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Effect of Glycerol on the Affinity of DnaA Protein for ATP in the Presence of Cardiolipin[1]

Masakazu Hase,* Yuichi Ishikawa,† Kazuhisa Sekimizu,* Tomofusa Tsuchiya,‡ and Tohru Mizushima‡,[2]

**Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8581; †Faculty of Engineering, Oita University, Oita 870-1192; and ‡Faculty of Pharmaceutical Sciences, Okayama University, Okayama 700-8530*

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Acidic phospholipids, such as cardiolipin, decrease the affinity of DnaA protein for adenine nucleotides and can activate the inactive form of DnaA protein *in vitro*. In this study, we examined the effect of glycerol on the affinity of DnaA protein for ATP in the presence of cardiolipin. High concentrations of glycerol (34%) restored the affinity of DnaA protein for ATP, which was decreased by cardiolipin. Glycerol inhibited the binding of cardiolipin with DnaA protein. Glycerol had little effect on membrane fluidity, which is essential for the interaction between cardiolipin and DnaA protein, whereas it increased the K_d value of DnaA protein for ATP in the absence of cardiolipin. These results suggest that glycerol causes DnaA protein to become insensitive as to the interaction with cardiolipin by changing the conformation of the protein without altering the physical nature of the phospholipid.

Key words: ATP-binding, cardiolipin, DNA replication, DnaA protein, glycerol.

DnaA protein, the initiator of DNA replication in *Escherichia coli*, specifically binds to *oriC*, the unique sequence for the initiation of chromosomal DNA replication, and causes duplex opening (*1-3*). DnaA protein exhibits a high affinity for ATP and ADP; the ATP-binding form is active in an *oriC* replication system *in vitro*, while the ADP-binding form is inactive (*4*). Synthesized organic compounds designed to block the ATP-binding to DnaA protein specifically inhibited *oriC* DNA replication *in vitro* (*5*). We recently showed that the induction of an artificially constructed mutant DnaA protein with decreased ATPase activity led to overinitiation of DNA replication in cells, resulting in a dominant lethal phenotype (*6*). These results suggest that adenine-nucleotides bound to DnaA protein regulate the initiation of chromosomal DNA replication in *E. coli* cells.

The replication of chromosomal DNA starts in coordination with cell division. A replicon model has been proposed to explain this coordination: DNA replication in bacterial cells starts on membranes, and the activity of the initiator protein for DNA replication is regulated by membrane components (*7*). The finding that DnaA protein interacts with membrane acidic phospholipids may provide biochemical support for the replicon model. Acidic phospholipids, in particular cardiolipin (CL), decrease the affinity of DnaA protein for adenine-nucleotides (*8-10*), and change the DnaA protein from the ADP-binding form to the ATP-binding form in the presence of high concentrations of ATP by stimulating the exchange of ADP with ATP (*8*). We reported that artificial membranes consisting of a mixture of acidic and basic phospholipids, which mimic biological membranes, can decrease the affinity of DnaA protein for adenine-nucleotides under the conditions under which acidic phospholipids form cluster structures (*11*). Thus, it was proposed that acidic phospholipids regulate the activity of DnaA protein in cells (*8-11*).

We recently reported that some temperature-sensitive *dnaA* mutants (*dnaA508* and *dnaA167* mutants) showed phenotypes that were sensitive to organic solvents, such as glycerol (*12*). Since organic solvents are known to affect the activities of some proteins bound to biological membranes (*13*), we proposed that organic solvents inhibit the interaction of DnaA protein with membrane phospholipids, and that mutations in the *dnaA* gene also affect the interaction, resulting in phenotypes of the *dnaA* mutants that are sensitive to organic solvents (*12*). In this study, we examined the effect of glycerol on the interaction of DnaA protein with CL. The results obtained suggest that high concentrations of glycerol affect the conformation of the protein so that it becomes less sensitive as to the interaction with CL.

