Conserved hydrophobic amino acid residues in the N-terminal region of DnaA protein are involved in DnaA–DnaA interaction

Shinji MIMA*, Masaki MAKISE*, Motohiro KOTERASAWA*, Tomofusa TSUCHIYA* and Tohru MIZUSHIMA*†¹

*Faculty of Pharmaceutical Sciences, Okayama University, 1-1-1, Tsushima-naka, Okayama 700-8530, Japan, and †PRESTO, Japan Science and Technology Corporation, Okayama 700-8530, Japan

We previously reported that a leucine-zipper-like structure (I26, L33 and L40) located in the N-terminal region of DnaA is essential for the duplex opening at *oriC* by DnaA. In this study, we focused on three other conserved hydrophobic amino acid residues, L3, L10 and L17, and examined the function of DnaA proteins mutated in these amino acid residues. DnaA427 (L17S) and DnaA413 (L3S, L10S and L17S) were inactive for *oriC* DNA replication both *in vitro* and *in vivo*. Although these mutant DnaA proteins maintained their binding activities for both ATP and *oriC*, they were unable to induce the opening of

duplex DNA at *oriC*. Glutathione-S-transferase (GST)-fused wild-type DnaA interacted with wild-type DnaA but not with DnaA427 and DnaA413. Based on these results, we propose that conserved hydrophobic amino acid residues in the N-terminal region of DnaA are involved in DnaA oligomerization, in which DnaA–DnaA interaction is required.

Key words: DnaA oligomerization, DNA replication, hydrophobic amino acid residues, N-terminal.

INTRODUCTION

DnaA is the initiator of chromosomal DNA replication in *Escherichia coli* [1]. It binds to five DnaA boxes located in *oriC*, oligomerizes to open up duplex DNA at *oriC*, and recruits DnaB (DNA helicase) into *oriC* [1–4]. For performing these initiation reactions, activities of DnaA for *oriC*-specific DNA-binding, oligomerization (DnaA–DnaA interaction), and DnaB binding are required [1]. DnaA also plays important roles in the regulation of DNA replication. DnaA tightly binds to ATP and ADP, and the resulting ATP·DnaA is active, whereas ADP·DnaA is inactive in an *oriC* DNA replication system *in vitro* [5,6]. Acidic phospholipids interact with DnaA and activate ADP·DnaA to ATP·DnaA in the presence of high concentrations of ATP *in vitro* [7–10]. After initiation of DNA replication, DnaA must be inactivated in order to suppress the re-initiation of DNA replication. The intrinsic ATPase activity of DnaA is involved in this inactivation [11–14]. Therefore, the activities of DnaA for adenine-nucleotide-binding, acidic phospholipid-binding, and ATPase are important in the regulatory mechanism of DNA replication. Although DnaA also changes DNA supercoiling by sequence-independent DNA binding activity [15–17], its role in DNA replication is unknown.

Since DnaA has multiple activities, determination of the functional domain (identification of essential amino acid residues) for each activity is important in order to fully understand the function and structure of DnaA. Examining the function of DnaA with mutations in the essential amino acid residues for each activity is an effective way of revealing the role for each activity in DNA replication in cells [18]. We identified amino acid residues that are essential for ATP binding, ATPase activity and acidic phospholipid-binding by site-directed mutational analysis, and examined the functions of these mutant DnaA proteins *in vivo* and *in vitro* [13,18–24]. Functional domains for membrane-

binding, *oriC*-specific DNA-binding and DnaA–DnaB interaction have been reported by other groups [25–28].

Previously we have examined the role of the N-terminal region of DnaA in chromosomal DNA replication, by using site-directed mutational analysis. We focused on conserved hydrophobic amino acid residues (I26, L33 and L40), which were predicted to form a leucine-zipper structure [29]. We showed that their mutant DnaA proteins were active for *oriC* binding, but not for the duplex opening at *oriC*, suggesting that these hydrophobic amino acid residues are involved in DnaA–DnaA interaction (DnaA oligomerization) [30]. Weigel et al. [31] supported this notion, by directly examining DnaA–DnaA interaction of N-terminal- and C-terminal-deleted DnaA.

