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 *

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Tohru Mizushima‡‡, Tohru Takaki‡‡, Toshio Kubota‡‡, Tomofusa Tsuchiya‡‡, Takeyoshi Miki‡, Tsutomu Katayama‡‡, and Kazuhisa Sekimizu‡‡

From the ‡Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582 and the §Faculty of Pharmaceutical Sciences, Okayama University, Okayama 700-8530, Japan

DnaA protein, the initiator of chromosomal DNA replication in Escherichia coli, is activated by binding to ATP in vitro. We introduced site-directed mutations into two amino acids of the protein conserved among various ATP-binding proteins and examined functions of the mutated DnaA proteins, in vitro and in vivo. Both mutated DnaA proteins (Lys-178 → Ile or Asp-235 → Asn) lost the affinity for both ATP and ADP but did maintain binding activity for oriC. Specific activities in an oriC DNA replication system in vitro were less than one-tenth those of the wild-type protein. Analysis by site-directed mutation of Walker A or B motif, respectively, and examined functions of DnaA proteins, revealed a defect in induction of the replication origin in vitro. On the other hand, expression of each mutated DnaA protein in the temperature-sensitive dnaA46 mutant did not complement the temperature sensitivity. We suggest that Lys-178 and Asp-235 of DnaA protein are essential for the activity needed to initiate oriC DNA replication in vitro and in vivo and that ATP binding to DnaA protein is required for DNA replication-related functions.

Initiation of DNA replication, a key step in the regulation of cell proliferation, seems to be regulated by the control of activity of initiator proteins. DnaA protein, the initiator of chromosomal DNA replication in Escherichia coli (1–4), has a high affinity for adenine nucleotides; the ATP-binding form of DnaA protein is active, whereas the ADP-binding form is inactive in an oriC DNA replication system in vitro (5). Synthesized organic compounds designed to block the ATP binding to DnaA protein specifically inhibited oriC DNA replication in vitro (6). These observations mean that adenine nucleotide binding to DnaA protein may regulate the activity of the protein in vivo. There is genetic evidence to support the notion that initiation of DNA replication is regulated by adenine nucleotide binding to DnaA protein in vivo; (i) spontaneous dnaA46 and dnaA5 mutants show recessive lethality and DNA synthesis in dnaA46 and dnaA5 proteins have a decreased affinity for ATP and ADP (7, 8); (ii) a spontaneous dnaAcos mutant shows dominant lethality due to overinitiation of DNA replication and DnaAcos protein loses the affinity for ATP and ADP (9, 10); (iii) site-directed mutation (Glu-204 → Gln) also causes dominant lethality, and the mutated protein, DnaA E204Q, has decreased intrinsic ATPase activity (11).

DnaA protein has Walker A and B motifs that are common to a number of nucleotide-binding proteins (12). Analysis by site-directed mutation for conserved amino acids located in Walker A and B motifs of these nucleotide-binding proteins revealed the necessity of these amino acids for nucleotide binding and also for functions related to nucleotide-binding activity. Substitution of conserved amino acids located in Walker A and B motifs of SecA, an essential protein for protein translocation across cytoplasmic membranes in E. coli (13), showed that ATP binding and ATPase activity of this protein are essential for protein translocation, in vitro and in vivo (14–17). To obtain more direct evidence that the activity of DnaA protein is regulated by ATP binding, analysis by site-directed mutation of amino acids essential for the ATP-binding activity should be rewarding. We introduced site-directed mutations into two amino acids (Lys-178 or Asp-235) of DnaA protein located in Walker A or B motif, respectively, and examined functions of the mutated DnaA proteins in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials—Wild-type DnaA protein was purified, as described (18), but with some modifications (19–21). Specific activity of the protein was 0.3 × 10⁶ units/mg. Purity of the fraction exceeded 95%, as determined by SDS-polyacrylamide (10%) gel electrophoresis. A crude extract was prepared from WM433 strain as described (22, 23).

| a-32P|ATP (3000 Ci/mmol) and [3H]ADP (40 Ci/mmol) were purchased from Amersham Pharmacia Biotech and DuPont. P1 nuclease and trypsin were from Yamasa Co. and Worthington Biochemical Co., respectively.

‡‡ Bacterial Strains—JM109 (recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δlac-proAB/F′traD36, proAB+, lacIq, lacZAM15), KS1001 (W3110, lacZ), KS1003 (KS1001, dnaA46) (24, 25), and WM433 (dnaA46, leu19, pro19, trp25, his347, thyA59, arg82, met55, deoB23, lac11, strA56, sul1, hsdR12) were from our laboratory stock.

