SecA Insertion into Phospholipids Is Stimulated by Negatively Charged Lipids and Inhibited by ATP: A Monolayer Study†

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ABSTRACT: SecA–lipid interactions are believed to be important for the translocation of precursor proteins across the inner membrane of *Escherichia coli* [Lill, R., Dowhan, W., & Wickner, W. (1990) *Cell 60*, 271–280]. SecA insertion into the phospholipid bilayer could play a role in this process. We investigated this possibility by studying the interactions between SecA and different phospholipids using the monolayer technique. It was established that SecA is surface-active and can insert into lipid monolayers. This insertion was greatly enhanced by the negatively charged lipids DOPG and *Escherichia coli* cardiolipin. Insertion of SecA into these negatively charged lipids could be detected up to initial surface pressures of 34 mN/m for DOPG and 36 mN/m for *Escherichia coli* cardiolipin, implying a possible role for negatively charged lipids in the insertion of SecA in biological membranes. High salt concentrations did not inhibit the SecA insertion into DOPG monolayers, suggesting not only an electrostatic but also a hydrophobic interaction of SecA with the lipid monolayer. ATP decreased both the insertion (factor 2) and binding (factor 3) of SecA to DOPG monolayers. ADP and phosphate gave a decrease in the SecA insertion to the same extent as ATP, but the binding of SecA was only slightly reduced. AMP-PNP and ATP-γ-S did not have large effects on the insertion or on the binding of SecA to DOPG monolayers. The physiological significance of these results in protein translocation is discussed.

Proteins destined for the outer membrane or periplasmic space of *Escherichia coli* are synthesized in the cytosol as precursors with an N-terminal extension, the signal sequence. The export of a subset of these precursors requires recognition by a soluble cytosolic component, SecB (Kumamoto & Beckwith, 1985), and subsequent targeting to the export machinery located at the inner membrane consisting of at least two integral membrane proteins, SecE (Schatz et al., 1989) and SecY (Shiba et al., 1984; Fandl et al., 1987), and one peripherally membrane-associated protein, SecA (Oliver & Beckwith, 1982).

The SecA protein couples ATP hydrolyses to the translocation process. It possesses three ATP-binding sites (Lill et al., 1989), and its ATPase activity probably provides the energy necessary for initiating the translocation of a precursor protein (Schiebel et al., 1991). The interaction of SecA with almost all components involved in the translocation process has been studied. The SecY protein stimulates the SecA ATPase activity (Lill et al., 1989); SecA recognizes SecB and forms a high-affinity binding site for SecB at the membrane (Hartl et al., 1990). SecA–precursor interactions are also reported by means of chemical cross-linking (Akita et al., 1990; Kimura et al., 1991) and precursor-dependent ATPase stimulation (Lill et al., 1990).

Several studies showed that negatively charged lipids are important for the translocation of precursor proteins [for a review, see De Vrije et al. (1990)]. One possibility is that SecA–lipid interactions are important for the translocation process. SecA only binds efficiently to liposomes containing negatively charged lipids, and this interaction results in a large increase in the SecA ATPase activity (Lill et al., 1990). Furthermore, recent data showed that the conformation of SecA is altered upon interaction with liposomes containing negatively charged lipids (Shinkai et al., 1991).

It has been suggested that SecA can insert into the bilayer, thereby initiating the translocation (Oliver et al., 1990a). In this study, we investigated the nature of the SecA–lipid interactions by means of the monolayer technique. This technique gives us the opportunity to follow both binding and insertion of a protein in a lipid monolayer. It is demonstrated that SecA is surface-active and inserts into the phospholipid monolayer, even at high initial pressures. This insertion was greatly enhanced by negatively charged lipids and decreased in the presence of ATP.

Materials and Methods

Materials. SecA was purified to >95% essentially as described (Cunningham et al., 1989) with one modification. The phosphocellulose column step was eluted with a less steep 1.6-L linear gradient of 0.01–0.3 M potassium phosphate, pH 6.5. SecA was stored as a 3 mg/mL stock solution in 50 mM Tris-HCl, pH 7.6, 10% glycerol, and 1 mM dithiothreitol at −80 °C. The purified SecA protein was active in stimulation of the translocation of ^{35}S-prePhoE according to Kusters et al. (1989).