EXPERIMENTAL PROCEDURES

Materials—DnaA protein was purified by the method described previously (*14*) except that a newly constructed overproducer was used (*11, 15, 16*). The specific activity of the protein was 0.7×10^6 units/mg. The purity of the fraction used exceeded 90%, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

CL was purchased from Sigma. [α-^{32}P]ATP (5 mCi/mmol) was obtained from Amersham.

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[2] To whom correspondence should be addressed. Tel/Fax: +81-86-251-7958, E-mail: mizushima@pheasant.pharm.okayama-u.ac.jp

Fluorescence Polarization (P Value)—Fluorescence polarization was determined as described (*11*). A stock solution of 1,6-diphenyl-1,3,5-hexatriene (1 mM) was prepared in THF (tetrahydrofurane). An aliquot of the THF solution (10 ml) was, after removal of the solvent, mixed with lipids (1 mM) in the presence of various concentrations of glycerol, and then the mixtures were lightly sonicated. The fluorescence intensity was monitored using a Hitachi F-4500 under conditions of excitation at 363 nm and emission at 434 nm. The P value was calculated with the commonly used equation (*11, 17*).

Order Parameter—This value was determined by using 5-doxyl-stearic acid as an ESR spin probe. The powdery stable radical was dissolved in deionized water by mixing with equimolar NaOH at room temperature. This aqueous solution of the amphiphilic spin probe (0.05 mM, 0.5 ml) was co-sonicated with an aqueous dispersion of CL yielding a [spin probe]/[CL] molar ratio of 1/300. ESR spectra for CL dispersions containing the spin probe at 30°C showed a typical anisotropic nitrooxide signal reflecting the perpendicular and parallel components of the g-values. By using the observed superhyperfine (shf) constants for the parallel and perpendicular components, $A_{//}$ and $A_{\perp}$, the order parameter was calculated according to the following ordinary equation:

$$(A_{//}-A_{\perp})/[A_{zz}-(A_{xx}+A_{yy})/2],$$

where A_{zz} (33.6 gauss), A_{yy} (5.8 gauss), and A_{xx} (6.3 gauss) are shf constants obtained from ESR spectra of the single crystal (*18*).

Influence of CL on Binding of ATP to DnaA Protein—DnaA protein and CL were mixed at 0°C for 15 min in 40 μl of buffer G [50 mM HEPES-KOH (pH 8.0 at 1 M), 0.5 mM magnesium acetate, 0.3 mM EDTA, 5 mM dithiothreitol, 10 mM ammonium sulfate, 17% (v/v) glycerol, and 0.005% Triton X-100]. [α-^{32}P]ATP (5 mCi/mmol) was then added to give a final concentration of 2 μM, followed by incubation at 0°C for 15 min. The solution was then passed through a membrane filter (Millipore) that had been presoaked in buffer G. The filter was washed with 6 ml of ice-cold buffer G and then dried under an infrared lamp. The radioactivity retained on the filter was measured with a liquid scintillation counter.

Influence of CL on the Release of ATP from the ATP-DnaA Complex—DnaA protein was pre-incubated with 2 μM [α-^{32}P]ATP (5 mCi/mmol) in 40 μl of buffer G at 4°C for 15 min. CL was added and then the mixture was incubated at 37°C, followed by passage through a nitrocellulose membrane filter. The radioactivity on the filter was then measured.

Measurement of the Interaction of CL with DnaA Protein—DnaA protein was incubated with CL in 40 μl of buffer G at 4°C for 15 min. Samples were centrifuged at 14,000 rpm for 30 min at 4°C (KUBOTA 1700) and the precipitate was suspended in the SDS-sample buffer and then subjected to SDS-polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

Effect of Glycerol on the Affinity of DnaA Protein for ATP in the Presence of CL—Acidic phospholipids, such as CL, interact with DnaA protein and decrease the affinity of