In this study, we have identified other conserved amino acid residues in the N-terminal region of DnaA (L3, L10 and L17). Site-directed mutational analysis of these amino acid residues suggests that they, and especially L17, are involved in DnaA–DnaA interaction (DnaA oligomerization).

MATERIALS AND METHODS

Materials

[α -³²P]ATP (3000 Ci/mmol), [α -³²P]dCTP (6000 Ci/mmol) and [γ -³²P]ATP (6000 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). P1-nuclease was purchased from Boehringer Mannheim (Mannheim, Germany). The wild-type DnaA was purified as described previously [32]. DnaA413 and DnaA427 were purified as described previously for DnaA126S and DnaA40S [30]. Purities of DnaA413, DnaA427 and the wild-type protein fractions were more than 80%, as judged from densitometric scanning. Concentrations of DnaA413, DnaA427 and the wild-type protein fractions were 40,

Abbreviations used: DTT, dithiothreitol; GST, glutathione S-transferase.

¹ To whom correspondence should be addressed (e-mail mizushima@pheasant.pharm.okayama-u.ac.jp).

60 and 120 $\mu\text{g/ml}$. HU protein was purified from over-producing cells, as described previously [33]. Glutathione S-transferase (GST)-DnaA was purified from *E. coli* cells (JM109) transformed with overproducing plasmids by use of glutathione-agarose-column chromatography, as described previously [34]. Approximately 10% of the total protein in the crude extract was GST-DnaA; the yield was approximately 30%. Briefly, cells were lysed by digestion with lysozyme, centrifuged and the supernatant subjected to glutathione-agarose chromatography as specified by the Sigma Corporation. The GST-DnaA was eluted with 10 mM reduced form of glutathione and the protein was dialysed to remove glutathione with buffer A [20 mM Hepes/KOH, pH 7.6, 0.1 M KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 20% (v/v) glycerol]. A crude extract for an *oriC*-complementation assay was prepared from the WM433 strain of *E. coli* as described previously [35].

Bacterial strains

Five strains of *E. coli* from our laboratory stocks were used in this study. They were JM109 [*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, Δ (*lac-proAB*)/F'(*traD36*, *proAB*⁺, *lacIq*, *lacZ* Δ M15), KS1001 (W3110 *lacZ*), KS1007 (KS1001, *dnaA508*) [36], KA450 (Δ *oriC1071::Tn10*, *rnhA199*(Am), *dnaA17*(Am), *trpE9828*(Am), *tyrA*(Am), *thr*, *ilv*, and *thyA*) [37], and WM433 (*dnaA204*, *leu19*, *pro19*, *trp25*, *his47*, *thyA59*, *arg28*, *met55*, *deoB23*, *lac11*, *strA56*, *sul1*, *hsdSK12*).

Site-directed mutagenesis and plasmid constructions

Site-specific mutagenesis was performed as described previously [13]. Briefly, uracil-containing single-stranded DNA of M13 phage, containing the coding region of the *dnaA* gene, was hybridized with oligonucleotide primers representing each mutation. The complementary DNA strand was synthesized *in vitro* and the resultant double-stranded DNA was introduced into *E. coli* JM109. Direct DNA sequencing confirmed the mutation, and double-stranded DNA containing the mutation was prepared.

