The effect of nonlamellar-prone lipids, diacylglycerol (DG) and phosphatidylethanolamine (PE), on the ATPase activity of SecA was examined. When *Escherichia coli* PE of the standard vesicles composed of 60 mol% of this lipid and 40 mol% of dioleoylphosphatidylglycerol (DOPG) is gradually replaced with either dioleoylglycerol (DOG) or dioleoyl PE (DOPE), the ATPase activity of SecA present together increased appreciably. When DOPE or *E. coli* PE was added to PC vesicles, the SecA ATPase activity was enhanced only slightly, suggesting that the hexagonal II structure per se is not important for the ATPase activity increase. It was observed that DOG induced phase separation of PG and, the lamellar-hexagonal II (L-HII) transition temperature of vesicles decreased by about 10 °C. The DOG analogs had no effect on these properties, suggesting the importance of the phase separation of PG and the decrease of L-HII transition temperature of lipid bilayers to the SecA ATPase activity. The phase separation of PG by Ca$^{2+}$ also brought about increased ATPase activity of SecA, underlining the importance of phase separation of PG for the enzyme activity. The incorporation of DOG or DOPE in the vesicle also increased the amount of SecA bound to model membranes and the extent of SecA penetration into the membrane. Studies with vesicles without SecA showed increased exposure of hydrophobic acyl chains when the DOG was present. Taken together, these observations suggest that the phase separation of PG and/or the bilayer penetration of SecA are mainly responsible for the enhanced SecA-vesicle interaction with concomitant increase in SecA ATPase activity.

SecA is the central protein component of the translocation machinery for the newly synthesized proteins in *Escherichia coli*. This protein exists as a homodimer of a 102-kDa subunit and is distributed in vivo about equally between the inner membrane and cytosol (1). The protein translocation is brought about by a close cooperation between SecA, an ATPase, and other Sec proteins, such as SecB and SecYE, and hydrolysis of ATP is required for this process (2, 3). SecA was also found to have another major function, an RNA helicase activity accompanying ATP hydrolysis, which may be necessary for the regulation of protein translocation (4).

SecA binds to various phospholipid vesicles including those of phosphatidylcholine (PC). In solution, SecA has only a residual ATPase activity (5). Although no discernible increase in this enzyme activity can be seen in the presence of PC vesicles, a tremendous enhancement of its activity is observed in the presence of vesicles, which has similar composition as the *E. coli* inner membrane, 60% phosphatidylethanolamine (PE) and 40% phosphatidylglycerol (PG) (5). This differential effect of the vesicle composition seems to be related to the extent of binding and penetration of SecA to the vesicles. More SecA binds to vesicles containing *E. coli* lipids than those of PC (6). SecA also penetrates deep into the lipid bilayer (7), and we have also found that SecA traverses the lipid bilayer (8, 9). These observations suggest that the nonlamellar-prone lipid, PE, in the vesicles promotes the binding and penetration of SecA into the bilayer, which enhances the ATPase activity. In this connection, it is of interest that the *E. coli* membrane also contains another nonlamellar-prone lipid, diacylglycerol (DG), although the precise role of this lipid is not known. DG is a neutral lipid that can lower the lamellar to hexagonal II phase transition (L-HII) temperature (TH) of PE significantly (10, 11). Even at low (2–3 mol%) concentrations, the DG stabilizes the HII structure of PE (11, 12). In *E. coli*, DG accounts for about 1% of total membrane lipid, which depends on the growth conditions (13). However, in some mutant strains, about 8% of DG is present in the inner membrane (14).

The nonlamellar structure of membrane has been shown to play important roles in various cell functions. Several in vivo and in vitro studies indicated the involvement of nonlamellar structure of membrane in the translocation of secretory proteins across the plasma membrane (15). Also, it was shown that nonlamellar lipid structure is induced by signal peptides (16). In particular, such activities as the ATP exchange by mitochondrial proteins (17) and Ca$^{2+}$-ATPase activity increase with increasing PE content (18), suggesting importance of nonlamellar structure.