SecA was ^{14}C-labeled according to the method of reductive methylation of the α- and ϵ-amino groups (Dottavio-Martin & Ravel, 1978). Shortly, the Tris buffer in which SecA was stored was replaced by a 40 mM phosphate buffer, pH 7.0, using the FPLC fast desalting column of Pharmacia (Sweden); 960 μg of SecA was added to 20 mM $NaCNBH_3$ and 0.32 mM (final concentrations) [^{14}C]formaldehyde (10 Ci/mol), dissolved in 40 mM phosphate, pH 7.0, in a total volume of 850 μL. After 30-min incubation at room temperature, the labeled SecA was separated from unbound label on the fast

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desalting column. The resulting SecA preparation had a specific radioactivity of 71 000 dpm/nmol, which corresponds to 3.2 methylated amino groups per protein. The radiolabeled SecA was stored as a stock solution of 0.4 mg/mL in 40 mM phosphate, pH 7.0, 10% glycerol, and 1 mM dithiothreitol and stored at −20 °C.

The radiolabeled SecA did not lose any of its activity during the labeling as was determined by a translocation experiment with ^{35}S-prePhoE according to Kusters et al. (1989) using fully active and nonradiolabeled purified SecA as a standard.

Protein concentrations were assayed according to Bradford (1976) using bovine serum albumin as a standard.

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was synthesised as described (van Deenen & De Haas, 1964), 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG), and 1,2-dielaidoyl-*sn*-glycero-3-phosphoglycerol (DEPG) were synthesized from their diacyl-PC analogues by phospholipase D mediated base exchange (Comfurius & Zwaal, 1977). *E. coli* cardiolipin (*E. coli* CL) was purchased from Sigma (St. Louis, MO). Phospholipid was determined as inorganic phosphate, after destruction with perchloric acid (Rouser et al., 1970).

E. coli phospholipids from wild-type strain SD12 (Shibuya et al., 1985) and PG-depleted strain HDL11 (Kusters et al., 1991) were isolated by Bligh and Dyer extraction (Bligh & Dyer, 1959) of inner membrane vesicles followed by column chromatography on Polygosil (60-4063, Macherey-Nagel). Neutral lipids and other contaminants were eluted with chloroform and the phospholipids with chloroform/methanol, 50/50 (v/v).

Anhydrous sodium cyanoborohydride ($NaCNBH_3$) was purchased from Aldrich; [^{14}C]formaldehyde was from NEN (New England Nuclear, U.K.). AMP-PNP and ATP were purchased from Sigma; ATP-γ-S was purchased from Boehringer (Germany). All other chemicals were of analytical grade or better.

Monolayer Experiments. Monolayer surface pressure was measured by the (platinum) Wilhelmy plate method (Demel, 1974) in a thermostatically controlled box, using a Cahn 2000 microbalance. The subphase was continuously stirred with a magnetic bar. SecA was added to the subphase through a hole in the edge of the dish. The pressure changes were followed until the surface pressure increase had reached a maximal value, usually within 30–40 min. Unless stated otherwise, the measurements were performed with the following parameters. The monomolecular lipid layers were spread from a chloroform or chloroform/methanol, 75/25 (v/v), solution to give an initial surface pressure of 25 mN/m. A subphase buffer of 50 mM Tris-HCl, pH 7.6, and 100 mM NaCl was used at a temperature of 31 ± 0.5 °C. The Teflon dish had a volume of 5 mL and a surface area of 8.81 cm^2. The buffer used for all monolayer experiments was filtered through a 22-μm pore filter and degassed prior to use.

In the case of experiments with radioactively labeled SecA, the Teflon dish had a surface area of 29.61 cm^2 and a volume of 20 mL. Surface radioactivity was determined with a Berthold LB 203E gas flow counter. After the final surface pressure and surface radioactivity were reached, monolayers were collected with about 90% efficiency by aspiration through a glass capillary directly into a scintillation vial, while manually decreasing the molecular area with a movable barrier; the result was corrected for the value measured in an equal volume of subphase.

