DnaA protein, the initiator of chromosomal DNA replication in *Escherichia coli*, seems to be regulated through its binding to acidic phospholipids, such as cardiolipin. In our previous paper (Hase, M., Yoshimi, T., Ishikawa, Y., Ohba, A., Guo, L., Mima, S., Makise, M., Yamaguchi, Y., Tsuchiya, T., and Mizushima, T. (1998) *J. Biol. Chem.* 273, 28651–28656), we found that mutant DnaA protein (DnaA431), in which three basic amino acids (Arg<sup>360</sup>, Arg<sup>364</sup>, and Lys<sup>372</sup>) were mutated to acidic amino acids showed a decreased ability to interact with cardiolipin in vitro, suggesting that DnaA protein binds to cardiolipin through an ionic interaction. In this study, we construct three mutant dnaA genes each with a single mutation and examined the function of the mutant proteins in vitro and in vivo. All mutant proteins maintained activities for DNA replication and ATP binding. A mutant protein in which Lys<sup>372</sup> was mutated to Glu showed the weakest interaction with cardiolipin among these three mutant proteins. Thus, Lys<sup>372</sup> seems to play an important role in the interaction between DnaA protein and acidic phospholipids. Plasmid complementation analyses revealed that all these mutant proteins, including DnaA431 could function as an initiator for chromosomal DNA replication in vivo.

DnaA protein, the initiator of chromosomal DNA replication in *Escherichia coli*, specifically binds to the origin of chromosomal DNA, forms oligomers to open up the duplex DNA, and recruits DnaB protein (DNA helicase) (1). The regulation of the activity of DnaA protein, which plays a major role in the control of DNA replication in cells, seems to be mediated by adenine nucleotides bound to DnaA protein. DnaA protein has a high affinity for ATP and ADP and the ATP binding form is active (2). Recent biochemical and genetic studies suggest that the regulation mechanism of DnaA protein is as follows. ATP-bound DnaA protein causes the duplex opening of DNA in order to initiate DNA replication (2–5). After initiation of DNA replication, DnaA protein is inactivated to the ADP bound form by stimulating the exchange reaction of ATP with ADP (11–14). Some genetic evidence supports the idea that DnaA protein is activated by acidic phospholipids to initiate DNA replication in vivo (15–19). In order to better understand how DnaA protein interacts with phospholipids and how it is involved in the regulation of DNA replication, identification of the amino acids of DnaA protein that are involved in membrane binding is important. A potential amphipathic helix (from Asp<sup>357</sup> to Val<sup>374</sup>) was suggested to be involved in the membrane binding of DnaA protein (20, 21). We recently reported that mutant DnaA protein (DnaA431), in which three basic amino acids in the helix (Arg<sup>360</sup>, Arg<sup>364</sup>, and Lys<sup>372</sup>) were mutated to acidic amino acids, had a decreased ability to interact with CL (22), suggesting that this amphipathic helix region is a membrane-binding domain of DnaA protein and that the functional interaction between DnaA protein and acidic phospholipids is mediated by an ionic interaction. We also found that another potential amphipathic helix (from Lys<sup>327</sup> to Ile<sup>345</sup>), which is located very close to the amphipathic helix from Asp<sup>357</sup> to Val<sup>374</sup> is also involved in membrane binding (23). In order to identify the important amino acids in the Asp<sup>357</sup>–Val<sup>374</sup> helix that are important for the interaction, we here construct three mutant dnaA genes each with a single mutation (R360E, R364E, and K372E) and examine the activities of mutant proteins in vitro and in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials—**A crude extract for an oriC complementation assay was prepared from the WM433 strain of *E. coli* as described previously (24). CL was purchased from Sigma. [α<sup>32</sup>P]ATP (10 mCi/mmol) and [3H]ADP (40 Ci/mmol) were purchased from Amersham Pharmacia Biotech. and DuPont, respectively. The mutant DnaA protein and the wild-type DnaA protein were purified, as described (22).

**Site-directed Mutagenesis and Plasmid Construction—**Site-specific mutation was performed using the method of Kunkel (25). In brief, uracil-containing single-stranded DNA of M13 phage, which contains the coding region of the dnaA gene, was hybridized with each of the

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*The abbreviations used are: CL, cardiolipin; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.*

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**Identification of Amino Acids Involved in the Functional Interaction between DnaA Protein and Acidic Phospholipids**

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oligonucleotide primers containing each mutation. 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 DNAs that contain the mutation were prepared.

For overproduction of the mutant DnaA protein, we used the pMZ001 plasmid (7), which contains the arabinose promoter. The EcoRI-HindIII regions of the double-stranded DNAs were ligated with pMZ001. The resultant plasmids were used for overproduction of the mutant DnaA proteins.

For analysis of the function of the mutant dnaA gene in vivo, we introduced the coding regions of the mutant dnaA genes under the promoter of the wild-type dnaA gene. The BamHII-HindIII fragments of the double-stranded DNAs were ligated to pMZ002, which contains the wild-type promoter of the dnaA gene (7).

