

Influence of Cluster Formation of Acidic Phospholipids on Decrease in the Affinity for ATP of DnaA Protein*

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DnaA protein is the initiator of chromosomal DNA replication in *Escherichia coli*. We examined the influence of artificial mixed membrane composed of synthetic acidic (phosphate) lipid and basic (ammonium) lipid on the affinity of DnaA protein for ATP. Two sets of acidic and basic lipids with distinguishable numbers of hydrophobic alkyl chains were devised. Synthetic membranes made of the sole acidic lipid but not the basic bilayers inhibited the ATP binding to DnaA protein and stimulated the release of ATP from the ATP-DnaA complex. The basic bilayer-forming compounds served as the matrix for the guest acidic lipids. Acidic lipids dispersed in the basic matrix membrane had little effect on ATP binding and on ATP release. Conversely, acidic lipids forming cluster structures in the mixed artificial membranes inhibited the ATP binding and stimulated the release of ATP. These observations suggest that in mixed lipid bilayers, a cluster structure of acidic lipids seems to be an important parameter to decrease the affinity of DnaA protein for ATP.

Chromosomal DNA replication in *Escherichia coli* initiates at a unique site, *oriC*, the origin of chromosomal DNA replication, depending on function of the initiator protein, DnaA protein (1–3). DnaA protein seems to be a key factor in mechanisms controlling DNA replication. The replication activity of DnaA protein is likely to be controlled by a number of regulatory factors functioning in concert. A protein factor that negatively regulates the activity of DnaA protein has recently been reported (4, 5). There is a line of evidence that suggests that transcription by RNA polymerase facilitates the function of DnaA protein to open up the *oriC* region for entrance of other replication proteins (6–8). The contribution of DNA supercoiling to control the capacity for initiation of DnaA protein has also been suggested (9, 10).

DnaA protein has a high affinity for ATP ($K_d = 0.03 \mu\text{M}$) and ADP ($K_d = 0.1 \mu\text{M}$) (11). ATP bound to DnaA protein hydrolyzes slowly to ADP in the presence of DNA (4). ADP also tightly binds to DnaA protein and is slowly replaced by ATP, and the

ATP-bound form is active in an *oriC* replication system reconstituted with purified proteins, whereas the ADP-bound form is inactive (11). Thus, adenine nucleotide binding is likely to be a primary process involved in the regulation of the activity of DnaA protein.

Lipid interaction of DnaA protein accompanied by a decrease in the affinity for ATP is an important clue for investigators examining the initiation of DNA replication (12–14). The initiation of chromosomal DNA replication in bacterial cells seems to occur on membranes (15–19). To assume that the activity of DnaA protein is regulated by phospholipids in biological membrane seems reasonable, and this notion is supported by the genetic studies of Xia and Dowhan (20). They showed that a lethal phenotype of a mutation in the *pgsA* gene, which is responsible for the synthesis of phosphatidylglycerol, is suppressed by a mutation in the *rnhA* gene. Because the mutation of this gene induces stable DNA replication, which is independent on DnaA protein and *oriC* DNA, they concluded that phosphatidylglycerol is necessary for the initiation of *oriC* DNA replication.

The bilayer membrane of *E. coli* is comprised of various phospholipids, such as four-chained cardiolipin, double-chained phosphatidylglycerol, and double-chained phosphatidylethanolamine. These natural lipids are differentiated by the number of alkyl chains, not only the charge of the hydrophilic group. Most studies concerning the interaction between lipids and DnaA protein have focused less on the unique property of mixed membrane and more on single component amphiphilic assemblage. When the numbers of hydrophobic chains are different among the bilayer-forming lipids, there is usually a tendency to take place a phase separation (cluster formation) in mixed membranes (21–24). This phase separation phenomena is a fundamental and unique property of mixed membranes.

Our objective in this study was to examine the importance of the phase separation in mixed membrane for a decrease in the affinity of DnaA protein for ATP. A precise system of regulation for phase separation in mixed membrane with synthetic lipids is a *sine qua non* for such studies. Through synthesis and characterization of hundreds of non-natural bilayer-forming lipids, we prepared a molecular design for bilayer assemblage, including the regulation of phase separation (25–30). A combination of a single-chained lipid containing azobenzene and glutamate-based double-chained amphiphile makes available a systematic mixed membrane system in which the cluster formation is controlled (21–24). Furthermore, the glutamate dialkyl lipids have actually been used for modifying the nature of aqueous proteins such as myoglobin and cytochrome *c* (31–33). We describe here how the phase separation phenomena of mixed bilayer membrane affects the affinity between DnaA protein and ATP.

