# SecA restricts, in a nucleotide-dependent manner, acyl chain mobility up to the center of a phospholipid bilayer

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**Abstract The effects of SecA–lipid interactions on lipid mobility were studied by electron spin resonance (ESR) spectroscopy in bilayer systems containing phospholipids spin-labeled at different positions along the acyl chain. The SecA protein, which functions in protein translocation at the cytosolic side of the *E. coli* inner membrane, was found to decrease the mobility of the lipids upon its interaction with the membrane. The restriction of lipid motion, at all chain positions measured, reflects the ability of SecA to penetrate the membrane. At a 49:1 lipid/protein molar ratio, a second, motionally more restricted component is observed in ESR spectra of phospholipids spin-labeled close to the methyl ends of the chains (12th and 14th positions). Furthermore, SecA was found to eliminate the order-to-disorder phase transition of 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol bilayers. A remarkably strong reduction in the ability of SecA to penetrate the membrane was found when the nucleotides ATP and ADP + $P_i$ were present. The presence of the non-hydrolyzable analogue AMP-PNP had no effect. These results clearly demonstrate that SecA perturbs, in a nucleotide dependent manner, lipid mobility upon insertion into the bilayer. The implications of these findings for translocation of precursor proteins across the *E. coli* inner membrane are discussed.**

*Key words:* Protein translocation; SecA; ESR; Protein–lipid interaction; Nucleotide

## 1. Introduction

SecA is a dimeric protein that is both present in the cytosol and localized peripherally at the cytoplasmic membrane in *E. coli* [1,2]. This protein functions as an ATPase and couples ATP hydrolysis to the translocation of precursor proteins across the *E. coli* inner membrane [3]. SecA interacts functionally with many components of the protein translocation machinery [1], including the negatively charged phospholipids [4]. Experiments with model membrane systems have provided some insight into the mode and consequences of the SecA–anionic phospholipid interaction [5–7]. Insertion of SecA into a phospholipid monolayer was enhanced by negatively charged phospholipids, and both its binding and insertion were modulated by nucleotides [5]. These factors were also found to be important for SecA-mediated vesicle aggregation [6], and led to the proposal of the existence of two lipid-association sites in both monomers of the dimer. One of these sites reflected an almost purely electrostatic interaction, and the other site exhibited both an electrostatic and a hydrophobic mode of interaction. These observations [5] led further to the proposal of a functional SecA insertion–deinsertion cycle, a hypothesis which was supported by analysis of the membrane behaviour of SecA from *B. subtilis* [8].

Very little is known about the extent of SecA insertion into a phospholipid membrane, which is an essential consideration with respect to the mechanism of SecA function in protein translocation. The only study reported so far used the intrinsic tryptophan fluorescence of SecA to demonstrate membrane penetration and yielded information on the localization of these aromatic residues [7]. Due to the interference of nucleotides with the tryptophan fluorescence, the effect of ATP on insertion of the Trp residues cannot be evaluated. What the consequences of the SecA insertion are for lipid mobility is completely unknown, but these are relevant to the mechanism. Changes in lipid mobility could directly or indirectly be necessary for the protein translocation process. We have attempted to obtain information on these questions by using spin-label ESR spectroscopy. This technique has been proven to sensitively monitor different aspects of lipid–protein interactions [9,10]. We were able to detect a strong propensity of the SecA protein to penetrate into a phospholipid bilayer, which led to a reduction in acyl chain mobility in a way that was modulated by different nucleotides.

## 2. Materials and methods

### 2.1. Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), 1,2-dioleoyl-*sn*-glycero-3-phospho-choline (DOPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) were obtained from Avanti (Birmingham, AL). 1-Acyl-2-stearoyl-phosphatidylglycerol spin-labeled at either the 5-, 12- or 14-position in the *sn*-2 chain (5-, 12- and 14-PGSL respectively), were synthesized by B. Angerstein as described in Marsh and Watts [9]. SecA was purified as described [5] and stored as a 3 mg/ml stock solution in 50 mM Tris-HCl, pH 7.6, 10% (w/v) glycerol, 1 mM dithiothreitol at −80°C. AMP-PNP, ADP and ATP were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade or better.

### 2.2. ESR sample preparation

Preparation of samples was performed essentially as described [10,11]. A dried lipid film (0.4 mg) with 1 mol% spin-labeled lipid was hydrated in 6.0 ml 50 mM Tris, 100 mM NaCl, pH 7.6, in the presence of SecA (3.5 mg). The nucleotides (0.5 M) ADP + $P_i$, ATP and AMP-PNP with $MgCl_2$ were added to a final concentration of 2 mM. After incubation for 60 min at 25°C, the lipid–protein complexes were

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*Abbreviations:* ESR, electron spin resonance; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; *n*-PGSL, 1-acyl-2-[*n*-(4,4-dimethyl-oxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphoglycerol; L/P, molar lipid-to-protein ratio.