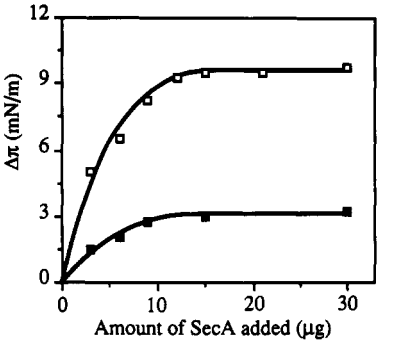

FIGURE 1: Monolayer surface pressure increase (Δπ) as a function of the amount of SecA injected in the subphase (5 mL). Initial pressure, 20 mN/m DOPG monolayer (□); DOPC monolayer (■). Further experimental details are given under Materials and Methods.

For experiments with azide-treated SecA, the protein was incubated with 10 mM sodium azide as described in Oliver et al. (1990b) before each experiment.

RESULTS

Surface pressure increases of lipid monolayers after injection of proteins into the subphase can be interpreted as the result of actual *insertion* of the proteins between the phospholipids of the monolayer, since Demel et al. (1973) established that agents known for interacting only with the phospholipid headgroup did not induce a surface pressure increase in a monolayer. We tested whether SecA could induce such a pressure increase in a DOPG monolayer and indeed found that the surface pressure of a monolayer was increased upon injection of SecA into the subphase. No pressure increase was observed when only the SecA–buffer was injected (data not shown).

To get more insight into the lipid specificity of the SecA interaction, the insertion of SecA into a DOPC and DOPG monolayer (initial surface pressures 20 mN/m) was measured as a function of the amount of SecA injected in the subphase (Figure 1). The pressure increase for DOPG was approximately 3 times higher than for DOPC, at all SecA concentrations, indicating a higher amount of insertion of SecA into a monolayer of negatively charged lipids. Maximal effects were observed when at least 15 μg of SecA was added to a subphase of 5 mL. This amount was used in further experiments although the effective subphase concentration was reduced to 65% of its original value due to SecA adsorption to the Teflon dish as will be discussed below.

More insight in the lipid specificity of the SecA insertion can be gained by varying the initial surface pressure of the phospholipid monolayers. SecA itself is surface-active, as in the absence of a phospholipid monolayer it gives rise to a surface pressure increase of 18.5 mN/m. Therefore, the initial surface pressure was always kept at or above 20 mN/m. Figure 2 shows that the enhanced insertion of SecA into monolayers of negatively charged lipids as compared to zwitterionic phospholipids was not dependent on the initial surface pressure. When DOPS monolayers were used, the same result was observed as compared to DOPG or *E. coli* CL (data not shown), indicating that the nature of the negatively charged phospholipid headgroup does not have a large influence on the final pressure increase. The limiting insertion pressure, defined as the maximal surface pressure allowing SecA insertion, is significantly higher when monolayers of DOPG or *E. coli* CL were used as compared to DOPC or DOPE monolayers. There was a large difference observed between the insertion of SecA into DOPE as compared to the insertion into DOPC while both are zwitterionic phospholipids.

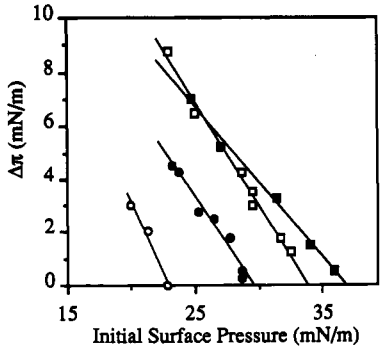

FIGURE 2: Insertion of SecA into a monolayer as a function of the initial pressure. Monolayers of DOPG (□), *E. coli* CL (■), DOPE (●), and DOPC (○). Fifteen micrograms of SecA was injected into a subphase of 5 mL.