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EXPERIMENTAL PROCEDURES¹

Materials—DnaA protein was purified by the method described previously (34), except that a newly constructed overproducer was used.² Specific activity of the protein was 0.7×10^6 unit/mg. Purity of the fraction used exceeded 90%, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The four non-natural lipids shown in Fig. 1 were synthesized (1N³ (24, 35), 2N (36), 1P (37), and 2P (38–40)). Powdery lipid was sonicated in a Branson Sonifier 185 (sonic power 30, 2 min, 1 ml) in distilled water to yield a translucent dispersion (10–20 mM). When acidic⁴ lipids were used, equimolar tris(hydroxymethyl)aminomethane was added for purposes of neutralization. Sonication of the powdery basic and acidic lipids (sonic power 40, for 3 min, 1 ml) in water led to mixed membrane dispersions (10–20 mM). This co-sonication method was used to avoid a nonfusion state where an aggregate of the acidic lipid and of the basic compound independently co-exist. These aqueous dispersions were used as stock solutions for the following assays. [α -³²P]ATP (5 mCi/mmol) was obtained from Amersham Corp.

Differential Scanning Calorimetry—Differential scanning calorimetry (DSC) was conducted on a Seiko SSC-5200 instrument equipped with the DSC-120 thermal balance. The lipid dispersions (10–55 mM, 52 μ l in 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% (v/v) Triton X-100), which were prepared by the co-sonication method, were sealed in silver sample pans. The buffer mentioned below was mounted on the thermal balance as a control sample. All DSC thermograms were scanned at a rate of +1 °C/min from 0 to 100 °C. The thermograms were reproducible for the cooled samples.

Fluorescence Polarization (P value)—A stock solution of 1,6-diphenyl-1,3,5-hexatriene (1 mM) was prepared in tetrahydrofuran. An aliquot of the tetrahydrofuran solution (10 μ l) was, after solvent removal, mixed with aqueous glycerol dispersions of lipids (1 mM, 1 ml), and the mixture was 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 common equation (21).

Assay for Influence of Lipids on ATP Binding to DnaA Protein—DnaA protein (2 pmol) and lipids were mixed at 0 °C for 15 min in 40 μ l of buffer G. [α -³²P]ATP (5 mCi/mmol) was then added to give the final concentration of 2 μ M and incubated at 0 °C for 15 min. The binding of ATP to DnaA protein reached equilibrium within 5 min (data not shown). The solution was passed through a membrane filter (Millipore HA 0.45 μ m pore) presoaked in buffer G. The filter was washed with 6 ml of ice-cold buffer G and dried under an infrared lamp. The retained radioactivity was measured in a liquid scintillation counter.

Assay for Influence of Lipids on Release of ATP from DnaA Protein—DnaA protein (2 pmol) was preincubated with 2 μ M [α -³²P]ATP (5 mCi/mmol) in 40 μ l of buffer G at 4 °C for 15 min. Lipids were added, and the mixture was incubated at 37 °C, followed by passage through a nitrocellulose membrane filter. Radioactivity on the filter was then measured.

RESULTS AND DISCUSSION

Design of Mixed Membranes to Control Phase Separation—To examine the importance of a cluster of acidic lipids in mixed membrane on the affinity of DnaA protein for ATP, we first selected appropriate synthetic lipids that satisfied the following criteria: (a) a guest amphiphile should be capable of interacting with DnaA protein and reducing the affinity of DnaA protein for ATP, (b) a matrix membrane should not affect