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isolated by ultracentrifugation at 45,000 rpm for 45 min at 4°C. After centrifugation, the pellets were transferred to ESR capillaries and concentrated further by centrifugation (Labofuge II, Heraeus, 15,000 × *g*, 3 min). After the ESR measurements, the lipid–protein complexes were dissolved in 25 ml 1 M NaOH for determining the lipid and protein contents. The phospholipid concentration was determined according to Rouser [12] and the protein concentration according to the modified method of Lowry [13] with bovine serum albumin as a standard.

*2.3. ESR spectroscopy*

The ESR measurements were performed on a Varian E-12 Century Line 9 GHz ESR spectrometer. The temperature was regulated with a pure nitrogen gas flow system and the sample capillaries were centered in a standard 4 mm quartz tube containing light silicone oil for thermal stability. Conventional, in phase, absorption spectra were recorded with a modulation frequency of 100 kHz and a modulation amplitude of 1.25 gauss peak-to-peak. The total scan width was 100 gauss. In the case of the 5-PGSL, the ESR spectra were quantitated in terms of the hyperfine splitting between the outer extrema ($2A_{max}$), and for 14-PGSL by spectral subtraction [14].

## 3. Results and discussion

*3.1. SecA complexes with lipid bilayers composed of DOPG or DMPG*

The ESR spectra of negatively charged phosphatidylglycerols spin-labelled at the 5th, 12th or 14th C-atom position in the *sn*-2 chain (5-, 12- and 14-PGSL) incorporated in DOPG, with and without SecA, are given in Fig. 1. In the absence of SecA (dashed lines), the spectra showed the mobility gradient within the bilayer, with the most anisotropic (motionally restricted) spectrum arising from the spin-label at the C5 position and the most nearly isotropic (least motionally restricted) spectrum from the C14 position. The binding of SecA causes several effects. A clear increase in anisotropy of the hyperfine splittings was observed in the spectrum for the 5-PGSL spin-label. A similar effect was observed also for one of the two components (the more mobile) in the spectra from the 12-PGSL and 14-PGSL spin-labelled phosphatidylglycerols. In addition, a second, more motionally restricted component was observed in the spectra from these latter two spin-labelled lipids. This second component, which is visible as partially resolved peaks or shoulders in the outer wings of the spectra, is attributed to lipid chains that directly contact membrane-penetrating parts of the protein [9,10].

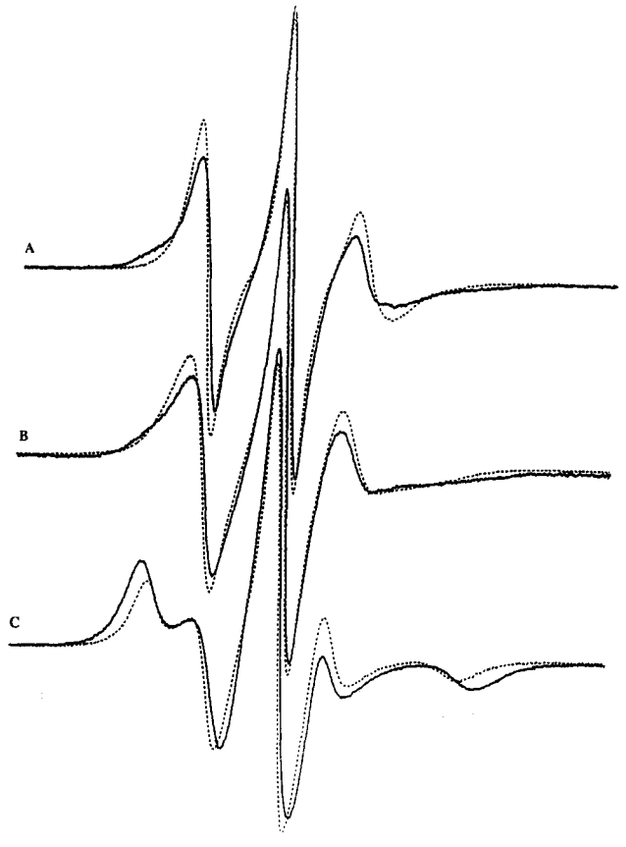


Fig. 1. Effect of SecA binding on the ESR spectra of DOPG dispersions containing spin-labeled PG. ESR spectra of spin-labeled phospholipids 14-PGSL (A), 12-PGSL (B) and 5-PGSL (C) in complexes of DOPG with SecA. Spectra of DOPG lipid dispersions alone are given as dashed lines. SecA was bound to DOPG bilayers at a lipid-to-protein ratio of 49 mol/mol. The spectra are recorded at 20°C. Total scan width = 100 gauss.