Table I: SecA Insertion and Binding to Monolayers of DOPG and DOPE at an Initial Pressure of 25 mN/m

lipid	surface pressure increase (mN/m)	collected monolayer radioactivity (dpm)
DOPG	6.50	5044 ± 83
DOPE	3.50	300 ± 44

This unexpected result demonstrates that SecA may initially interact with PE, the most abundant lipid in *E. coli*, via hydrogen bonding to the lipid headgroup, leading to a more efficient insertion as compared to DOPC.

To get more insight into the different interactions of SecA between zwitterionic phospholipids and negatively charged lipids, radioactively labeled SecA was used to quantify the amount of SecA present at the interface. The surface radioactivity will register both the insertion of SecA in and the adsorption of SecA to the monolayer while the radioactivity in the subphase is not detected (Demel, 1982). The surface pressure increases were identical for both ^{14}C-labeled SecA and unlabeled SecA. Monolayer experiments were performed with DOPG and DOPE monolayers at an initial pressure of 25 mN/m. The rise in the surface pressure parallells the rise of the surface radioactivity, indicating that binding to and insertion of SecA in the phospholipid monolayer occur coincidentally. The surface radioactivity in the experiments with the DOPG monolayers was much higher as compared to the experiments with the DOPE monolayer, implying that there is less binding of SecA to DOPE as compared to DOPG. Washing the subphase after the surface pressure and radioactivity had reached a maximum did not result in a change in surface radioactivity or surface pressure, demonstrating that SecA is not loosely bound to the monolayer so that it could be washed away. The amount of binding of SecA to the monolayers was quantified by collecting the monolayer (Table I). These data are consistent with the earlier reported data of Lill et al. (1990) that SecA only binds to liposomes of negatively charged lipids. The effective SecA concentration in the subphase could be determined by counting the radioactivity of a sample of the subphase before the subphase was washed and was established to be 65% of the added SecA.

The more efficient insertion of SecA in negatively charged phospholipid monolayers is indicative of an interaction based on electrostatic attraction. However, high salt concentrations had only a minor effect on the insertion of SecA into a DOPG monolayer (Figure 3), suggesting that the interaction as reflected by the pressure increase is not dominated by electrostatic interactions and that hydrophobic interactions are evidently involved. The insertion rate of SecA into the DOPG monolayer was increased at higher salt concentrations. This

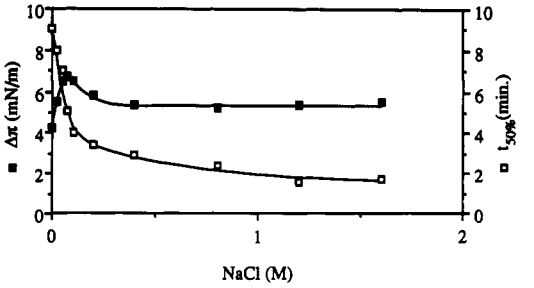

FIGURE 3: Influence of the ionic strength of the subphase on the extent of insertion and insertion rate of SecA in a DOPG monolayer, initial surface pressure 25 mN/m. Final pressure increase (■); time required to obtain 50% of the final increase (□). SecA was injected into a subphase of 50 mM Tris-HCl containing the indicated concentrations of NaCl.

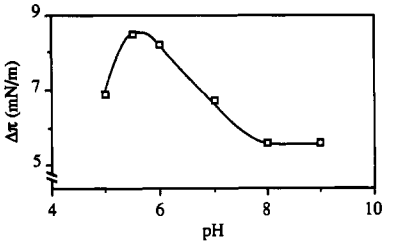

FIGURE 4: Effect of the subphase pH on the surface pressure increase induced by SecA into monolayers of DOPG, initial surface pressure 25 mN/m. A 100 mM NaCl/50 mM phosphate buffer was used as subphase at the indicated pH values.