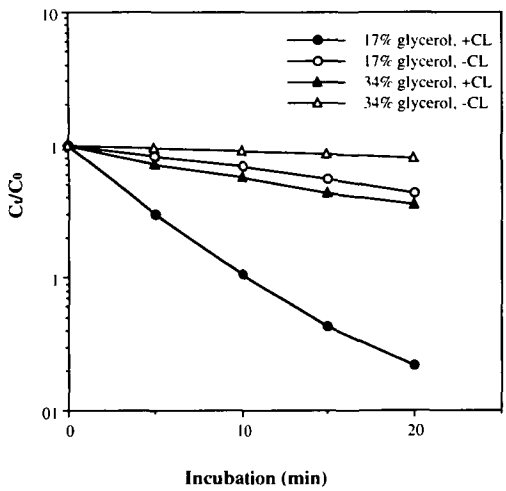

Fig. 2. **Influence of glycerol on the release of ATP from the DnaA-ATP complex in the presence and absence of CL.** The dissociation of ATP from the ATP-DnaA complex (2 pmol) in the presence of CL (0 or 3.75 μM) and glycerol (17 or 34%) at 37°C was examined, as described under "EXPERIMENTAL PROCEDURES." Ct and Co denote the concentrations of ATP-DnaA retained and of initial ATP-DnaA, respectively.

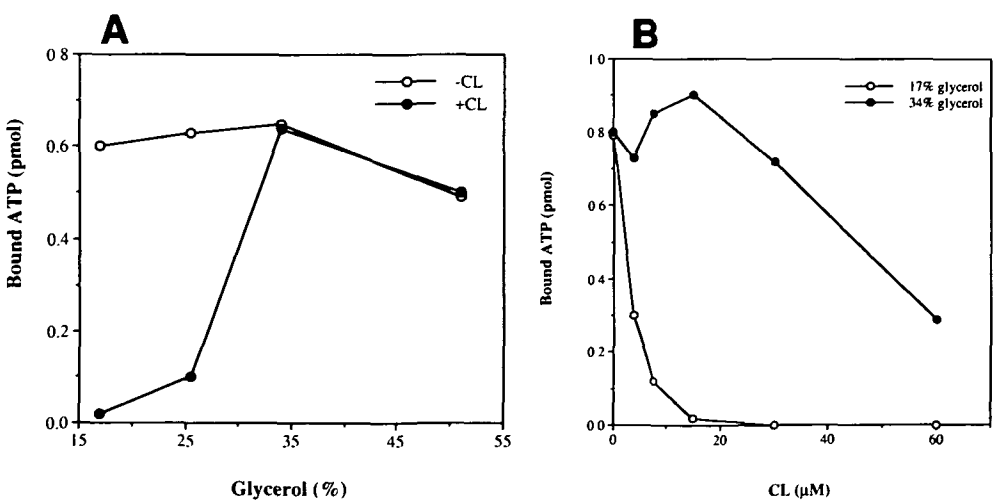

Fig. 1. **Influence of glycerol on the ATP binding activity of DnaA protein in the presence and absence of CL.** DnaA protein (2 pmol) was incubated with CL (A, 0 or 15 μM; B, at the indicated concentrations) in the presence of glycerol (A, at the indicated concentrations; B, 17 and 34%). The mixtures were further incubated with 2 μM [α-^{32}P]ATP (5 mCi/mmol). Bound ATP was determined as described under "EXPERIMENTAL PROCEDURES."

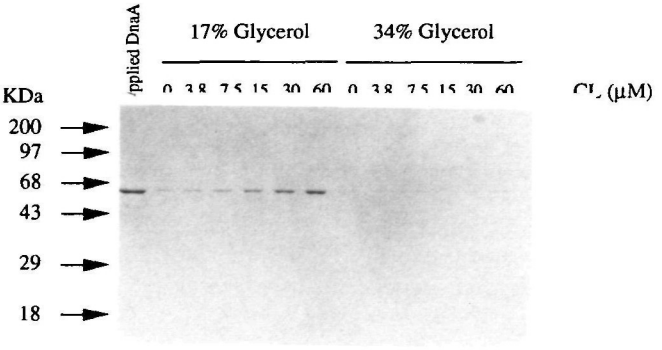

Fig. 3. **Influence of glycerol on the binding of CL with DnaA protein.** DnaA protein (5 pmol) was incubated with various concentrations of CL in the presence of 17 or 34% glycerol. Samples were centrifuged and the precipitates were subjected to SDS-polyacrylamide gel (10%) electrophoresis, followed by staining with Coomassie Brilliant Blue R-250.