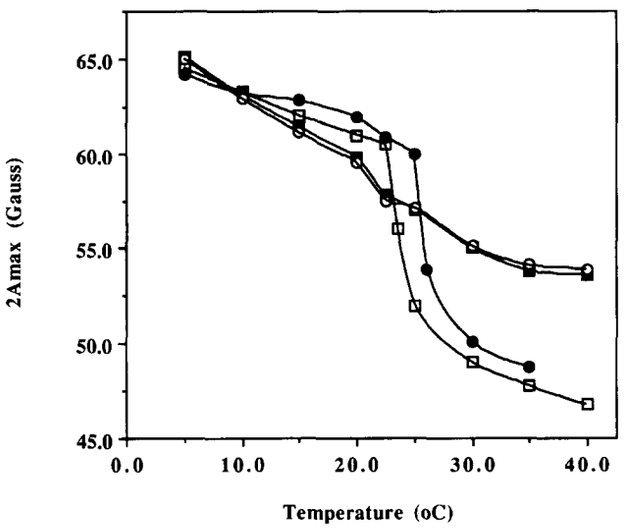


Fig. 2. Phase behaviour of DMPG dispersions doped with 1 mol% 5-PGSL, in the absence and presence of SecA and nucleotides. The temperature dependence of the outer hyperfine splitting ($2A_{max}$) of 5-PGSL is shown for DMPG dispersions in the absence of SecA (□) and in the presence of SecA (■), and upon addition of ADP + $P_i$ (●) or AMP-PNP (○). The lipid-to-protein ratio of the samples containing SecA was 47 mol/mol.

The gel-to-liquid crystalline bilayer phase transition of DMPG offers another tool to study the membrane interaction of SecA. In the absence of SecA, the outer hyperfine splitting ($2A_{max}$) of 5-PGSL in DMPG bilayers changes abruptly at around 23°C, which is the phase transition temperature of that lipid (Fig. 2). Binding of the SecA protein led to a complete elimination of this chain-melting phase transition and resulted instead in a steady decrease of the outer hyperfine splitting with increasing temperature. This result again is consistent with the penetration of (part of) the SecA protein into the hydrophobic region of the bilayer, hence disrupting the cooperative packing of the lipid chains in the gel phase. The ESR results with spin-labelled lipids are therefore in agreement with the interpretation of previous experiments using monolayers [5], and measurements of fluorescent probe anisotropy [6] and intrinsic fluorescence [7], as regards the potential for membrane penetration by SecA. Furthermore, the results with 14-PGSL indicate that the SecA protein penetrates deeply into the membrane bilayer,

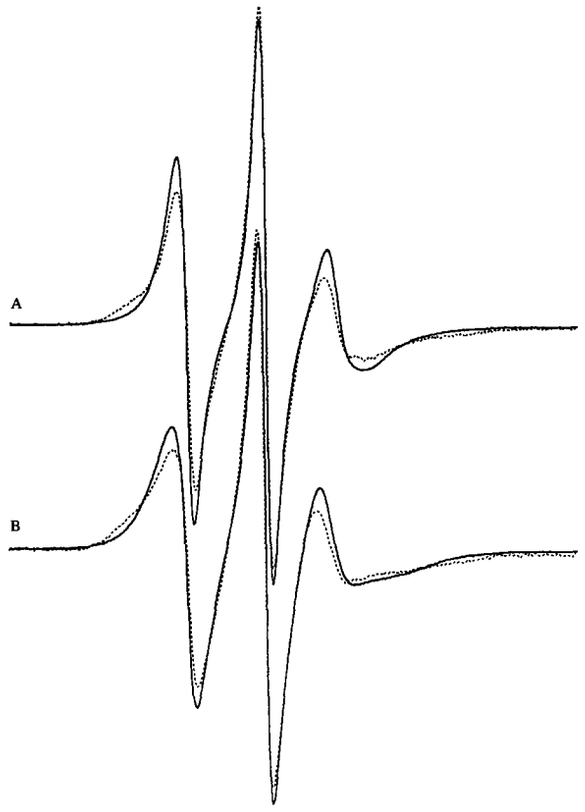


Fig. 3. ESR spectra of spin-labeled phosphatidylglycerols in complexes of SecA with DOPG dispersions in the absence and presence of ADP+$P_i$. ESR spectra of 14-PGSL(A) and 12-PGSL(B) in complexes of SecA with DOPG dispersions, in the absence (dashed lines) and presence of ADP+$P_i$ (solid lines). The spectra are recorded at 20°C. Total scan width = 100 gauss.

at least as far as the 14-C atom position of the lipid chains. Using a spectral subtraction program essentially as described [14], we could calculate from the experimental ESR spectra of 14-PGSL in DOPG (cf. Fig. 1A, solid line) that approximately 30% of the lipids are motionally restricted. This implies that one SecA interacts directly with 15 DOPG molecules, and that only a relatively small part of the 901-residue SecA protein is involved in deep penetration into the lipid membrane.