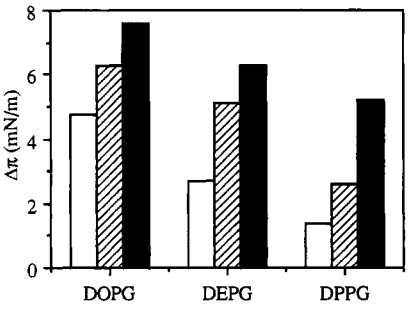

FIGURE 5: Effect of the temperature and the lipid acyl chain unsaturation on the insertion of SecA into DOPG, DEPG, and DPPG monolayers, initial surface pressure 25 mN/m, at 17 °C (white bars), 31 °C (striped bars), and 42 °C (black bars).

probably reflects a decrease of the initial electrostatic repulsion by shielding of the negative charges on the protein (SecA has an excess of 26 acidic amino acids; Schmidt et al., 1988) and the phospholipids. The effect of pH on the insertion of SecA into monolayers is given in Figure 4. In the pH range 5.0–9.0, especially the charge profile of the protein is predicted to be affected. To cover this pH range, a phosphate buffer was used. At pH 7.6, the surface pressure increase using a phosphate buffer was identical to that for the Tris buffer used in the other experiments. These results demonstrate that phosphate, a product of the SecA ATPase activity, does not influence SecA insertion into a DOPG monolayer. Lowering the pH from 8.0 to 5.5 gave a significant increase in the insertion of SecA into a DOPG monolayer. Also, the insertion rate of SecA was increased, comparable to the increase in the insertion rate observed at high ionic strength (data not shown). Lowering the pH beyond 5.5 caused a reduction of the insertion of SecA.

By comparing the insertion of SecA into DOPG, DEPG, and DPPG monolayers, insight could be gained into the effect of acyl chain packing in the monolayer on the insertion of SecA (Figure 5). With decreasing unsaturation, the molecular packing increases. SecA insertion into DEPG monolayers was lowered as compared to DOPG monolayers at 17, 31, and 42

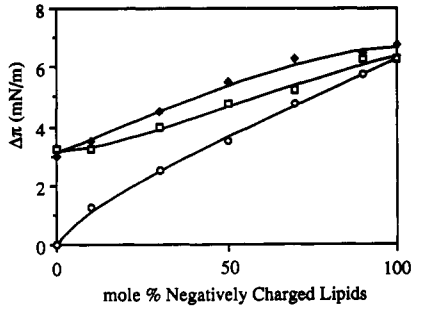

FIGURE 6: Insertion of SecA as a function of the amount of negatively charged lipids in the monolayer. Mixed monolayers of DOPE–DOPG (□), DOPE–*E. coli* CL (♦), and DOPC–DOPG (○) were spread at 25 mN/m initial pressure.

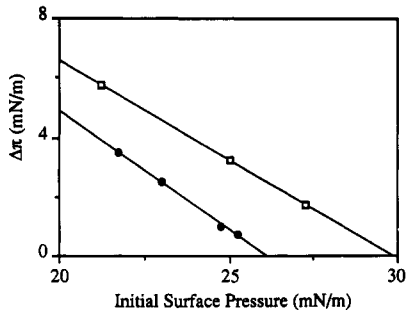

FIGURE 7: Insertion of SecA as a function of the initial surface pressure of monolayers of phospholipid extracts of wild-type inner membrane vesicles of *E. coli* strain SD12 (□) and of PG-depleted inner membrane vesicles of *E. coli* strain HDL11 (●).

°C. Although the molecular area of DEPG is reduced compared to DOPG, it is not in a condensed state at the conditions used. On the other hand, DPPG was found to be in a condensed state at 17 and 25 °C (data not shown). The SecA insertion in DPPG monolayers at 17 °C was, however, not completely ceased under these conditions.

To determine the anionic lipid concentration dependency of the SecA interaction, mixed monolayers of anionic and zwitterionic lipids were formed. The insertion of SecA showed a nearly linear dependency on the amount of DOPG in a DOPE or DOPC monolayer (Figure 6). There was only a minor deviation from linearity when DOPG was replaced by *E. coli* CL in mixtures with DOPE. These results show that there are only small differences in SecA interaction between these anionic lipids, also in mixtures with DOPE.