Site-directed Mutagenesis and Plasmid Construction—Site-specific mutation was carried out using the methods of Kunkel (26). In brief, uracil-containing single-stranded DNA of M13 phage, which contains the coding region of the dnaA gene was hybridized with the oligonucleotide primer, 5′-CCGGTCGTGATCGACTCACCTCTCAGTG-C3′, or 5′- CAAAACACTGTATATCQGATGACCTGACGTG-C3′, containing a mismatch sequence for replacement of Lys-178 with Ile or Asn, respectively (the changed bases are underlined). The complementary DNA strand was synthesized in vitro, and the resultant double-stranded DNA was introduced into JM109 cells. The mutation was confirmed by DNA sequencing, and double-stranded DNA (pMz1000-3 or pMz2000-4), with a mutation for replacement of Lys-178 with Ile or

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† To whom correspondence should be addressed: Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan. Tel.: 81-92-642-6641; Fax: 81-92-642-6646; E-mail: sekimizu@bisei.pharm.kyushu-u.ac.jp.

1 L. Guo, T. Miki, and K. Sekimizu, unpublished data.
Asp-235 with Asn, respectively, was prepared. For overproduction of the mutant DnaA protein, we used the pMZ001 plasmid (11), which contains the arabinose promoter (9). The EcoRI-HindIII region of pMZ000-3 or pMZ000-4 was ligated with pMZ001. The resultant plasmid was named pMZ001-3 or pMZ001-4, respectively, and used for overproduction of the mutant DnaA protein.

For analysis of the function of the mutant dnaA gene in vivo, we introduced the coding region of the mutant dnaA gene under the promoter of the wild-type dnaA gene. The BamHI-HindIII fragment of pMZ000-3 or pMZ000-4 was ligated to pMZ002 (11), which contains the wild-type promoter of the dnaA gene to construct pMZ002-3 (dnaA01) or pMZ002-4 (dnaA04), respectively.

**Filter-binding Assay for ATP and ADP Binding to DnaA Protein**—ATP- and ADP-binding activity of DnaA protein was determined by the 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, and 0.007% Triton X-100). Samples were passed through nitrocellulose membranes (Millipore HA, 0.45 mm) 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 sulfate, and 0.005% Triton X-100). The radioactivity remaining on the filters was counted in a liquid scintillation counter.

**RESULTS AND DISCUSSION**

**Construction and Purification of Mutant DnaA Proteins**—Yoshida and Amano (28) compared amino acid sequences of various ATP-binding proteins and predicted that Lys-178 and Asp-235 of DnaA protein, located in Walker A and B motifs, respectively, are essential for its ATP-binding activity (28). These residues for intrinsic ATPase activity of the protein (28). These described the function of the mutated DnaA proteins by immunoblotting with affinity-purified anti-DnaA serum (Table I). Since mutant DnaA proteins (DnaA K178I and DnaA D235N) were recovered in insoluble fractions after centrifugation of the cell lysate (data not shown), the mutant DnaA proteins were solubilized with gua-

**TABLE I**

| Fraction | Total protein | DnaA protein | Yield |
|----------|--------------|--------------|-------|
|          | mg           | mg           | %     |
| DnaA K178I |              |              |       |
| I. Insoluble | 8            | 1.0          | 100   |
| II. Guanidine | 2.4          | 0.8          | 80    |
| III. Superose gel | 0.15        | 0.13         | 13    |
| DnaA D235N |              |              |       |
| I. Insoluble | 9            | 0.9          | 100   |
| II. Guanidine | 2.3          | 0.8          | 89    |
| III. Superose gel | 0.15        | 0.12         | 13    |

**Strain KA450 transformed with pMZ001–3 or pMZ001–4 was grown in 20 liters of LB medium containing 25 μg/ml thymine at 37 °C until the optical density at 595 nm reached 0.5, then arabinose was added to a level of 1%. After 1 h of incubation at 37 °C, the cells were harvested by centrifugation, resuspended in buffer C (18) containing 250 mM KCl to an optical density at 595 nm of 220, and stored at −80 °C. Thawed cell suspension was diluted 2-fold with buffer C containing 250 mM KCl, and spermidine-HCl and egg white lysozyme were added to the final concentrations of 20 mM and 400 μg/ml, respectively. After incubation at 0 °C for 30 min, the sample was frozen in liquid nitrogen. The lysate was thawed and centrifuged for 30 min at 20,000 rpm in a Beckman TL100.3 rotor. The precipitates (Fraction I) were washed twice with buffer C containing 250 mM KCl and finally resuspended in buffer C containing 4 mM spermidine-HCl, 250 mM KCl. Insoluble materials were removed by centrifugation for 30 min at 50,000 rpm in a rotor Beckman TLA100.3. The supernatant (Fraction II) was gel-filtered on a Superose 12 column (Pharmacia fast protein liquid chromatography HR10/30) equilibrated with buffer D (18) at a flow rate of 0.5 ml/min, and DnaA fractions were pooled (Fraction III). Through purification, DnaA protein was monitored and determined by immunoblotting (18).