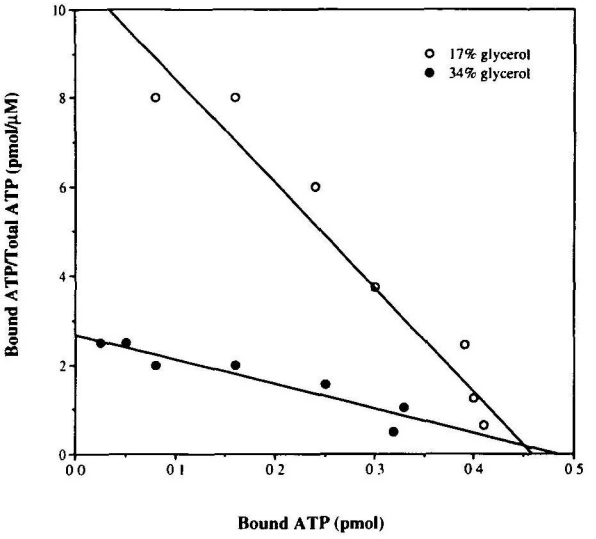

Fig. 4. **Influence of glycerol on the K_d value of DnaA protein for ATP.** DnaA protein (1 pmol) was incubated at 4°C with various concentrations of [α-^{32}P]ATP for 15 min in the presence of 17 or 34% glycerol. The amount of bound ATP was determined by filter-binding assay, as described under "EXPERIMENTAL PROCEDURES," and then a Scatchard plot was prepared.

TABLE I. **Effect of the glycerol on the fluidity of CL vesicles.** The P value and order parameter of CL vesicles in the presence of glycerol were determined as described under "EXPERIMENTAL PROCEDURES." The averages of duplicate measurements are given, and the deviation was less than 5%.

	Glycerol (%)		
	0	17	34
P value	0.08	0.07	0.10
Order parameter	0.629	0.666	0.675

DnaA protein for ATP; CL inhibits the binding of ATP to DnaA protein and stimulates the release of ATP from the ATP-DnaA complex (*8*). In this study, we examined the effect of glycerol on the ATP-binding and the ATP-release in the presence of CL. Since 17% (v/v) glycerol is necessary to protect DnaA protein from denaturation, we examined the effect of glycerol at concentrations higher than 17%.

CL (15 μM) completely inhibited the binding of ATP to DnaA protein as described previously (*8*), and glycerol (more than 34%) prevented the inhibition (Fig. 1A). Glycerol had little effect on the binding of ATP to DnaA protein in the absence of CL (Fig. 1A). Even in the presence of 30 μM CL, 34% (v/v) glycerol restored the ATP-binding activity of DnaA protein which had been inhibited by CL (Fig. 1B).

We further examined the effect of glycerol on the stimulation by CL of the release of ATP from the ATP-DnaA complex. DnaA protein was pre-incubated with [α-^{32}P]ATP to form the [α-^{32}P]ATP-DnaA complex, followed by incubation at 37°C with and without CL. The addition of CL (3.75 μM) accelerated the dissociation of ATP from the ATP-DnaA complex in the presence of 17% glycerol (Fig. 2), as described previously (*8*). The effect of CL was greatly reduced in the presence of 34% glycerol (Fig. 2). These results indicate that glycerol restores the high-affinity binding of DnaA protein to ATP, which is inhibited by CL.