In the present investigation, the possible effect of nonlamellar structure of model membrane to the SecA-membrane inter-
action is examined by increasing the nonlamellar-prone lipids such as dioleoylglycerol (DOG) and dioleoylphosphatidylethanolamine (DOPE) in the lipid vesicles. For this, we gradually replaced the E. coli PE in liposomes initially consisting of 60% E. coli PE and 40% dioleoylphosphatidylglycerol (DOPG) with DOG or DOPE and measured the SecA ATPase activity (5). DOPE has a lower lamellar to hexagonal II transition temperature (12 °C) than the E. coli PE (about 55 °C) and assumes the HII conformation at room temperature. Our results show that the ATPase activity of SecA is able to recognize the nonlamellar structure of membrane and its ATPase activity increases with increasing nonlamellar-prone lipid content. However, the bulk of ATPase activity increase accompanying the enhanced SecA binding to the vesicles seems to come from the phase separation of PG present together.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phospholipids and DOG were purchased from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. Fluorescence probes were from Molecular Probes. Chloroform solutions of lipids were stored in sealed ampules under argon at −20 °C. All other chemicals were of the highest grade commercially available.

**SecA Preparation and ATPase Activity Assay**—SecA protein was purified from a SecA-overproducing strain (RR1/pMAN400) (19) as described (9). The ATPase assay was performed in 100 μl of reaction buffer containing 100 mM NaCl, 1 mM dithiothreitol, 2 mM ATP, and 2 mM MgCl₂. The sample solutions containing 1.5 μg of SecA protein and liposomes were incubated at 37 °C for 40 min prior to the analysis of the released inorganic phosphate (Pi) as described (20). The reactions were stopped by the addition of a color reagent (0.034% malachite green and 10.5 g/liter ammonium molybdate in 1 v 1 HCl and 0.1% Triton X-100) and 100 μl of 34% citric acid solution. After standing for 30 min at room temperature, the absorbance was measured at 660 nm. One unit of ATPase activity is defined as the hydrolysis of 1 pmol of ATP per minute. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard (21).

**RESULTS**

**Effects of Nonlamellar-prone Lipids on the SecA ATPase Activity**—Fig. 1A shows that the ATPase activity of SecA increased linearly with increasing concentration of DOG in the vesicles by as much as about 50% when DOG content reached 5 mol%. Above 5 mol% of DOG, the lipid vesicle did not form. Fig. 1B shows that the ATPase activity was enhanced by about 50% when all of the E. coli PE in the standard vesicles was replaced by DOPE in the absence of DOG. However, only a slight increase in the ATPase activity was seen when egg PE was used as the substitute instead of DOG or DOPE.
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Differential ATPase activity increase seems to be related to the ease with which the L-H transition occurs because the \( T_H \) of DOPE, egg PE, and *E. coli* PE are 12, 45, and 55 °C, respectively (26).

Next, the effect of PE on the ATPase activity of SecA in the absence of acidic phospholipid, PG, was determined. Fig. 1C shows that, when the content of *E. coli* PE or DOPE in 1-palmityl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) vesicles is increased, the ATPase activity of SecA increased linearly but with a much smaller extent than the case of standard vesicles containing PG. Here, DOPE showed a more pronounced effect on the activity than *E. coli* PE, which may arise from different \( T_H \) values of these PEs. This result suggests that PE, a nonlamellar-prone lipid, itself is able to increase the ATPase activity of SecA only slightly in the absence of acidic phospholipids. Also, it should be noticed that vesicles containing DOPE, which is in the \( H_{II} \) state at the experimental temperature of 37 °C, give only a slightly higher ATPase activity than the vesicles with *E. coli* PE, which is in the lamellar form.

The effect of DOG analogs on the ATPase activity of SecA was also examined. Fig. 2A shows that the ATPase activity decreased slightly with increasing vesicle concentrations of DG analogs. The DG analogs used here are 2-monoooleoylglycerol, 1-monoooleoylglycerol, dioleoyl-1-flouro-2,3-propanediol, and dioleoyl-1-glyceramide.

The ATPase activity also decreased when *E. coli* PE in the standard vesicles was replaced by PC, which is known to be a bilayer-forming lipid (Fig. 2B). It is clear, therefore, that the propensity of \( H_{II} \) formation as well as acidic phospholipids is essential for the increased ATPase activity of SecA. Rietveld et al. found that dioleoylphosphatidylglycerol or dimyristoylphosphatidylethanolamine, both of which prefer a lamellar structure in the membrane, could not restore protein translocation in *E. coli* AD93 strain, which cannot synthesize PE when these lipids were incorporated into the inner membrane; however, DOPE did (15).