To construct plasmids for overexpression of DnaA, the *EcoRI*-*HindIII* region of each *dnaA* gene was ligated into pMZ001 [13], which contains the arabinose promoter. Plasmids for complementation analysis were constructed by ligating the coding regions of the mutant *dnaA* gene (*Bam*HI-*Hind*III fragment) into pMZ002 [13], which contains the wild-type promoter of the *dnaA* gene.

oriC DNA replication in a crude extract

Replication of minichromosomes in a crude extract (fraction II) was assayed as described previously [35]. Template DNA (M13E10, 200 ng, 600 pmol of nucleotides), 200 μg of fraction II from WM433 (*dnaA204*) and DnaA were mixed with reaction cocktails [35] and incubated for replication at 30 °C for 20 min. The reaction was terminated by chilling on ice and adding 10% (w/v) trichloroacetic acid. Samples were then passed through Whatman GF/C glass-fibre filters. The radioactive counts on the filter were measured in a liquid scintillation counter and the amount of DNA synthesized (pmol of nucleotides) was calculated, as described previously [35].

Filter-binding assay for ATP binding to DnaA

DnaA was incubated with [α -³²P]ATP at 4 °C for 20 min in 40 μl of buffer G [50 mM Hepes/KOH, pH 7.6, 0.5 mM magnesium acetate, 0.3 mM EDTA, 5 mM DTT, 17% (v/v) glycerol, 10 mM ammonium sulphate, and 0.005% (v/v) Triton X-100]. Samples were filtered onto nitrocellulose membranes (Millipore HA,

0.45 μm) and washed with ice-cold buffer G. The radioactivity remaining on the filter was counted with a liquid scintillation counter.

Filter-binding assay for *oriC* binding to DnaA

Specific 294 bp DNA fragments containing *oriC* were synthesized by PCR using a template (pBSoriC) [38] and primers ACCTGGGATCCTTGGGTATTA and ATGTGGATAACTCTGTCA-GG [39]. DNA fragments were radiolabelled by T4 polynucleotide kinase and [γ -³²P]ATP. The specific activity of each probe was 10000 c.p.m./fmol DNA. Binding of DnaA to *oriC* DNA fragments (20 fmol) was measured by a filter-binding assay. DnaA and DNA were incubated for 5 min at 37 °C in buffer G with 5 mM ATP. Samples were filtered through Millipore HA, 0.45 μm nitrocellulose membranes (Millipore, Bedford, MA, U.S.A.), and the retained radioactivity was counted in a liquid scintillation counter.

Assay for generation of *oriC* sites sensitive to P1-nuclease

Generation of *oriC* sites sensitive to P1-nuclease was measured according to a modification of an original method described by Bramhill and Kornberg [4]. DnaA was incubated with 45 fmol of pTB101 [40] and 20 ng of HU protein in 16 μl of buffer [60 mM Hepes/KOH, pH 8.0, 320 $\mu\text{g/ml}$ BSA, 8 mM magnesium acetate, 30% (v/v) glycerol, and 5 mM ATP], for 5 min at 37 °C. P1-nuclease (3 units) was added and the P1-cleavage reaction was terminated after 1 min by adding 20 μl of 50 mM EDTA and 1% (w/v) SDS. Samples were separated by agarose (0.8%, w/v) gel electrophoresis and stained with ethidium bromide.

Glutathione-beads-precipitation assay (GST pull-down assay)

DnaA and GST-DnaA were incubated at 37 °C for 5 min in 40 μl of buffer G containing 1 mM ATP. The mixture was diluted and 40 μl of glutathione-agarose beads (Sigma, 50% slurry) was then added. After 2 h rotation at 4 °C and three washes with the same buffer, the precipitates were suspended with SDS-sample buffer and separated by SDS/PAGE (7.5% gel), followed by immunoblotting with anti-DnaA polyclonal antibody.

RESULTS AND DISCUSSION

Activities of mutant DnaA proteins for *oriC* DNA replication *in vivo* and *in vitro*

In addition to a leucine-zipper-like structure (I26, L33 and L40), which was shown to be important for the duplex opening at *oriC in vitro* [30], we have found three other hydrophobic amino acid residues in the N-terminal region of DnaA (L3, L10 and L17) which may form this structure (Figure 1). These amino acid residues are well conserved among various species of DnaA as hydrophobic amino acid residues (Figure 1). Furthermore, these two leucine-zipper-like structures (six conserved hydrophobic amino acid residues) are included in the N-terminal DnaA fragment (1–86 amino acid residues of DnaA), which was shown to be necessary and sufficient for the DnaA–DnaA interaction [31].