Effect of Glycerol on the Binding of CL with DnaA Protein—The results described above suggest that the interaction of CL with DnaA protein is inhibited by high concentrations of glycerol, resulting in recovery of the high-affinity binding of DnaA protein to ATP in the presence of CL. To test this notion, we directly measured the binding of CL with DnaA protein in the presence of high concentrations of glycerol. DnaA protein was incubated with various concentrations of CL in the presence of 17 or 34% glycerol, and then the mixtures were centrifuged. The DnaA protein precipitated was determined by SDS-polyacrylamide gel electrophoresis, followed by densitometric scanning. As shown in Fig. 3, less DnaA protein was precipitated with CL in the presence of 34% glycerol than with 17% glycerol. Densitometric scanning revealed that 70% of DnaA protein was precipitated with 60 μM CL in the presence of 17% glycerol, whereas only 15% of DnaA protein was precipitated with 60 μM CL in the presence of 34% glycerol. This result supports the notion that the interaction of CL with DnaA protein is inhibited by high concentrations of glycerol.

Effect of Glycerol on Lipid Vesicles—Organic solvents are known to affect the physicochemical properties of phospholipid vesicles, such as membrane fluidity. It was reported that the interaction of phospholipid vesicles with DnaA protein depended on the high fluidity of the membranes (*10*). Thus, it was reasonable to postulate that the effect of glycerol on the interaction of DnaA protein with CL is due to its action on CL vesicles. To test this possibility, we examined the effect of glycerol (34%) on the fluidity and morphology of CL vesicles. The fluorescence polarization (P value) of 1,6-diphenyl-1,3,5-hexatriene incorporated into lipid vesicles reflects the membrane fluidity, especially for the hydrophobic center of the lipid assembly (*17*), since this fluorescence probe is quite hydrophobic. A higher P value means lower fluidity (*17*). As shown in Table I, the P value of CL vesicles was not significantly affected by the addition of glycerol under the present conditions.

In order to determine the change in fluidity around the membrane surface, we selected 5-doxyl-stearic acid as the ESR spin probe, whose radical moiety is fixed at the fifth carbon from the hydrophilic group. ESR measurement using the spin probe allowed us to determine the effect of

glycerol on membrane fluidity in the hydrophilic area as a change in the order parameter (*18*). A higher order parameter value means lower fluidity (*18*). Similar to the P value, the order parameter was not altered significantly on the addition of glycerol (Table I). From these two spectroscopic parameters for the fluidity of the CL membrane, both the hydrophobic and hydrophilic areas of the membrane seem not to be influenced significantly by glycerol at concentrations up to 34%.

We also observed the structure of CL vesicles under an electron microscope in the presence of 34% glycerol and found that glycerol did not damage the lipid bilayer structure of CL vesicles; in the presence of 34% glycerol, the typical vesicle structure was observed, as in the case of the control (without glycerol) (data not shown). This result suggests that glycerol had little effect on the morphology of CL vesicles under the present conditions.

Effect of Glycerol on the Affinity of DnaA Protein for ATP—The preceding observations suggested that the inhibitory effect of glycerol on the interaction of CL with DnaA protein was not due to its action on CL vesicles. Thus, we considered the possibility that glycerol induces a conformational change in DnaA protein and inhibits its interaction with CL. To test this possibility, we determined the K_d value of DnaA protein for ATP in the presence of 34 or 17% glycerol as an index of the structural change of DnaA protein. DnaA protein was incubated with various concentrations of [α-^{32}P]ATP and then the bound ATP was determined by filter-binding assay. As shown in Fig. 4, the slope of the line on Scatchard plot analysis decreased with an increase in the glycerol concentration. The K_d values of DnaA protein for ATP in the presence of 34 and 17% glycerol were calculated to be 180 and 40 nM, respectively. The determined K_d value in the presence of 17% glycerol coincides with that reported previously (*4*). A high concentration of glycerol may destabilize the ATP-DnaA complex. Based on these observations, we consider that glycerol induces a conformational change in DnaA protein and makes the protein insensitive as to the interaction with CL. To investigate the specificity of the action of glycerol toward DnaA protein, the influence of glycerol on the K_d values of other ATP-binding proteins for ATP should be examined.

Since the ATP-binding (Fig. 1) and the ATP-release (Fig. 2) experiments were performed in the presence of 2 μM ATP, this being much higher than the K_d value of DnaA protein for ATP even in the presence of 34% glycerol, glycerol (34%) did not significantly affect the reactions in the absence of CL (Figs. 1 and 2).

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