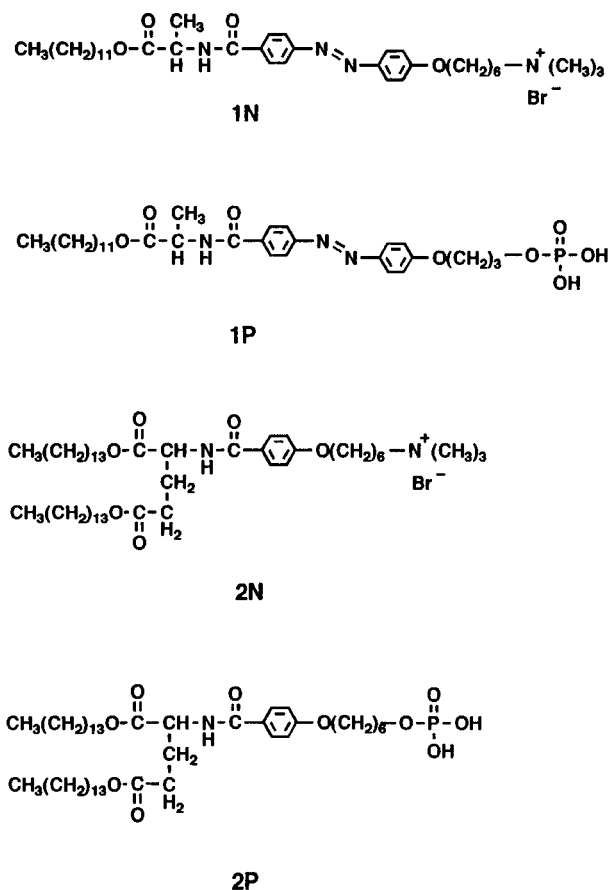


FIG. 1. Molecular structure of non-natural lipids used in this experiment. Single-chained alanate and double-chained glutamate compounds are abbreviated as 1 and 2, respectively. Basic and acidic hydrophilic groups are expressed as capital N and P, respectively.

the affinity, (c) both the guest and matrix membranes should be miscible, (d) a system by which both states, phase separation and monomeric dispersion, should be controllable, (e) amphiphiles that met these criteria should make available a bilayer membrane in aqueous glycerol, which is essential for maintenance of ATP-binding activity of DnaA protein, and (f) the membrane should represent a clear gel to liquid crystal phase transition so as to be detectable with thermal analysis.

Previous studies revealed that anionic residues in lipids are indispensable for a decrease in the affinity of DnaA protein for ATP, and cationic and zwitter ionic (neutral) lipids do not influence the affinity (12–14). Therefore, selecting negatively and positively charged amphiphiles as guest and matrix membranes, respectively, is a prerequisite for the basic demands (a) and (b). Moreover, opposite charge mixing makes guest and matrix membranes miscible (requirement (c)). The requirements (d), (e), and (f) are discussed in the following sections.

On the basis of a heterogeneous combination, in terms of the number of hydrophobic alkyl chains, we devised two pairs of amphiphiles; one is single-chained (1N and 1P), and the other is a double-chained amphiphile (2N and 2P), as shown in Fig. 1. Double-chained 2N and 2P and single-chained 1N and 1P provide typical non-natural bilayer membranes, both possessing sharp gel to liquid crystal phase transitions when the hydrophobic force due to the alkyl chain is strengthened with aromatic stacking force (37–40). The acidic compounds, 1P and 2P, are comprised of hydrophobic structures similar to the basic correspondents 1N and 2N, respectively. We previously reported that phase separation in a mixed membrane is caused by the heterogeneous combination of hydrophobic alkyl chains

¹ Dr. Kazuhisa Sekimizu and Dr. Yuichi Ishikawa accept responsibility for assays of DnaA protein and for non-natural bilayer membrane, respectively.

² T. Katayama, unpublished data.

³ The abbreviations used are: 1N, *O*-dodecyl-*N*-[1-carbonyl-4-(6-trimethylammoniohexyloxy)azobenzene]-(L)alanate bromide; 2N, *O,O'*-ditetradecyl-*N*-[1-carbonyl-4-(6-trimethylammoniohexyloxy)-benzene]-(L)glutamate bromide; 1P, *O*-dodecyl-*N*-[1-carbonyl-4-(3-phosphoropropoxy)azobenzene]-(L)alanate bromide; 2P, *O,O'*-ditetradecyl-*N*-[1-carbonyl-4-(6-phosphoropropoxy)benzene]-(L)glutamate bromide; DSC, differential scanning calorimetry.

⁴ The terms used herein, "acidic" and "basic," refer to the negatively charged (anionic) phosphate and the positively charged (cationic) trimethylammonium groups, respectively.

TABLE I
DSC parameters and fluorescence polarization (*P* value) of 1,6-diphenyl-1,3,5-hexatriene for dispersions of bilayer-forming amphiphiles

The parentheses indicate the absence of glycerol. DSC parameters: [amphiphile] = 10 mM; scanning rate, +1 °C/min; scanning range, 0–100 °C. Fluorescence polarization: [amphiphile] = 1 mM; [fluorescence probe] = 0.01 mM; excitation, 363 nm; emission, 434 nm. Dispersion conditions are: pH 8.0, HEPES-KOH buffer (50 mM) containing 17% (v/v) glycerol and 0.005% (w/v) Triton X-100.