Translocation of a precursor protein into PG-depleted inner membrane vesicles of *E. coli* is severely hampered (De Vrije et al., 1988; Kusters et al., 1991). One possible explanation could be reduced SecA insertion into the bilayer. Therefore, we examined the insertion of SecA into phospholipid extracts of wild-type and PG-depleted [composition (mol % on P_i basis): 90.5% PE, 1.8% PG, 1.3% CL, and 6.3% PA; Kusters et al., 1991] inner membrane vesicles from *E. coli* strains SD12 and HDL11 (a strain in which PG synthesis can be regulated; Kusters et al., 1991), respectively. We found indeed a reduced insertion of SecA into monolayers of lipid extracts of PG-depleted vesicles as compared to the lipid extracts of wild-type inner membrane vesicles (Figure 7). The absolute difference increased with increasing initial surface pressure, and the limiting pressure for SecA insertion was higher for monolayers formed of lipid extracts of wild-type inner membrane vesicles.

The ATPase activity of SecA is stimulated by negatively charged lipids (Lill et al., 1990). It is suggested that ATP hydrolysis and ATP binding of SecA have different functions in the translocation of precursor proteins (Schiebel et al., 1991). Therefore, we examined the effect of ATP on the

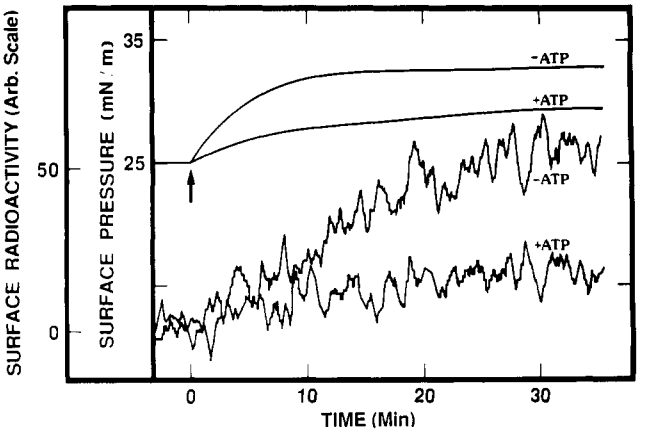

FIGURE 8: Effect of ATP, present in the subphase before SecA was injected, on the insertion of SecA in a DOPG monolayer. ATP (2 mM final concentration) was added to the subphase buffer, containing 2 mM $MgCl_2$, from a stock solution of 0.7 M before the lipid monolayer was spread at an initial surface pressure of 25 mN/m. ATP and $MgCl_2$ present (+ATP); no ATP or $MgCl_2$ present (−ATP).

Table II: Effect of ATP and Analogues on the Insertion and Binding of SecA to DOPG Monolayers at an Initial Pressure of 25 mN/m[a]

addition	surface pressure increase (mN/m)[b]	collected monolayer radioactivity (dpm)[c]
none	6.2 ± 0.2	2131 ± 44
ATP	2.9 ± 0.4	622 ± 58
ADP + P_i	3.1 ± 0.4	1789 ± 50
AMP-PNP	5.7 ± 0.1	1828 ± 89
ATP-γ-S	5.5 ± 0.1	1755 ± 78

[a] The SecA in these experiments had a specific activity of 95 500 dpm/nmol. In order to use a minimum amount of radiolabeled SecA, the Teflon dish was used with a surface area of 8.81 cm^2 and a subphase volume of 6 mL. [b] These data include both radioactive and nonradioactive SecA. [c] These data represent the mean of two experiments and the spread of the data.

interaction of SecA with DOPG monolayers. In Figure 8, we show two experiments in which radioactively labeled SecA was injected (indicated by the arrow) in the subphase buffer which either contained no ATP or $MgCl_2$ (−ATP) or was supplemented with 2 mM ATP and $MgCl_2$ (+ATP). Under these conditions, less than 1% of the ATP was converted into ADP and phosphate (P_i) by the SecA protein (data not shown). Both surface pressure and surface radioactivity were recorded simultaneously. It is clear from the results that SecA insertion into a DOPG monolayer is reduced in the presence of ATP and $MgCl_2$. The amount of SecA detected by the surface radioactivity is even more strongly reduced in the presence of ATP and $MgCl_2$. This indicates that also the binding of SecA to the DOPG monolayer is reduced in the presence of ATP. The quantification of these experiments by collecting the monolayer and counting the radioactivity is presented in Table II. It could be argued that the reduction of the SecA penetration is the result of the presence of $MgCl_2$ in the subphase, which has a condensing effect on the DOPG monolayer. However, in the presence of $MgCl_2$ in the subphase, the insertion of SecA to the DOPG monolayer was less than 10% reduced (data not shown).