**FIG. 1**

**Isolation of DnaA K178I and D235N.** Protein fractions from Table I were applied on SDS-polyacrylamide gel electrophoresis, stained with Coomassie Brilliant Blue R-250. A, DnaA K178I; B, DnaA D235N.
FIG. 2. ATP and ADP binding to DnaA K178I and D235N, measured by filter-binding assay. DnaA K178I and DnaA D235N or the wild-type DnaA protein (2 pmol) was incubated with various concentrations of [α-32P]ATP (A) or [3H]ADP (B) for 15 min at 0 °C. The amount of bound ATP or ADP was determined by filter-binding assay, as described under “Experimental Procedures.”

vidine HCl, followed by gel filtration column chromatography. Aggregated and monomer forms of the mutant proteins were recovered (data not shown) as in the case of the wild-type DnaA protein (18). The monomer form of DnaA K178I and DnaA D235N was purified to apparent homogeneity, with a 13% recovery (Table I). Purity of each final fraction (fraction III) exceeded 90%, as determined by SDS-polyacrylamide gel electrophoresis (Fig. 1).

Characterization of ATP- and ADP-binding Activity of DnaA K178I and DnaA D235N—DnaA protein (DnaA K178I, DnaA D235N, or the wild-type protein) and various concentrations of [α-32P]ATP were incubated, and the amount of ATP bound to the protein was determined by filter-binding assay (5). As shown in Fig. 2A, the mutant DnaA proteins were defective in high affinity binding to ATP; even in the presence of 1 μM ATP, a negligible amount of ATP bound to the mutant DnaA proteins. As for the wild-type DnaA protein, the Kd value of the protein for ATP was 30 nM, a value much the same as noted earlier (5). We also examined high affinity binding of the mutant DnaA proteins for ADP. As shown in Fig. 2B, both mutant DnaA proteins were inert for the high affinity binding for ADP. The Kd value of the binding of ADP to the wild-type DnaA protein was 100 nM, that is much the same as reported elsewhere (5).

Since the low affinity binding of ATP to DnaA protein could not be detected by the filter-binding assay we used, the observations described in Fig. 2 did not exclude the possibility that mutant DnaA protein can bind to ATP or ADP in the presence of high concentrations of ATP or ADP. Since ATP concentrations in cells and in an oriC DNA replication system in vitro are relatively high (more than 1 mM), ATP binding of these proteins should be examined in the presence of high concentrations of ATP. We investigated the ATP- or ADP-binding activity of the mutant DnaA proteins (DnaA K178I and DnaA D235N) by the ATP- or ADP-dependent formation of trypsin-resistant peptide (27, 29). DnaA K178I, DnaA D235N, or the wild-type protein was preincubated with various concentrations of ATP or ADP at 4 °C and then further incubated with trypsin. Limited trypsinolysis of the wild-type DnaA protein produced a predominant 30-kDa peptide, in an ATP- or ADP-dependent manner (Fig. 3), much the same as noted elsewhere (27, 29). In the case of DnaA K178I and DnaA D235N, the 30-kDa peptide was not detected, even in the presence of 2 mM ATP or ADP (Fig. 3). Therefore, the mutant DnaA proteins apparently could not bind to ATP or ADP, even in the presence of high concentrations of ATP or ADP.

Replication Activity of DnaA K178I and DnaA D235N in Vitro—We measured the replication activity of DnaA K178I and DnaA D235N in an oriC complementation assay (22, 23). As shown in Fig. 4, DnaA K178I and DnaA D235N were less active than the wild-type protein for DNA replication. The specific activity of these mutant proteins was less than one-tenth that of the wild-type protein. DnaA A184V, DnaA46, and DnaA5 required longer incubation periods for expression of replication activity; the time lag for DNA replication reaction for these mutant DnaA proteins has been reported (16, 17, 27).

In the case of DnaA K178I and DnaA D235N, the time course of DNA replication is approximately linear, as is the case for the wild-type protein (Fig. 4B). Preincubation of the wild-type DnaA protein with 1 μM ADP (but not the mutant proteins) inhibited replication activity (Fig. 4A), findings consistent with our observations that these mutant proteins cannot bind to ADP (Figs. 2 and 3). Therefore, these amino acids (Lys-178 and Asp-235) are apparently needed for replication activity of DnaA protein in vitro.