### 3.2. *Effect of nucleotides on the insertion of SecA into DOPG bilayers*

In view of the ATPase activity of SecA and the reported [5] nucleotide-mediated insertion of SecA into lipid monolayers, we were particularly interested to study the effect of nucleotides on SecA–lipid interactions. As shown in Fig. 2, the presence of ADP+$P_i$, the products of the ATPase reaction, nearly completely eliminated the large effect that SecA has on the phase transition of DMPG. Only a small upward shift of the transition temperature is apparent that probably results from the peripheral association of SecA. Strikingly, addition of AMP-PNP, a non-hydrolysable ATP analog but which, like ATP, binds to the protein, did not reverse the strong effects that SecA alone has on the phase transition. Addition of 2 mM $MgCl_2$ alone, which was included with the added nucleotides, also had no effect (data not shown). The ESR spectra of the 12- and 14-PGSL spin-labels in DOPG dispersions together with SecA in the presence or absence of nucleotides are given in Fig. 3. The effect of the presence of nucleotides is immediately clear from these spectra. The second broader component that is induced in the outer wings of the spectra upon addition of the protein is completely absent in the presence of ADP+$P_i$. Similar results were observed on the addition of ATP (data not shown). The presence of nucleotides under the conditions used did not significantly alter the lipid-to-protein ratio (49:1 mol/mol) of the SecA–DOPG complexes. Therefore the disappearance of the motionally restricted lipid component is most likely due to a conformational change that reduces the membrane penetration of the SecA protein. Protease experiments have suggested a conformational change of SecA upon binding of ATP [15]. Control samples containing 2 mM $MgCl_2$ in the presence or absence of SecA did not show any change in the ESR spectra by $Mg^{2+}$ alone, just as with the DMPG phase transition (data not shown). Table 1 summarizes the results obtained with the 5-PGSL spin-label in DOPG; for comparison the data in DMPG are also included. It is found that the presence of the nucleotides ADP+$P_i$ and ATP decreased the outer hyperfine splitting of the 5-PGSL spin-label in the SecA–DOPG complex. These results demonstrate that the deep insertion of SecA was strongly reduced if not eliminated by ADP+$P_i$ and by ATP, while AMP-PNP had no effect. This is in agreement with the conformational changes proposed to take place in SecA upon binding and hydrolysis of ATP [15].

## 4. Concluding remarks

SecA was found to be able to insert up to the 14-position of the acyl chains of PG bilayers such that approximately 15 DOPG molecules are affected directly in their mobility upon insertion of one SecA molecule. Very recent trypsin accessibility experiments suggests that SecA can even (partially) traverse a lipid bilayer [16].

Most strikingly the deep insertion of the SecA molecule is (nearly) completely blocked by ATP or the products of the ATPase reaction, whereas ATP binding (mimicked by AMP-PNP) does not interfere with deep insertion. These data support and extend the previous proposed cycle of SecA binding, insertion, deinsertion and dissociation [5]. This cycle could facilitate translocation of precursors across the bacterial inner membrane, for instance by pushing segments of the mature region of a precursor across the membrane in a cyclic manner. The SecY and E proteins could control this process. Very recently, support for this model was obtained in studies in the prokaryotic secretion pathway [17,18].

Finally SecA can be considered to be a member of a novel

Table 1
Outer hyperfine splittings, $2A_{max}$, of 5-PGSL incorporated in DOPG (or DMPG) dispersions on binding of SecA at L/P = 49 mol/mol in the absence and presence of 2 mM nucleotides*

| Sample | $2A_{max}$ (Gauss) |
|---|---|
| Lipid alone | 48.5 (49.0) |
| + SecA | 53.1 (55.0) |
| + SecA + [ADP+$P_i$] | 51.1 (50.2) |
| + SecA + [ATP] | 50.4 (n.d.) |
| + SecA + AMP-PNP | n.d. (55.1) |

*Samples were measured at 30°C. Values in parentheses correspond to DMPG, and those without to DOPG.

class of membrane interacting proteins which can switch between a loosely associated and deeply membrane embedded form. The mitochondrial precursor apocytochrome *c* and the C-terminal fragment of colicin Ia might be members of that family [19,20].

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