Addition of ATP after the surface pressure increase due to SecA insertion in the DOPG monolayer, in the presence of 2 mM $MgCl_2$ had reached a maximal value, did not result in a significant decrease of the surface pressure and the surface radioactivity (data not shown).

To test the effect of only ATP binding to SecA without hydrolysis, nonhydrolyzable analogues of ATP (AMP-PNP and ATP-γ-S) were used. The presence of AMP-PNP (5

mM) and $MgCl_2$ (2 mM) in the subphase before SecA addition gave only a small effect on the insertion or binding of SecA to a DOPG monolayer as compared to the SecA insertion and binding with no addition. ATP-γ-S (5 mM) gave similar results (Table II).

The effects of ADP and P_i on the SecA–lipid interaction were also studied, since they are the products of the SecA ATPase activity and also could influence the SecA–lipid interaction. When ADP and P_i (2 mM final concentration) were present in the subphase before SecA was injected, a similar decreased SecA insertion as compared to the presence of ATP could be observed (Table II). However, the binding of SecA in the presence of ADP and P_i was similar to the binding of SecA in the presence of the nonhydrolyzable analogues of ATP.

It is reported that azide has an inhibitory effect on the precursor-stimulated SecA ATPase activity in the presence of inner membrane vesicles (Oliver et al., 1990b). We examined the effect of azide treatment of SecA on the insertion into DOPG monolayers. No effect of azide (10 mM) on the SecA insertion was found both in the presence and in the absence of ATP and $MgCl_2$ (data not shown). Therefore, we conclude that azide treatment of SecA has no effect on SecA–lipid interactions.

Discussion

Until now, no study of the SecA–lipid interactions concerned actual SecA insertion between the phospholipids. The main result of this study is that SecA *can* insert into a phospholipid monolayer and this insertion is enhanced by negatively charged lipids. SecA is an acidic protein; the sum of the Glu plus Asp residues exceeds the sum of the Lys and Arg by 26 residues (Schmidt et al., 1988). How does such a negatively charged protein preferentially insert into monolayers of negatively charged lipids? A possible explanation for this is the presence of a region rich in positively charged residues. In the amino acid sequence of SecA, the region of the first 167 residues has an excess of 7 positively charged residues. This might be the region interacting with the phospholipids. The repulsion between the overall negative charge on SecA and the negative charges on DOPG is probably the first barrier to be overtaken by SecA before it can bind to the monolayer, since high ionic strength shields the negative charges, thereby lowering the barrier, and the rate of SecA insertion was enhanced. It is shown that SecA binding to liposomes of negatively charged lipids results in an enhanced protease sensitivity (Shinkai et al., 1990), indicating that a conformational change of SecA takes place at the membrane surface. This conformational change could expose hydrophobic regions of SecA, allowing the interaction of SecA with the monolayer to become more hydrophobic and as a result the SecA–lipid interaction can be less influenced by high ionic strength.

The insertion of SecA into monolayers of negatively charged lipids could be detected up to initial surface pressures of 34 mN/m for DOPG and 36 mN/m for *E. coli* CL. The biological membrane pressure (for an erythrocyte membrane) is established to be between 31 and 34 mN/m (Demel et al., 1975). Thus, SecA has the potency to insert into a biological membrane. If SecA insertion into the inner membrane is physiologically relevant, the negatively charged lipids in the inner membrane would play an important role. Using phospholipid extracts of PG-depleted and wild-type inner membrane vesicles, we could establish that the insertion of SecA was higher in monolayers of the wild-type extract as compared to the extract of the PG-depleted inner membrane vesicles. Therefore, the possibility exists that the diminished protein translocation in mutants with a low content of PG (and CL) is caused by a reduced ability of SecA to penetrate the lipid bilayer.