**Influence of DOG and Its Analogs on the Phase Transition Temperature**—Diacylglycerols are known as potent promoters of nonlamellar phases in various phospholipid systems. Therefore, we also investigated the effect of DOG and its analogs on the transition temperature of vesicle systems. Fig. 3 shows that DOG decreased the \( T_{II} \) of the standard vesicles by about 10 °C when 5 mol% DOG was incorporated. This result agrees well with the published data that, upon incorporation of as little as 1 mol% diolein or diilinolen, the \( T_{II} \) of fully hydrated POPE is lowered by approximately 9 °C (27–29). The effect of DOG analogs on the \( T_{II} \) was also examined, and the results are shown in the inset of Fig. 3. It is clear that the presence of these analogs in the vesicle did not affect the \( T_{II} \). It should be remembered that these analogs did not influence the ATPase activity of SecA.

**Phase Separation of Lipids Induced by DOG**—Acidic phospholipids are essential components for the ATPase activity of SecA, and they promote SecA binding and insertion into model membranes (5, 29). It may be expected, therefore, that the local concentration of acidic phospholipids is one of the important factors determining the SecA ATPase activity and that this local concentration might be regulated by nonlamellar-prone lipids. To test this possibility, we utilized the self-quenching of the fluorescence of NBD-labeled phospholipids (30) at 30 °C. Fig. 4 shows that the increased DOG concentration in lipid bilayers brought about 13% quenching of the fluorescence of liposomes containing 10 mol% of NBD-PG (A) or NBD-PE (B) as compared with the value of the sample without DOG. This suggests that DOG promotes the phospholipid clustering in lipid bilayers inducing the formation of domains enriched with either PE or PG in the membrane. This also suggests that the increase of the ATPase activity of SecA by nonlamellar-prone lipids could arise from the clustering of acidic phospholipid, PG. In fact, we measured enzyme activity at 37 °C, which is below the L-\( H_{II} \) phase transition temperature of liposomes (Fig. 3). In other words, liposomes are still in the lamellar structure at this temperature. So, we may deduce that the effect of nonlamellar-prone lipids on the ATPase activity of SecA is because of the formation of lipid domains enriched with acidic phospholipids. However, we cannot still preclude the possibility that the “propensity” of \( H_{II} \) phase formation is important to regulate the ATPase activity of SecA from the observation that the presence of *E. coli* PE in PC vesicles showed slightly higher activity than the PC vesicle without PE (Fig. 1C). It is most interesting that the DOG analogs, which did not induce an enhanced ATPase activity of SecA (Fig. 2A), also did not promote the clustering of either NBD-PG or NBD-PE (Fig. 4).

**CaCl\(_2\)-induced Phase Separation and Its Effect on the SecA ATPase Activity**—To confirm the effect of phase separation of PG (Fig. 4) on the ATPase activity of SecA, we examined the influence of Ca\(^{2+}\)-induced PG clustering on the SecA ATPase activity. When CaCl\(_2\) was added to the standard liposomes (60 mol% *E. coli* PE, 40 mol% DOPG) containing 10 mol% of NBD-PG, the fluorescence intensities decreased as a function of CaCl\(_2\) concentration, indicating the formation of PG domains in the membrane (Fig. 5A). Fig. 5A also shows Ca\(^{2+}\)-induced phase separation of PG in the vesicles containing 60 mol% DOPE or 60 mol% POPC. DOPE is the most efficient in facilitating the formation of PG domains, and POPC exerted the least effect on the phase separation.

Ca\(^{2+}\) stimulated the ATPase activity of SecA bound to the standard vesicles as well as to the vesicles that contain 60...
mol% of POPC instead of the E. coli PE in the standard vesicles (Fig. 5B). There is an initial sharp increase in the activity reaching a plateau around 1 mM of CaCl₂, the effect on the E. coli PE vesicles being greater than that on the POPC vesicles. These results confirm that increase in the local PG concentration enhances the ATPase activity of SecA. These observations also suggest that the change of local concentration of acidic phospholipid, PG, because of the presence of DOG, is directly involved in the regulation of the ATPase activity of SecA bound to membranes.

SecA Binding to Model Membranes Containing Nonlamellar-prone Lipids—The effect of nonlamellar-prone lipids and phase separation induced by Ca²⁺ on the binding of SecA to model membranes at 30 °C was measured by the quenching of Trp residues of SecA by pyS DHPE incorporated into the standard vesicles (7). This confirms that increase in the local PG concentration enhances the ATPase activity of SecA. These observations also suggest that the change of local concentration of acidic phospholipid, PG, because of the presence of DOG, is directly involved in the regulation of the ATPase activity of SecA bound to membranes.