We constructed four mutant *dnaA* genes by replacing these leucine residues with the hydrophilic amino acid residue, serine, using site-directed mutation (Figure 1). To determine whether these mutant *dnaA* genes could function *in vivo*, we conducted plasmid complementation experiments using a *dnaA508* mutant. The coding region of the mutant or the wild-type *dnaA* genes was conjugated with the wild-type *dnaA* promoter in pMZ002 [13].

Figure 1 Amino acid sequence and strategy for site-directed mutations in the N-terminal region of DnaA

Amino acid sequences of the N-terminal regions of DnaA (from M1 to W50) are shown. The * indicates an amino acid residue that is identical to that found in *E. coli*. These data are from [1]. eco, *E. coli*; sty, *Salmonella typhimurium*; sma, *Serratia marcescens*; pmi, *Proteus mirabilis*; bap, *Buchnera aphidicola*; ppu, *Pseudomonas putida*; sco, *Streptomyces coelicolor*; mlu, *Micrococcus luteus*; bsu, *Bacillus subtilis*; bbu, *Borrelia burgdorferi*. The conversion of amino acid residues in the mutant DnaA proteins is shown.

Table 1 Complementation analysis of temperature-sensitivity of a *dnaA508* mutant with plasmids carrying the mutant *dnaA* genes

KS1007 (*dnaA508*) cells were transformed with pMZ002 with *dnaA*⁺ (wild-type), *dnaA425*, *dnaA426*, *dnaA427*, or *dnaA413*. Cultures were diluted appropriately and spread on Luria–Bertani agar plates containing 100 µg/ml ampicillin. Plates were incubated at 30 °C or 42 °C, the number of colonies was counted and the ratio of transformation efficiencies (42 °C/30 °C) was determined.

Plasmid-borne allele	Transformation efficiency (42 °C/30 °C)
None	1.2×10^{-5}
<i>dnaA</i> ⁺	1.0
<i>dnaA425</i>	0.9
<i>dnaA426</i>	1.0
<i>dnaA427</i>	1.7×10^{-4}
<i>dnaA413</i>	1.0×10^{-5}

Each resultant plasmid was introduced into a high-temperature-sensitive *dnaA508* mutant (KS1003), which grows at 30 °C but not at 42 °C. As shown in Table 1, the ratio of transformation efficiency of *dnaA413* and *dnaA427* at 42 °C to that at 30 °C was less than 0.001, whereas that of *dnaA425*, *dnaA426* and the wild-type *dnaA* was approx. 1.0. These results suggest that DnaA413 and DnaA427 are inactive for *oriC* DNA replication *in vivo*. Since *dnaA425* and *dnaA426* were active *in vivo*, L3 and L10 seem not to be important for the function of DnaA. However, because the ratio of transformation efficiency of *dnaA413* at 42 °C to that

at 30 °C was less than 10 % of that of *dnaA427*, these amino acid residues may play an auxiliary role in DNA replication in cells.

We purified both DnaA413 and DnaA427 from overproducing cells and examined their activities for *oriC* DNA replication *in vitro*, using an *oriC* complementation assay [35]. As shown in Figure 2, DNA replication was lower with DnaA413 and DnaA427 than with the wild-type protein. The specific activities of these mutant proteins were less than one-tenth of that of the wild-type protein. DnaA A184V, DnaA46 and DnaA5 required longer incubation periods for expression of their replication activity [41–43]. In the case of DnaA413 and DnaA427, the time course of DNA replication was approx. linear as in the case of the wild-type protein (results not shown). These results demonstrate that DnaA413 and DnaA427 are inactive for *oriC* replication *in vitro*.

