipids [36]. Thus DnaA433 and DnaA434 proteins seemed to lose an affinity for phospholipids, as in the case of DnaA431 [26]. In the following experiments, we compared the activities of purified DnaA433 and DnaA434 with those of DnaA431 and the wild-type protein.

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

The functional interaction of DnaA protein with acidic phospholipids can be monitored by phospholipid-dependent stimulation of the release of ATP (or ADP) from DnaA protein or phospholipid-dependent inhibition of the binding of ATP (or ADP) to DnaA protein [16]. Thus the mutant DnaA proteins were required to maintain their ATP- or ADP-binding activities for examination of their functional interaction with acidic phospholipids. The ATP-binding activities of these mutant proteins were examined by a filter-binding assay [5]. As shown in Figure 3, top panel, DnaA433 and DnaA434 showed ATP-binding activities. We also examined the ATP-binding activities of both mutant DnaA proteins in the presence of various concentrations of ATP. Scatchard-plot analysis revealed that the K_d values of DnaA433, DnaA434, DnaA431 and the wild-type protein for ATP were 59, 38, 19 and 30 nM, respectively. The numbers of ATP-binding sites per DnaA433, DnaA434, DnaA431 and the wild-type proteins were calculated to be 0.44, 0.38, 0.46 and 0.42, respectively, by the Scatchard-plot analysis.

Figure 5 Replication activity of the mutant DnaA protein in a crude extract

DnaA433, DnaA434, DnaA431 and the wild-type DnaA protein were incubated with 1 μ M ATP for 15 min at 0 °C. DNA replication in a crude extract was carried out for 20 min as described in the Materials and methods section.

Figure 6 Schematic representation of a plausible electrostatic interaction between the cationic residues on the helices and a multi-anionic lipid

The K_d value and the number of ATP-binding sites of the wild-type protein were nearly the same as reported previously [5].

We also examined the ADP-binding activities of mutant DnaA proteins in the same manner. As shown in Figure 3, bottom panel, all of the mutant DnaA proteins showed ADP-binding activity in the presence of various concentrations of ADP. Scatchard-plot analysis revealed that the K_d values of DnaA433,

DnaA434, DnaA431 and the wild-type protein for ADP were 220, 150, 160 and 230 nM, respectively. The numbers of ADP-binding sites per DnaA433, DnaA434, DnaA431 and the wild-type proteins were calculated to be 0.38, 0.21, 0.46 and 0.34, respectively, by the Scatchard-plot analysis. The results suggest that these three amino acids in the third amphipathic helix are not necessary for the adenine nucleotide-binding activity of DnaA, as in the case of the basic amino acids in the fourth helix.

Inhibition of ATP-binding to DnaA proteins by CL

As described above, the functional interaction between DnaA and CL can be measured by two methods, stimulation of release of ATP from DnaA protein and inhibition of ATP binding to DnaA protein by CL. At first, we examined the effect of CL on the release of ATP from the mutant DnaA proteins. The complex of the wild-type protein with ATP was stable, i.e. a very small amount of ATP was released from DnaA protein in the absence of CL and CL stimulated the release as described previously [16]. However, the complex of DnaA433 with ATP was unstable, i.e. ATP bound to DnaA433 was easily released, even in the absence of CL (results not shown). This may be due to the fact that DnaA433 is unstable at 37 °C, which is required for the stimulation of the ATP release by CL [16]. Thus we measured the functional interaction between the mutant DnaA proteins and CL by CL-dependent inhibition of ATP binding to DnaA protein, which was carried out at 4 °C. DnaA protein was pre-incubated with CL and further incubated with [α - 32 P]ATP. Bound [α - 32 P]ATP was determined by a filter-binding assay. As shown in Figure 4, ATP binding to each of mutant DnaA proteins (DnaA431, DnaA433 and DnaA434) was not inhibited strongly by CL. Interestingly, DnaA434, with mutations in both the third and fourth amphipathic helices, was the most resistant to the inhibition by CL (Figure 4). In the presence of 0.1 μ g of CL in a 40 μ l reaction mixture, ATP binding to the wild-type DnaA protein was inhibited by more than 80%, whereas ATP binding to DnaA434 was reduced by less than 50% (Figure 4). These results suggest that basic amino acids in the third amphipathic helix (Arg-328, Arg-334 and Arg-342) are involved in the functional interaction between DnaA protein and acidic phospholipids.

Replication activity of the mutant DnaA proteins *in vitro*

We measured the abilities of DnaA433 and DnaA434 proteins to initiate DNA replication with an *oriC* complementation assay [27,28]. As shown in Figure 5, DnaA434 showed DNA-replication activity, although the activity was less than the activities of DnaA431 and the wild-type protein. DnaA433 showed little replication activity in a crude extract (Figure 5). The specific activity of DnaA434 was about one-seventh of the activity of the wild-type protein. DnaA A184V, DnaA46 and DnaA5 required longer incubation periods for expression of their replication activity; a time lag for the DNA-replication reaction has been reported previously for these mutant DnaA proteins [7–9]. In the case of these mutants, the increase in DNA replication was approximately linear with time, as was the case for the wild-type protein (results not shown). Pre-incubation with each of the mutant proteins with 1 mM ADP inhibited the replication activity (results not shown), as is the case with the wild-type protein [5].

The relatively low specific activities of the mutant DnaA proteins seems not to be due to denaturation as a result of the purification procedures, because the specific activities of the crude-extract fractions of the mutant DnaA proteins were also

lower than the specific activity of the wild-type protein. The third amphipathic helix seems to be important for maintenance of the higher-order structure of DnaA protein, which is required for its replication activity as predicted previously [37]. Thus we should rule out the possibility that the insensitivity of these mutant DnaA proteins to CL is due to the non-specific structural changes by these mutations.

In this study, we have shown that in DnaA433, a mutant DnaA protein in which cationic residues in the third amphipathic helix are replaced with anionic residues, the binding to anionic CL is disturbed (Figure 4). This result is similar to that observed with DnaA431, which has similar mutations in the fourth helix [26]. In addition, when there are mutations of both the third and fourth helices, as occurs in DnaA434, the interaction with lipid is perturbed even more effectively (Figure 4). These results enable us to propose an electrostatic interaction between the protein and acidic lipids (Figure 6). CL, which possesses two anionic sites, might form a bridge over the cationic residues of the third and fourth helices. Besides the first priority situation, it is also possible that binding of an anionic lipid to the cationic residues of the fourth helix may be disturbed by a structural change in the protein arising from a mutation of the third helix, even though the lipid does not bind to the third helix. In any case, it can be concluded that the third amphipathic helix plays an important role in the binding of DnaA protein to membranes.

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