**RESULTS AND DISCUSSION**

**Strategy for Site-directed Mutation and Purification of Mutant DnaA Proteins**—In our previous paper (22), we showed that mutant DnaA protein (DnaA431) with a triple substitution of Arg360, Arg364, and Lys372 of DnaA protein with Glu had a decreased ability to interact with CL. To determine which amino acid is important for the interaction, we constructed three mutant dnaA genes with single mutations as shown in Fig. 1. The coding region of each mutant dnaA gene was conjugated with the promoter of the arabinose operon to construct a plasmid for overproduction of the mutated DnaA protein (DnaAR360E, DnaAR364E, DnaAK372E, or DnaA431). To avoid contamination of the wild-type DnaA protein in the fraction of the mutant DnaA protein, the KA450 strain (oriC1071::Tn10, rnhA199(Am), dnaA17(Am), trpE9828(Am), tyrA(Am), thr, ilv, and thyA) was used as host cells for overproduction. The viability was not dependent on the function of DnaA protein in KA450. Addition of 1% arabinose caused overexpression of each mutant DnaA protein (data not shown).

Purification of each mutant DnaA protein was done as described previously (22). All mutant DnaA proteins were purified to homogeneity (Fig. 2) with approximately the same recoveries (7–9%). The migration of DnaA431 was a little slower than other proteins (Fig. 2) and the wild-type protein (data not shown), as reported previously (22).

**Characterization of ATP and ADP Binding Activity of the Mutant DnaA Proteins**—The functional interaction of DnaA protein with acidic phospholipids was estimated by acidic phospholipid-dependent stimulation of the release of ATP (or ADP) from DnaA protein (11). The activation of the ADP binding form of DnaA protein by acidic phospholipids is mediated by this function of acidic phospholipids (11). Thus, it is necessary for the mutant DnaA proteins to maintain their ATP binding activities in order to examine their functional interaction with acidic phospholipids. The ATP binding activities of these mutant proteins were examined by a filter binding assay (2) and a Scatchard plot analysis. As shown in Fig. 3, each mutant DnaA protein showed nearly the same ATP binding activity as the wild-type protein. The \( K_d \) values of DnaAR360E, DnaAR364E, DnaAK372E, and the wild-type protein for ATP were determined to be 56, 82, 84, 87, and 66 nM, respectively. The \( K_d \) values of DnaAR360E, DnaAR364E, DnaAK372E, or DnaA431. To avoid contamination of the wild-type DnaA protein in the fraction of the mutant DnaA protein, the KA450 strain (oriC1071::Tn10, rnhA199(Am), dnaA17(Am), trpE9828(Am), tyrA(Am), thr, ilv, and thyA) was used as host cells for overproduction. The viability was not dependent on the function of DnaA protein in KA450. Addition of 1% arabinose caused overexpression of each mutant DnaA protein (data not shown).

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DnaA431, and the wild-type protein for ADP were determined to be 121, 151, 137, 84, and 127 nM, respectively. The numbers of ADP-binding sites per DnaAR360E, DnaAR364E, DnaAK372E, DnaA431, and the wild-type proteins were calculated to be 0.52, 0.88, 1.00, 0.48, and 0.90, respectively. The $K_d$ value and the number of ADP-binding sites of the wild-type protein were nearly the same as reported previously (2). These results enabled us to examine their functional interaction with acidic phospholipids. The results also suggest that these amino acids are not necessary for the adenine-nucleotide binding activity of DnaA protein and that these mutations do not drastically affect the higher order structure of DnaA protein.

Replication Activity of the Mutant DnaA Proteins in Vitro—

We measured the activities of DnaAR360E, DnaAR364E, DnaAK372E, DnaA431, and the wild-type proteins for initiation of DNA replication in an oriC complementation assay in a

![Figure 3](image1)

**FIG. 3.** ATP binding to mutant DnaA proteins measured by a filter binding assay. DnaAR360E, DnaAR364E, DnaAK372E, DnaA431, and the wild-type proteins were incubated with various concentrations of [$\alpha$-32P]ATP for 15 min at 0 °C. The amount of bound ATP was determined, as described under “Experimental Procedures” and analyzed by the Scatchard plot method.

![Figure 4](image2)

**FIG. 4.** ADP binding to mutant DnaA protein measured by a filter binding assay. DnaAR360E, DnaAR364E, DnaAK372E, DnaA431, and the wild-type proteins were incubated with various concentrations of [3H]ADP for 15 min at 0 °C. The amount of bound ADP was determined, as described under “Experimental Procedures” and analyzed by the Scatchard plot method.
as shown in Fig. 5, all the proteins supported the oriC DNA replication in vitro. The specific activities of DnaAR360E, DnaAR364E, DnaAK372E, DnaA431, and the wild-type protein were 0.38, 0.20, 0.24, 0.19, and $0.41 \times 10^6$ units/mg of protein (1 unit of protein promotes the incorporation of 1 pmol of nucleotides/min at 30°C). DnaAA184V, DnaAR360E, DnaAR364E, DnaAK372E, and the wild-type protein were incubated with 1 μM ATP for 15 min at 0°C. DNA replication in a crude extract was done for 20 min as described under “Experimental Procedures.” ■ DnaA+; □, DnaAR360E; ○, DnaAR364E; △, DnaAK372E; ◦, DnaA431.
DnaA46, and DnaA5 required longer incubation periods for expression of their replication activity; a time lag for the DNA replication reaction has been previously reported for these mutant DnaA proteins (3). In the case of these mutants, the time course of DNA replication was approximately linear as is the case of the wild-type protein (data not shown). Preincubation with each of the mutant proteins with 1 μM ADP inhibited the replication activity (data not shown) as is the case of the wild-type protein (2). These results suggest that Arg260, Arg264, and Lys372 of the DnaA protein are not essential for its activity for DNA replication and provide further evidence that these mutations do not drastically affect the higher order structure of DnaA protein.