Activities of mutant DnaA proteins for the duplex opening at *oriC*

The duplex opening activity of DnaA can be monitored by generation of sites at *oriC* that are sensitive to P1-nuclease [4]. In these experiments we have compared the ability of DnaA413 and DnaA427 to open up the DNA duplex. As shown in Figure 3, the generation of *oriC* sites sensitive to P1-nuclease (appearance of linear DNA) was observed with the wild-type DnaA protein but not with DnaA413 or DnaA427, suggesting that neither of the mutant proteins could open up the duplex DNA at *oriC*. The appearance of linear DNA with the wild-type DnaA was lower than that previously reported [4,6]. This may be due to the background of experiments (without DnaA) being much lower

Figure 2 Replication activities of mutant DnaA proteins in a crude extract

DNA replication activities of mutant proteins DnaA427, DnaA413, and the wild-type DnaA (DnaA⁺) were measured using the *oriC* DNA replication system of a crude extract. DNA replication was expressed as the amount of DNA synthesized (pmol, nucleotides) at 30 °C in 20 min.

Figure 3 Activities of mutant DnaA proteins with respect to the DNA duplex opening at *oriC*

DnaA427, DnaA413, and the wild-type DnaA (DnaA⁺) were incubated with an *oriC* plasmid (pTB101) for 5 min at 37 °C and then incubated with P1-nuclease (3 units) for 1 min at 37 °C. Samples were subjected to agarose (0.8%) gel electrophoresis and stained with ethidium bromide (A). The ratio of linear DNA to total DNA was calculated based on densitometric scanning of the resulting bands (B).

Figure 4 *oriC*-binding activities of mutant DnaA proteins

DnaA427, DnaA413, and wild-type DnaA (DnaA⁺) were incubated with radiolabelled *oriC* DNA fragments (20 fmol) for 5 min at 37 °C. The amount of bound *oriC* was determined by a filter-binding assay.

than that previously reported [4,6]. When the wild-type DnaA was pre-incubated with ADP, the amount of linear DNA was decreased (results not shown), as described previously [6]. Based on these observations, we concluded that the low activity of DnaA413 and DnaA427 for *oriC* DNA replication *in vivo* (Table 1) and *in vitro* (Figure 2) is because of the inability of these proteins to open up the DNA duplex at *oriC*.

Activities of mutant DnaA proteins for binding to ATP or *oriC*

For the duplex opening at *oriC* by DnaA, not only *oriC*, but also ATP binding to DnaA is required [6]. Therefore, we examined the *oriC* and ATP binding activities of DnaA413 and DnaA427 using a filter-binding assay. As shown in Figure 4, DnaA413 and DnaA427 bound to *oriC* DNA fragments in a dose-dependent manner. Since the binding of these mutant DnaA proteins to labelled *oriC* DNA fragments was reduced by non-labelled *oriC* DNA fragments, but not by X174 DNA fragments (results not shown) that have no DnaA box (non-specific competitor), DnaA413 and DnaA427 bound to *oriC* DNA fragments in a sequence-specific manner under the conditions used in the experiments presented in Figure 4.

We also examined ATP-binding activities of DnaA413 and DnaA427. As shown in Figure 5, both mutant DnaA proteins showed ATP-binding activity. Scatchard-plot analysis of the data in Figure 5 gave the partition coefficient (K_d) value and the number of ATP-binding sites for each DnaA. The K_d values of DnaA413, DnaA427, and the wild-type protein for ATP were determined to be 120, 101 and 96 nM respectively. The numbers of ATP-binding sites per DnaA413, DnaA427, and the wild-type protein were calculated to be 0.28, 0.5, and 0.5, respectively. The K_d value and the number of ATP-binding sites of the wild-type protein were nearly the same as reported previously [23]. Since some DnaA mutants (such as DnaAR328E and DnaAR342E) showed DNA replication activity even with about half ATP-binding sites of the wild-type protein [23], we consider that the decrease in the ATP-binding sites of DnaA413 cannot explain the inability of this protein to initiate *oriC* DNA replication *in vivo* (Table 1) and *in vitro* (Figure 2).