Amphiphile	T_c	ΔH	Peak width ^a	P value at 37 °C
	°C	kJ/mol	°C	
1P	81, 85 ^b (77, 86) ^b	~11 (~11)	broad (broad)	^c
1N	80 (79)	31 (30)	5.3 (5.5)	^c
2P	61 (60)	28 (27)	3.2 (3.2)	0.31 (0.37)
2N	49 (51)	57 (54)	1.3 (0.9)	0.18 (0.26)

^a Peak to peak width of the first derivative for the observed peak.

^b Two broad endothermic peaks.

^c Fluorescence quenching due to absorption of amphiphile possessing azobenzene chromophore overlaps excitation and emission wavelength of the probe.

(21–24). Hence, when the acidic lipid is diluted with the basic matrix consisting of a similar hydrophobic moiety, namely 1P/1N and 2P/2N, the acidic lipid is likely to disperse monomerically into the basic matrix membrane due to electrostatic force. On the other hand, in the cross-bred combinations, 1P/2N and 2P/1N, the acidic amphiphile is segregated by the basic matrix to show phase separation, if the clustering force arising from the difference of alkyl chains exceeds the electrostatic force. These postulations are examined in the following sections.

Aggregation Properties of Non-natural Amphiphiles in Aqueous Glycerol—In the following experiments, we usually added 17% (v/v) glycerol to the buffer to protect DnaA protein from denaturation. Mixing distilled water with protic organic solvents, however, generally results in destabilization of membrane (41–45). Thus, we first determined the stability of the bilayer membrane in the buffer containing glycerol. Aqueous bilayers can undergo the gel to liquid crystal phase transition. This macroscopic phenomenon is one of the most fundamental membrane properties and causes microscopic physicochemical changes in membrane fluidity (24, 25). To estimate damage of bilayer structures by the addition of glycerol, DSC and fluorescence polarization (*P* value) of 1,6-diphenyl-1,3,5-hexatriene were used in characterizing phase transition and membrane fluidity, respectively.

Table I summarizes phase transition and membrane fluidity for aqueous dispersions of the four amphiphiles. Even in the presence of 17% glycerol, the dispersions of 1N, 2P, and 2N possessed symmetric and single endothermic peaks on heating at 80, 61, and 49 °C, respectively. For the dispersion of 1P, doublet endothermic peaks were observed around 80 °C, with and without glycerol. In addition to the peak shape, the presence and the absence of 17% glycerol made little difference on values for phase transition temperature (T_c), enthalpic change associated with the transition (ΔH), and peak width, without regard for amphiphile structures, except for peak width of 2N. *P* values for the dispersions of 2N and 2P lessened by 0.06–0.08 with 17% glycerol. The microscopic environment of the double-chained membrane interior is affected by glycerol, but this change is not so extensive as to conclude the amphiphiles exist in nonbilayer state such as micellar or monomeric dispersion. We actually confirmed the presence of vesicular (2N, diameter 40–400 nm) and tube-shaped (1N, length 500–3000 nm, width 150 nm) membranes even in the presence of 17% glycerol by transmittance electron microscopy for the dispersions stained with uranyl acetate (data not shown).

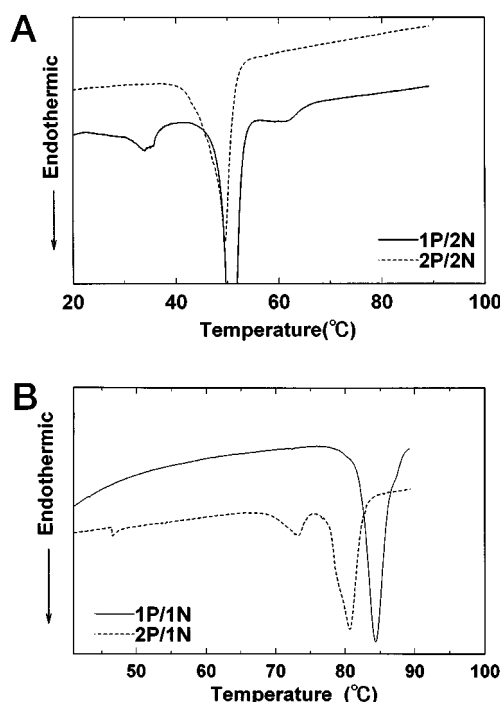


FIG. 2. DSC thermograms of aqueous dispersions of mixed membranes made of the single-chained basic matrix 1N (A) and 2N (B). The conditions were: [acidic lipid] = 5 mM, [basic lipid] = 50 mM, [tris(hydroxymethyl)aminomethane] = 5 mM, aqueous medium containing 17% (v/v) glycerol, and 0.005% (w/v) Triton X-100.