Functional Interaction of the Mutant DnaA Proteins with CL—To determine which mutation in DnaA431 is responsible for its decreased ability to interact with CL, the effects of CL on the release of ATP from DnaAR360E, DnaAR364E, DnaAK372E, DnaA431, and the wild-type protein were examined. DnaA protein bound to [γ-32P]ATP was incubated with CL at 37 °C and the remaining ATP was determined by a filter binding assay. As shown in Fig. 6, the rates of release of ATP from the mutant proteins in the absence of CL were nearly the same as that of the wild-type protein, suggesting that the Kd value for ATP of DnaA protein was not significantly affected by these mutations, as described above. On the other hand, the release of ATP in the presence of CL was affected by the mutations. CL greatly stimulated the release of ATP from the wild-type but not from DnaA431 protein (Fig. 6), as reported previously (22). Among these three mutant proteins with a single mutation, DnaAK372E showed the lowest stimulation of the ATP release by CL (Fig. 6). The stimulation by CL of the ATP release from DnaAR364E was a little lower than the wild-type protein, whereas that from DnaAR360E was approximately the same as that of the wild-type protein (Fig. 6). The kapp (apparent rate constants) can be calculated from the slope (Fig. 6). The kapp values for DnaAR360E, DnaAR364E, DnaAK372E, DnaA431, and the wild-type protein in the presence of CL were 4.3 × 10−3, 2.9 × 10−3, 1.2 × 10−3, 1.6 × 10−3, 5.0 × 10−3 (s−1), respectively. Thus, K372E of DnaA protein is mostly responsible for the decreased activity of DnaA431 to interact with CL. Lys372 of DnaA protein seems to be important for its interaction with CL, being consistent with the previous result that proteolysis at Lys372 by protease was inhibited by acidic phospholipids (20).

In Vivo Activity of Mutant DnaA Proteins—The results described above suggest that DnaA431 and DnaA K372E have a decreased ability to interact with CL in vitro. Thus, it is interesting to know whether these mutant DnaA proteins can function in vivo as an initiator protein for DNA replication. We used a plasmid complementation method using temperature-sensitive dnaA mutants to address this question. The coding regions of these mutant and wild-type dnaA genes were conjugated with the wild-type dnaA promoter on pMZ002 (7). Each resultant plasmid was introduced into a high temperature-sensitive (100 °C) strain (KS1003) (28), which has mutations in the dnaA46, dnaA508 mutant (KS1007) (28), which has a mutation in the N-terminal region of DnaA protein. The results were similar to those with the dnaA46 mutant (data not shown). Since pMZ002 is a high copy number plasmid (a derivative of pBR322) (7), there was a possibility that overproduction of these mutant proteins suppressed their disability to initiate DNA replication in cells. However, this possibility may be unlikely, because these mutant dnaA genes in a low copy number plasmid (a derivative of mini-R plasmid) also could complement the temperature sensitivities of the dnaA46 and dnaA508 mutants (data not shown). Colony sizes and growth rates of the temperature-sensitive dnaA mutants carrying pMZ002 (or the low copy number plasmid) with dnaAK372E or dnaA431 were indistinguishable with those of cells carrying pMZ002 (or the low copy number plasmid) with the wild-type dnaA gene (data not shown). Thus, we consider that DnaA431 and DnaA K372E proteins, which have a decreased activity to interact with CL in vitro, are able to initiate DNA replication in cells. However, even in the case of the low copy number plasmid carrying the dnaAK372E (or dnaA431) gene, it is still possible that cell survival at 42 °C is mediated by K372E (or DnaA431)-DnaA46 or K372E-DnaA508 hybrid proteins. To address this question, one should create an E. coli carrying the dnaAK372E (or dnaA431) gene as the sole source of DnaA.

At present, we cannot use these data to rule against an essential role of DnaA membrane binding for its functioning in the cell or against an essential role of reactivation of ADP-bound DnaA protein by acidic phospholipids in regulating DNA replication. This is because it is possible that these mutant DnaA proteins can interact with membrane acidic phospholipids in vivo by the aide of other membrane components such as membrane proteins.

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Membrane Binding to DnaA Protein
Identification of Amino Acids Involved in the Functional Interaction between DnaA Protein and Acidic Phospholipids
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