SecA Penetration into Lipid Bilayer—The effect of DOG on the insertion of SecA into lipid bilayer was tested with resonance energy transfer between the fluorescence of Trp residues in SecA and pyrene-labeled PE at 30 °C. The emission of Trp fluorescence and the excitation of pyrene show strong spectral overlap. Because the pyrene group is attached to the end of the decanoyl chain at the sn-2 position of PE, it is possible to measure the extent of SecA insertion into lipid bilayers. The extent of energy transfer increased with increasing concentration of DOG in the membranes, as shown by increasing intensity of nonlamellar structure, is responsible for the enhancement of ATPase activity.

The decreased F/F₀ values with increasing Ca²⁺ concentration (Fig. 6C) indicates the enhanced SecA binding to lipid vesicles accompanying the phase separation. When the fact that DOG and Ca²⁺ effectively induce the phase separation of lipids in membranes is taken into account, this result strongly supports the notion that the change of local concentration of acidic phospholipids regulates the partitioning of SecA into the membranes. In other words, the formation of lipid domains enriched with acidic phospholipids enhances the amount of SecA bound to lipid bilayers and consequently increases the SecA ATPase activity.

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pyrene fluorescence for monomer at 375 nm (Fig. 7A). DOPE also increased the incorporation of SecA into the membrane (Fig. 7B), but there was little change of the energy transfer when egg PE was used. These results suggest that nonlamellar-prone lipids tested here promote the insertion of SecA into membrane.

As control experiments, we measured the excimer (E) and monomer (M) fluorescence of pyrene-PE and pyS DHPE in the membrane and determined the E/M ratio to examine the distribution of pyrene probes. The E/M ratio reflects the enrichment of its local concentration in the membrane (31). The E/M ratio did not change when PE was replaced by nonlamellar-prone lipids (data not shown), which indicates there was no redistribution of the probes in the presence of nonlamellar-prone lipids.

Influences of DOG on the Exposure of Phospholipid Acyl Chains—To examine possible structural change in the membranes induced by DOG, we measured the fluorescence intensity of pyrene-PE, incorporated into the membrane, at 376 nm at several temperatures. The fluorescence decreased with increasing concentration of DOG in the membrane within the temperature range studied (Fig. 8A). This result suggests that DOG induces the exposure of fatty acyl chains of lipids from the interior of the membrane to the surface. However, the fluorescence intensities at different DOG content converged at 50 °C, indicating that the extent of exposure is similar at this temperature regardless of the presence of DOG. To confirm this result, we used the E/M ratio change caused by the collision between pyrene-PE and pyS DHPE, incorporated into E. coli PE/PG and PC vesicles, respectively. Fig. 8B shows that the E/M ratio increased with increasing amount of DOG in the membrane at all temperatures tested.

DISCUSSION

SecA is an unusual water-soluble protein that readily penetrates the lipid bilayer. It shows only a basal ATPase activity in aqueous solution, but the activity increases dramatically in the presence of vesicles containing negatively charged phospholipids. In the presence of PC vesicles, however, there is no appreciable enzyme activity other than the basal activity.

In the present investigation, we observed that the DOG and DOPE, which are known to have a propensity of forming nonlamellar structure, when present in the vesicles with the lipid composition similar to the E. coli membrane, enhance the ATPase activity of SecA. Similar increase in biological activity by these nonlamellar-prone lipids was observed for a number of systems, and it was generally assumed that the phase transition from lamellar to HII is responsible for the increase. That this may not be the case is apparent in Fig. 1C, which shows only a marginal increase in ATPase activity or SecA binding when the content of E. coli PE below its phase transition temperature or DOPE above the transition temperature in PC

FIG. 5. Calcium-induced phase separation of PG and stimulation of SecA ATPase activity. The formation of PG domains in PE/PG vesicles at 30 °C was measured by using the fluorescence quenching of 10% NBD-PG as a function of CaCl₂ (A). The ATPase activity of SecA at 37 °C was also measured with increasing concentration of CaCl₂ (B). All other conditions are the same as described in Figs. 1 and 4. ○, 60 mol% E. coli PE/40 mol% DOPG; ○, 60% POPC/40% DOPG; □, 60% DOPE/40% DOPG.