oriC-binding Activity of DnaA K178I and DnaA D235N—DnaA protein, which specifically binds to the oriC sequence, causes duplex opening (4). The binding of ATP or ADP to wild-type DnaA protein does not affect binding to oriC (5). When we examined oriC-binding activity of the mutant DnaA proteins by filter-binding assay (20), the binding activity of DnaA K178I and DnaA D235N was indistinguishable from that of the wild-type protein, as shown in Fig. 5. Binding of the mutant DnaA proteins to the oriC DNA was competed for by nonlabeled or X174 DNA but not by dX174 DNA (data not shown), which has no DnaA box, as in the case of the wild-type DnaA protein. Thus, mutant DnaA proteins can specifically bind to oriC DNA. These observations suggest that mutation (K178I or D235N) in the ATP-binding domain does not affect the oriC-binding activity of DnaA protein. These results were also interpreted to mean that the mutation (K178I or D235N) specifically affects the ATP-binding activity of DnaA protein, the result being low replication activity of the proteins.

![Figure 2](image2.png)

![Figure 3](image3.png)
FIG. 4. Replication activity of DnaA K178I and DnaA D235N in a crude extract. K178I, D235N, or the wild-type DnaA protein (A; indicated amounts; B: DnaA*, 1 pmol; DnaA K178I and DnaA D235N, 1.5 pmol) was incubated with 1 μM ATP (A, B) or ADP (A) for 15 min at 0 °C. DNA replication in a crude extract was done (A, 20 min; B, indicated periods), as described under “Experimental Procedures.”

FIG. 5. oriC-binding activity of DnaA K178I and D235N. K178I, D235N, or the wild-type DnaA protein was incubated for 5 min at 30 °C with 25 fmol (10 000 cpm) of 3₂-end-labeled pBSorC (4). Samples were passed through membranes (Millipore, HAWP), and the retained radioactivity was counted. The amount of bound DNA to DnaA protein was determined as described (10).

Activity of DnaA K178I and DnaA D235N for Duplex Opening at oriC—Because our observations suggested that the mutant DnaA proteins showed low replication activity as a result of loss of the affinity for adenine nucleotides we asked which stage of DNA replication would defects in the mutant DnaA (DnaA K178I and DnaA D235N) be evident. It was reported that the ATP- but not ADP-binding form of DnaA protein is active for duplex opening, as monitored by generation of a sensitive site at oriC to P1 nuclease (4, 5). We compared the potential of mutant DnaA proteins to that of the wild-type protein with regard to duplex opening. As shown in Table II, generation of oriC sites sensitive to P1 nuclease (proportion of linear molecules) was stimulated by adding wild-type DnaA protein but not by exposure to DnaA K178I and DnaA D235N, which suggested that these mutant proteins are not active in duplex opening at oriC. When the wild-type DnaA protein was preincubated with ADP, the generation of oriC sites sensitive to P1 nuclease was not stimulated by the protein (Table II), as described (5). Preincubation of the mutant DnaA proteins with ADP did not alter their potential for generation of oriC sites sensitive to P1 nuclease (Table II). Based on these observations, we attribute the low activity of the mutant proteins (DnaA K178I and DnaA D235N) for DNA replication in vitro to their lack of potential for duplex opening at oriC. Thus, the thesis that the ATP binding to DnaA protein is required for duplex opening at oriC is given support.

Activity of DnaA K178I and DnaA D235N for DNA Replication in Vivo—To examine functions of these amino acids (Lys-178 and Asp-235) of DnaA protein in vivo, we did plasmid complementation experiments with the mutant dnaA genes (dnaA401 and dnaA402), using a temperature-sensitive dnaA46 mutant. Since results from biochemical studies on DnaA K178I and DnaA D235N revealed that both mutant proteins lose the affinity for ATP, examination of the activity of the mutant DnaA proteins in cells may yield important information on the physiological roles of ATP-binding activity of DnaA protein. The coding region of dnaA401 (DnaA K178I), dnaA402 (DnaA D235N), or the wild-type dnaA gene was conjugated with the wild-type dnaA promoter on pMZ002 (11), and each resultant plasmid was introduced into a temperature-sensitive dnaA46 mutant (KS1003) followed by incubation at 42 °C or 30 °C. As shown in Table III, the ratio of transformation efficiencies at 42 °C to that at 30 °C of pMZ002-3 (dnaA401) or pMZ002-4 (dnaA402) was less than 1/10⁵, whereas that of pMZ002-2 (wild-type) (11) was 0.65. We confirmed that approximately the same amount of the mutant and wild-type DnaA proteins was expressed in the KA450 strain (9), as based on immunoblotting analysis (data not shown). These results suggest that DnaA K178I and DnaA D235N cannot initiate DNA replication at 42 °C in the dnaA46 mutant and that Lys-178 and Asp-235 of DnaA protein are essential for activity for DNA replication in cells. The ATP-binding capacity...
of DnaA protein is apparently essential for replication activity of DnaA protein.

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