Also, the membrane fluidity influences protein translocation. This was demonstrated by the use of *E. coli* strains, auxotrophic for unsaturated fatty acids, in which a reduction of membrane fluidity resulted in a decreased translocation (Ito et al., 1977; Pagès et al., 1977). We showed that SecA insertion into condensed monolayers was reduced. This implies that the protein translocation in the unsaturated fatty acid auxotrophs is inhibited by the reduced ability of SecA to penetrate the bilayer. These results suggest that SecA insertion in the bilayer is of importance for the translocation of precursor proteins.

ATP binding was deduced to be in the first 217 amino acid residues from the amino terminus of SecA (Matsuyama et al., 1991). Interaction of SecA with ATP also resulted in a change of conformation, resulting in an increased resistance to protease digestion (Shinkai et al., 1991). The more rigid structure of SecA due to the interaction with ATP could cause a shielding of the putative domain of positive charges, thereby reducing the binding of SecA with the negatively charged lipids and as a result also reducing the insertion of SecA.

The effects of ATP and the products of the ATPase reaction (ADP + P_i) on the insertion of SecA into a DOPG monolayer were the same. ADP binding by SecA also resulted in an increase in protease resistance to the same amount as ATP did, also indicating a conformational change (Shinkai et al., 1991). These protease resistance studies implied that the SecA conformations in the presence of ATP and ADP may be similar (Shinkai et al., 1991). However, our results indicate that the SecA conformations in the presence of ATP or ADP must be different, since the binding of SecA to the DOPG monolayer in the presence of ADP was a factor of 3 higher.

The effect of ATP binding by SecA on the interaction with DOPG monolayers was examined using nonhydrolyzable analogues of ATP, AMP-PNP and ATP-γ-S. These experiments gave different results as compared to the results obtained with ATP, although also the protease resistance of SecA in the presence of ATP-γ-S was increased to the same extent as compared to the effect of ATP (Shinkai et al., 1991), suggesting that the SecA conformations in the presence of ATP or the nonhydrolyzable analogues are also different. Probably hydrolysis of the phosphate bond changes the SecA conformation. At the monolayer surface, the SecA ATPase activity is expected to be stimulated by the negatively charged lipids (Lill et al., 1990), but due to the relative low SecA concentration, large subphase volume, and low lipid concentration as compared to Lill et al. (1990), this stimulation can be calculated to be undetectable. However, hydrolysis must be present due to the differences in the results with ATP and the nonhydrolyzable analogues. This lead us to a difference in interpretation between ATP *binding* and ATP *hydrolysis*.

Although the presented results do not show actual SecA release due to ATP hydrolysis, we postulate, on the basis of the data obtained in the presence of nucleotides, a cycle of SecA binding, insertion, de-insertion, and dissociation from the membrane. ATP binding by SecA results in efficient binding and insertion of SecA in the membrane. Upon hydrolysis of ATP, SecA de-inserts and dissociates from the membrane. The products of the ATPase reaction, ADP and P_i, increase the affinity of SecA for the membrane, and SecA rebinds to the membrane but does not insert. Alternatively, SecA remains bound to the membrane after ATP hydrolysis. Upon binding of another ATP molecule, the cycle starts again.

Parallels to the in vitro experiments of Schiebel et al. (1991) and this SecA-binding cycle can be drawn. The nonhydrolyzable analogues of ATP (ATP binding) initiated partially the translocation of proOmpA into inverted inner membrane vesicles of *E. coli* (Schiebel et al., 1991). We showed that in the presence of nonhydrolyzable analogues of ATP the SecA insertion into DOPG monolayers was only slightly reduced. Therefore, ATP binding by SecA may initiate the translocation of a precursor protein by insertion of SecA into the bilayer. Upon hydrolyses of ATP, SecA releases the precursor (Schiebel et al., 1991) and probably dissociates from the membrane. Another ATP-binding step could further translocate the precursor.

Future experiments will address the question of whether SecA insertion into the lipid phase of the membrane is occurring during translocation of precursor proteins.

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