Distribution of Acidic Amphiphiles in Basic Matrix Membrane—Phase separation in the mixed membranes was examined using DSC and UV-visible absorption spectroscopy. It is important for the DSC study that phase transition parameters of a cluster domain differ from those of a matrix membrane. As shown in Table I, the single-chained bilayers represented a phase transition temperature higher by 20–40 °C over the double-chained bilayers. Also phase transition temperatures of the acidic bilayers being at pH 8.0 are 5–10 °C higher than those of the corresponding basic membranes. The four assemblies can be readily distinguished as different peaks on DSC thermograms.

Fig. 2A shows DSC thermograms of the double-chained basic membrane, 2N (matrix bilayer 50 mM) mixed with the acidic membrane, 1P or 2P (guest bilayer 5 mM). The mixed membrane 2P/2N has a single and broad endothermic peak at the same temperature as the T_c (49 °C) of the matrix bilayer 2N. The width of the peak of the dispersion 2P/2N is 5–6 times larger than that of the single component bilayer of 2N (~5 °C, Table I). On the other hand, the 1P/2N thermogram has two additional peaks below (~35 °C, 15% [ratio of area]) and above (62 °C, 5%) the major transition (51 °C, 80%). The width of the major peak (~2.2 °C) is nearly twice that of the bilayer 2N but about half as large as that of the mixed membrane 2P/2N.

The guest amphiphile 1P containing the azobenzene chromophore allows one to monitor aggregation properties through UV-visible spectroscopy (21–24, 37, 46, 47). The azobenzene amphiphile 1P in basic matrix 2N represented its λ_{max} at 336 nm at 37 °C, thereby indicating the presence of the face to face stacking (H-aggregate) (47). When raising the temperature to 70 °C, the λ_{max} shifted to 360 nm and reverted to the original wavelength by cooling to room temperature. This reversible spectral change is a typical instance of aggregation and disintegration of azobenzene amphiphiles associated with fluidity change of the matrix membrane (21–23). Despite the presence of electrostatic force, the single-chained anionic amphiphile

forms a cluster in the double-chained cationic matrix bilayer, as represented by small additional peaks. The spectral shift seen with cluster formation would be relevant to the multien-dothermic peaks.

The same situation of cluster structure formation arises for another matrix membrane. Fig. 2B shows DSC thermograms for the single-chained basic membrane 1N (50 mM) (matrix bilayer) mixed with the acidic membrane, 1P or 2P (5 mM) (guest bilayer). Like the double-chained matrix membrane, 2N, a system of different hydrophobic chains represents a set of broad peaks at 74 and 81 °C, whereas another combination for which similar hydrophobic modules are used led to a single peak at 84 °C (36 kJ/mol). Mixing the acidic amphiphile possessing a hydrophobic chain similar to that of the matrix compound with matrix lipids had little effect on the peak width (4–5 °C), the enthalpic change (31–36 kJ/mol), and the temperature range (around 80 °C) of the host membrane 1N. The acidic amphiphile containing azobenzene is miscible with the chromophoric matrix membrane. As opposed to the 1P, miscibility of the double-chained 2P is low enough to yield an additional peak below the main transition. This provides evidence for the presence of cluster 2P in the single-chained basic matrix membrane.

Cluster Size—The presence of multiple endothermic peaks does not necessarily mean that only the guest lipids converge on the acidic domains. This situation can be readily confirmed in the 2P/1N mixed membrane. Assuming that a phase-separated cluster is made up of only the 2P molecules, ΔH values for the additional peak (73 °C) and the main transition (81 °C) can be calculated to be 36 and 26 kJ/mol, respectively. The former value is nearly 30% more and the latter is almost 20% less than the ΔH values for 2P and 1N single-component bilayers, respectively (Table I). The excess of the additional peak over the hypothetical value can be offset by a decrease in the main transition. In other words, the acidic cluster domain of 2P contains matrix component 1N as minor ingredient. This situation can be confirmed also from the peak position in the DSC thermogram. In the case of the phase-separated cluster exists as the same assembly as the single-component 2P bilayer, the additional peak should appear close at the T_c of the 2P bilayer, 61 °C. The fact of showing the peak (73 °C) between the T_c values for the 2P (61 °C) and for 1N (80 °C) bilayers indicates that the phase transition property of the acidic cluster is affected by the mixing with matrix lipids.