Figure 5 ATP-binding activities of mutant DnaA proteins

DnaA427, DnaA413, and wild-type DnaA (DnaA⁺) (2 pmol of each) were incubated with indicated concentrations of [α -³²P]ATP for 20 min at 4 °C. The amount of bound ATP was determined by a filter-binding assay.

Figure 6 Activities of mutant DnaA proteins with respect to the DnaA–DnaA interaction

(A) Reaction mixtures, which contained the wild-type GST-DnaA (1 pmol), glutathione–agarose beads (40 μ l), or the wild-type DnaA (1 pmol) were incubated at 37 °C for 5 min as indicated. (B) Indicated amounts of DnaA427, DnaA413, and wild-type DnaA (DnaA⁺) were incubated with GST-DnaA (1 pmol) at 37 °C for 5 min. After precipitation with glutathione–agarose beads, the precipitates were subjected to SDS/PAGE (7.5% gel) followed by immunoblotting with anti-DnaA polyclonal antibody.

Activities of mutant DnaA proteins for the DnaA–DnaA interaction

The duplex opening reaction can be divided into at least three steps. The first step is the ATP binding to DnaA, since ADP·DnaA and adenine-nucleotide-free DnaA were inactive for the duplex opening [4,6]. The second step is the DnaA

binding to *oriC*. The third step is DnaA oligomerization at *oriC*, in which a DnaA–DnaA interaction is required. Since we showed that DnaA427 and DnaA413 were active for both ATP binding (Figure 5) and *oriC* binding (Figure 4), both mutant DnaA proteins appear to be able to perform the first and second steps. Therefore, it is reasonable to predict that both mutant DnaA proteins have a defect in the third step, DnaA oligomerization (DnaA–DnaA interaction), because both proteins were inactive for the duplex opening reaction (Figure 3).

In order to examine the direct DnaA–DnaA interaction, we developed a GST pull-down assay. In the GST pull-down experiments, near stoichiometric amounts of DnaA (wild-type or mutant) and GST-DnaA (wild-type) were incubated and the GST-DnaA was precipitated with glutathione–agarose beads. The presence of DnaA in the precipitates was determined by immunoblotting. The wild-type DnaA was precipitated with glutathione–agarose in a manner dependent on GST-DnaA (Figure 6A). The wild-type DnaA precipitation was dependent on glutathione–agarose beads (Figure 6A) and was not inhibited by addition of excess amounts of BSA (results not shown). GST alone did not pull-down DnaA (results not shown). Based on these results, we concluded that we could use the GST pull-down experiments to examine the specific DnaA–DnaA interaction. As shown in Figure 6(B), compared to the wild-type DnaA, less amounts of both DnaA427 and DnaA413 were precipitated with GST-DnaA. These results indicate that mutations in DnaA427 and DnaA413 affect the DnaA–DnaA interaction, suggesting that the conserved hydrophobic amino acid residues in the N-terminal region of DnaA (L3, L10 and L17) are involved in DnaA–DnaA interaction.

In the present study we examined the function of the N-terminal region of DnaA, using DnaA proteins with mutations in conserved hydrophobic amino acid residues (L3, L10 and L17). DnaA427 (L17S) and DnaA413 (L3S, L10S and L17S) were inactive for *oriC* DNA replication both *in vitro* and *in vivo*. Both of the mutant DnaA proteins were active for the binding to *oriC* and ATP but not for the opening of duplex DNA at *oriC*. Furthermore, GST pull-down experiments showed that both of the mutant proteins have a defect in the DnaA–DnaA interaction. Therefore, these conserved hydrophobic amino acid residues, especially L17, are important for the DnaA–DnaA interaction and DnaA oligomerization, which cause the duplex opening at *oriC*. Thus, results in this study support the idea that the N-terminal region of DnaA is involved in DnaA–DnaA interaction. The N-terminal region of other initiators of DNA replication, RepE and RepA, were shown to be essential for their self-interaction and for the function as the initiator of DNA replication [44,45]. Therefore, it seems that the N-terminal regions of initiators of DNA replication seem to be generally involved in their self-interaction.