FIG. 6. Effects of nonlamellar-prone lipids on the SecA binding associated to model membranes. The amount of SecA bound to the membrane was determined from the quenching of SecA Trp fluorescence by 2 mol% of pyS DHPE incorporated into lipid bilayers. 0.6 μM of SecA was incubated with model membrane at 30 °C for 10 min, and the fluorescence intensity of Trp residues of SecA was measured at 340 nm. F/F₀ represents the fluorescence intensity ratio for the sample with (F) and without (F₀) pyS DHPE. The E. coli PE in the standard vesicle was replaced by DOG (A) or DOPE or egg PE (B). In C, F value was determined as a function of CaCl₂ concentration in the solution containing standard vesicles.
vesicle is increased. A number of additional experimental results, such as enhancement of ATPase activity when phase separation in the standard vesicle was induced by Ca\(^{2+}\), suggest that the phase separation rather than phase transition is mainly responsible for the increased enzyme activity. It seems that the DOG, a nonlamellar-prone lipid, promotes phase separation, although the precise reason of this is not clear. The DOG analogs, which do not induce phase separation, also do not affect the ATPase activity of SecA. In this connection, it is of interest that the cluster formation of acidic phospholipids decreases the DnaA protein affinity for ATP, suggesting that the phase separation of anionic phospholipids regulates DnaA protein activity (32).

At the moment, we do not have a satisfactory explanation for the increased ATPase activity of SecA accompanying phase separation of PG. The experimental results show that SecA binding and penetration into the membrane are enhanced by the phase separation. This may mean that SecA penetration prefers the PG clusters, and the increased ATPase activity is simply the consequence of increased SecA population that penetrates the membrane. The presence of DOG also induced the exposure of phospholipid acyl chains to the surface. This may be the result of lamellar to H\(_{11}\) transition, but the exposure may not be directly related to the phase separation. The exposure should certainly promote the SecA binding to the vesicles.

It was shown that the C-terminal end of SecA is less stable than the rest of the SecA and that this is the domain penetrating the membrane (32). The penetration of the C-terminal end will bring about its separation from the N-terminal region of SecA. The N-terminal two-thirds of the SecA sequence contains the ATP binding sites, and the ATPase activity is known to be inhibited by the C-terminal end (33) when SecA assumes the native structure. The insertion of C-terminal end into the membrane eliminates this inhibiting effect. Taken together, the presence of nonlamellar-prone lipid promotes L-H\(_{11}\) transition, bringing about phase separation of PG that, in turn, promotes the insertion of the C-terminal end of SecA. The removal of the inhibitory effect of the C-terminal end by its membrane insertion enhances the ATPase activity of the N-terminal side of SecA.

Many natural membranes are rich in lipids that have strong propensities to form H\(_{11}\) phases or nonlamellar membrane structures. The importance of these lipids in membranes on the enzyme activities and protein functions has already been suggested for ubiquinol-cytochrome c reductase and mitochondrial H\(^-\)ATPase (17), rhodopsin (34), and alamethicin conductance states (35). Although these observations demonstrated the important role of nonlamellar membrane structures in the enzyme activities or protein functions, it is not yet clear whether phase separation is also involved in these systems.

In relation to the translocation of preproteins in _E. coli_, Rietveld et al. (15) provided the first direct evidence for the role of nonlamellar-prone lipids to facilitate the passage of preproteins through the cell membrane (15). Another example of the involvement of nonlamellar structure in the translocation is that functional signal peptides can induce the H\(_{11}\) structure of membrane (16). However, the detailed role of nonlamellar-prone lipids in the _E. coli_ translocation has not been elucidated.

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**FIG. 7. Penetration of SecA into lipid bilayers.** All experimental conditions are the same as in Fig. 4 except that 2 mol% of pyrene-PE instead of pyS DHPE was used as the membrane probe. Fluorescence intensity was measured at 375 nm with 342 nm of excitation wavelength. _E. coli_ PE was replaced with DOG (A) or with DOPE and egg PE (B) as described previously. I and I\(_0\) represent the fluorescence intensity for the sample with (I) and without (I\(_0\)) SecA protein.

**FIG. 8. Effect of DOG on the exposure of fatty acyl chains of lipid molecules to membrane surface.** The extent of exposure of fatty acyl chains was measured by the decrease of pyrene-PE fluorescence (A) or by the collision between pyrene-PE and pyS DHPE incorporated into lipid bilayers (B): 2 mol% of pyrene-PE and pyS DHPE were incorporated into standard vesicles containing each indicated DOG amount and POPC vesicles, respectively. The fluorescence intensity in the range of 360–500 nm was measured with 342 nm of excitation wavelength with increasing temperature.
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