Effect of Single Component Membrane on the Affinity of DnaA Protein for ATP—We explored the single component membrane from the viewpoint of requirements (a) and (b) in the initial section. We used filter binding assay for examination of ATP binding to DnaA protein (11). Fig. 3 shows the influence of the bilayer-forming lipid on ATP binding to DnaA protein. The two basic lipids, 1N and 2N, had little effect on the ATP binding to DnaA protein over the range of the lipid concentration from 1 to 1000 μM . On the contrary, in case of acidic lipids, 1P and 2P, the amount of bound ATP decreased with increase in the lipid concentration. The curve shifted toward the direction of low concentration of lipid when 2P replaced 1P, thereby suggesting the superior aggregation stability of the double-chained lipid, as compared with the single-chained compound. Because the addition of acidic lipids to the DnaA-ATP complex without incubation at 37 °C did not affect the amount of ATP on the filter (Fig. 4), the inhibitory effect of acidic lipids on ATP binding to DnaA protein cannot be explained by the release of DnaA protein from the filter by acidic lipids.

The interaction between DnaA protein and lipid was also evaluated through the ATP release from DnaA protein-ATP complex (Fig. 4). The k_{app} (apparent rate constants) values for

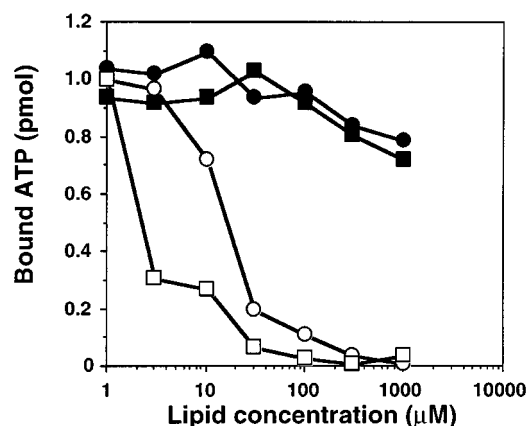


FIG. 3. Influence of synthetic lipids on the ATP binding reaction to DnaA protein. Assay of ATP binding to DnaA protein (2 pmol) was done as described under "Experimental Procedures." \circ , 1P; \bullet , 1N; \square , 2P; \blacksquare , 2N.

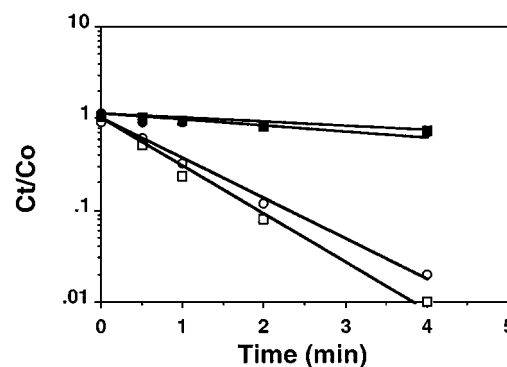


FIG. 4. Kinetic traces of the ATP release from DnaA-ATP protein representing effects of the change in lipids. Dissociation of the DnaA-ATP complex (1.1 pmol) in the presence of lipids (1P and 2P, 300 μM ; 1N and 2N, 30 μM) at 37 °C was examined, as described under "Experimental Procedures." C_t and C_o stand for concentrations of ATP-DnaA retained and of initial ATP-DnaA, respectively. \circ , 1P; \bullet , 1N; \square , 2P; \blacksquare , 2N.

2P, 1P, 2N, and 1N were calculated to be 1.1×10^{-2} , 8.5×10^{-3} , 9.6×10^{-4} (s^{-1}), and 9.6×10^{-4} (s^{-1}), respectively. The two acidic lipids (30 μM) accelerate the release of ATP 1 order of magnitude faster than does the basic amphiphile (300 μM). Moreover, the finding that the k_{app} value of 2P is slightly larger than that of 1P is consistent with the observation of ATP binding behavior. ATP release was nonexistent in the absence of lipid. These results indicate that the acidic lipid is much superior to the basic compound in stimulating ATP release from the DnaA-ATP complex and in inhibiting ATP binding to DnaA protein. These results are consistent with previous studies showing the importance of acidic residues in lipids for interactions with DnaA protein (12–14).