As described above, Weigel et al. [31] reported that the N-terminal-deleted DnaA (87–467 amino acid residues of DnaA) showed a defect in DnaA–DnaA interaction and the N-terminal fragment of DnaA (1–77 amino acid residues of DnaA) was active for DnaA–DnaA interaction (Figure 7). On the other hand, Sutton et al. [28] reported that the N-terminal-deleted DnaA (63–467 or 130–467 amino acid residues of DnaA) could open-up the duplex opening at *oriC* (Figure 7). In the previous study [30], we showed that mutant DnaA proteins with mutations in the N-terminal region (DnaA422 and DnaA424) were inactive for the duplex opening at *oriC* (Figure 7). In the present study we showed that mutant DnaA proteins with mutations in the N-terminal region (DnaA427 or DnaA413) were not active for either DnaA–DnaA interaction or the duplex opening at *oriC* (Figure 7). It is very difficult to explain all of these results. One

		<i>in vitro</i> duplex opening	<i>in vitro</i> DnaA-DnaA interaction	reference
DnaA ⁺	1	+	+	
DnaA [63-467]	63	+	N.T.	[28]
DnaA [130-467]	130	+	N.T.	
DnaA [1-77]	77	N.T.	+	[31]
DnaA [87-467]	87	N.T.	-	
DnaA422	I26S L40S	-	N.T.	[30]
DnaA424		-	N.T.	
DnaA427	L17S L23S L33S L40S	-	-	This study
DnaA413		-	-	

Figure 7 Summary of DnaA proteins, which have deletions or mutations in the N-terminal region of DnaA

Information for DnaA proteins which have deletions or mutations in the N-terminal region of DnaA are from [28,30,31] and this study. The *in vitro* duplex opening activity was monitored by P1-nuclease assay [28,30, and this study]. The *in vitro* DnaA-DnaA interaction was monitored by an GST-pull down experiment (this study) or experiments using biotinylated DnaA and DnaA coupled to streptavidin coated magnetic beads [31]. N.T., not tested.

possible explanation for the discrepancy between the results of Sutton et al. [28] and Weigel et al. [31] is that the P1-nuclease assay is not as sensitive as the DnaA-DnaA interaction assay. When the most N-terminal residues of the N-terminal region are divided into two domains (1–50 and 50–100), we mutated in only the former domain whereas Sutton et al. [28] deleted both of these domains (Figure 7). The discrepancy between these results may be explained by this difference. It was reported that some mutations in the N-terminal region of DnaA (DnaA508) affect the tertiary structure of DnaA [31]. Therefore, one can consider that the mutations in our studies may have an effect on the tertiary structure of DnaA, resulting in the defect for the duplex opening. We showed here, however, that both DnaA413 and DnaA427 could bind to both ATP and *oriC*, suggesting that both proteins maintained the normal tertiary structure of DnaA.

We examined previously the role of other conserved hydrophobic amino acid residues (I26, L33 and L40) in the N-terminal region of DnaA [30]. DnaA proteins mutated in these amino acid residues were active for the binding to *oriC* but not for the opening of duplex DNA at *oriC* [30], as is the case of DnaA427 and DnaA413. Therefore, not only L3, L10 and L17, but also I26, L33 and L40, seem to be involved in the DnaA-DnaA interaction (DnaA oligomerization). The inhibition of DnaA-DnaA interaction by mutations in DnaA413 and DnaA427 was partial; both mutant DnaA proteins can weakly interact with GST-DnaA at higher concentrations (Figure 6). The involvement of I26, L33, and L40 in the DnaA-DnaA interaction may explain the residual activity of DnaA413 and DnaA427 for the DnaA-DnaA interaction.

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