Decrease in Affinity of DnaA Protein for ATP by Acidic Lipids Forming Cluster Structures in Mixed Bilayer Membranes—From the viewpoint of phase separation, we examined the interaction between mixed membrane and DnaA protein through the release and incorporation of ATP. Fig. 5 shows the inhibition for ATP incorporation by various mixed membranes. In the case of the single-chained phospholipid 1P, mixed with 1N possessing the same hydrophobic moiety, the inhibition curve shifted from that of sole 1P by 1 order of magnitude toward the direction of higher concentrations. The curve for the mixed membrane of the heteroalkyl combination (1P/2N), however, is coincident with that for the single acidic component membrane of 1P, within experimental error.

The same situation as the 1P system holds in case of the

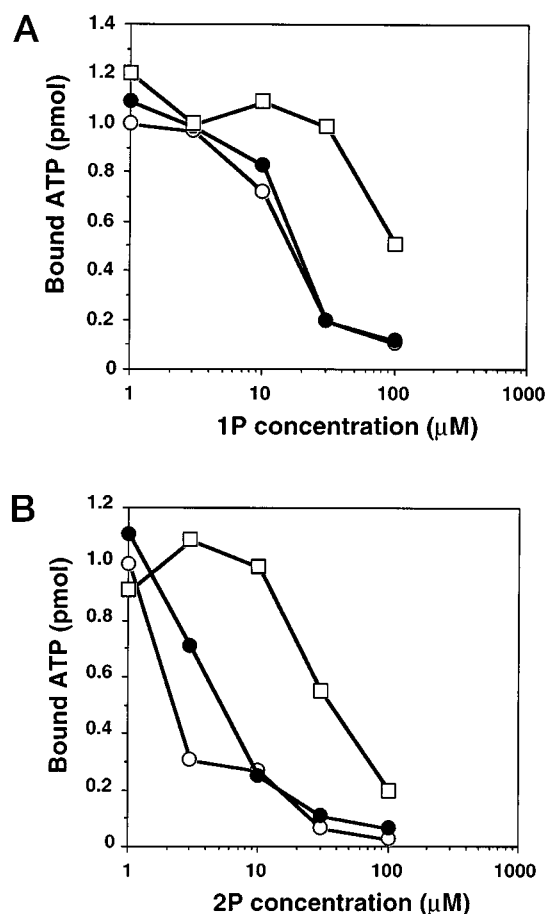


FIG. 5. Influence of mixed bilayer membranes on ATP binding reaction to DnaA protein. Mixed bilayer membranes of 1P and 2N or 1P and 2N were constructed by sonication, with the proportion of acidic and basic lipids being 1:10. The amount of ATP bound to DnaA protein (2 pmol) was determined as described in the legend of Fig. 3. A, ○, 1P; ●, 1P/2N; □, 1P/1N. B, ○, 2P; ●, 2P/1N; □, 2P/2N.

double-chained phospholipid 2P. Mixing with 2N weakened the inhibitory effect of 2P on the ATP binding to DnaA protein. On the contrary, the mixed membrane of the heteroalkyl combination (2P/1N) was more inhibitory than that of the homoalkyl combination (2P/2N). Because the two basic lipids had little effect on ATP incorporation over the concentration range of 1–100 μM (Fig. 3), the disparity in the inhibitory behavior between the mixed membranes of the heteroalkyl combination and of the homoalkyl combination should be considered from the viewpoint of acidic lipids.

Subsequently, ATP dissociation from the ATP-DnaA complex in the presence of added mixed membrane was monitored. Fig. 6 shows linear relations between time and logarithms for the amount of the dissociated ATP. k_{app} can be calculated from the slope. The k_{app} values for membranes 1P, 1P/1N, 1P/2N, 2P, 2P/2N, 2P/1N were determined to be 8.5×10^{-3} , 5.7×10^{-3} , 7.6×10^{-3} , 1.1×10^{-2} , 6.6×10^{-3} , and $9.2 \times 10^{-3} (\text{s}^{-1})$, respectively. The ATP release varies with the combination of the number of alkyl chains. The mixed membranes of heteroalkyl combination (1P/2N and 2P/1N) facilitate ATP release more rapidly than do those of the homoalkyl combination (1P/1N and 2P/2N). These observations on ATP release are consistent with results of its inhibitory effects on ATP binding to DnaA protein.

As mentioned in the previous section, mixed membranes of the heteroalkyl combination (1P/2N and 2P/1N) give rise to the phase separation (cluster formation) of acidic lipids. It seems apparent that a mixed membrane decreases the affinity of DnaA protein for ATP under conditions where acidic compo-

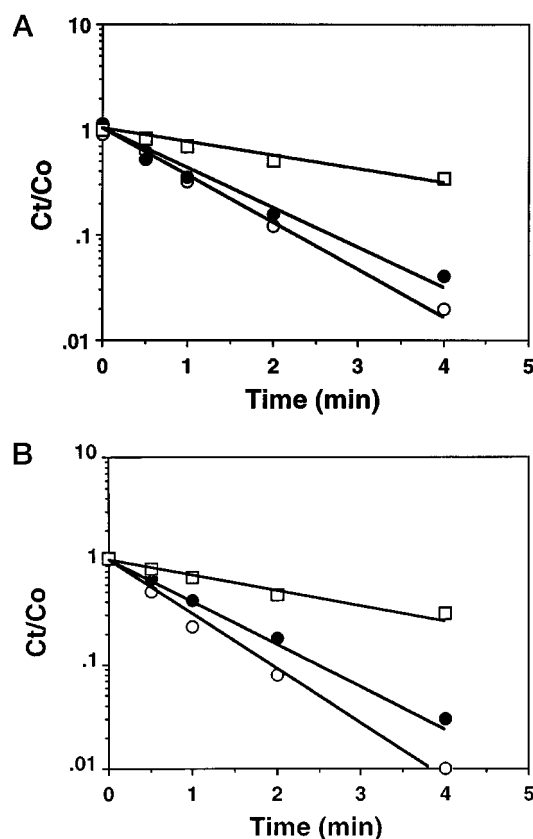


FIG. 6. A family of kinetic traces for the ATP release from DnaA-ATP protein depending on the mixed bilayer membrane. Dissociation of the DnaA-ATP complex in the presence of lipids was examined as described in the legend of Fig. 4. The conditions were: [initial DnaA-ATP] = 1.1 pmol/40 μl , [acidic lipid] = 30 μM , [basic lipid] = 300 μM , 37 $^{\circ}\text{C}$. A, ○, 1P; ●, 1P/2N; □, 1P/1N. B, ○, 2P; ●, 2P/1N; □, 2P/2N.

nents form cluster structures.

This study focused on phase separation (cluster formation) in mixed non-natural membranes and the impact on the interaction between lipid membrane and DnaA protein was apparent. The heterogeneous combination, in terms of the number of alkyl chains, for the mixed membrane (1P/2N and 2P/1N) gave rise to clusters abundant in acidic lipids, whereas similarity in the hydrophobic structure (1P/1N and 2P/2N) and the electrostatic interaction among the hydrophilic groups left the acidic lipids as a monomeric species in the matrix membrane. With this information one can examine the influence of the acidic cluster in the mixed membrane on the affinity of DnaA protein for ATP. The affinity change caused by the acidic lipids did lessen by diluting with matrix membranes; however, acidic lipids in a cluster state are as effective as those in the single component membrane in reducing the affinity. Diversity in size of the cluster and its purity might provoke a difference in the affinity change. Therefore, cluster formation of phospholipids in a mixed membrane may be one factor regulating the interaction between DnaA protein and lipids.

The heterogeneity in hydrophobic moiety between the component lipids is a key factor for formation of cluster domains in mixed artificial membranes (21–24). Partial hydrolysis and/or ester exchange for acyl chain of phospholipid by phospholipases could provide biological lipid membranes with heterogeneity in hydrophobic domains and might be a candidate offering a membrane signal to the DnaA protein. In fact, the rapid turnover of acidic phospholipids in *E. coli* has been reported (48, 49). Moreover, Ohki reported that breakdown of acidic phospholipids in

E. coli is apparently coupled with cycles of cell proliferation (50). All these results taken together lead to the thesis that the cluster structure of acidic phospholipids is controlled in concert with the cell cycle and that such a process may regulate DnaA protein activity.

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