Interaction of Phosphatidylserine Synthase from *E. coli* with Lipid Bilayers: Coupled Plasmon-Waveguide Resonance Spectroscopy Studies

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ABSTRACT The interaction of phosphatidylserine (PS) synthase from *Escherichia coli* with lipid membranes was studied with a recently developed variant of the surface plasmon resonance technique, referred to as coupled plasmon-waveguide resonance spectroscopy. The features of the new technique are increased sensitivity and spectral resolution, and a unique ability to directly measure the structural anisotropy of lipid and proteolipid films. Solid-supported lipid bilayers with the following compositions were used: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC); POPC-1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (POPA) (80:20, mol/mol); POPC-POPA (60:40, mol/mol); and POPC-1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG) (75:25, mol/mol). Addition of either POPA or POPG to a POPC bilayer causes a considerable increase of both the bilayer thickness and its optical anisotropy. PS synthase exhibits a biphasic interaction with the bilayers. The first phase, occurring at low protein concentrations, involves both electrostatic and hydrophobic interactions, although it is dominated by the latter, and the enzyme causes a local decrease of the ordering of the lipid molecules. The second phase, occurring at high protein concentrations, is predominantly controlled by electrostatic interactions, and results in a cooperative binding of the enzyme to the membrane surface. Addition of the anionic lipids to a POPC bilayer causes a 5- to 15-fold decrease in the protein concentration at which the first binding phase occurs. The results reported herein lend experimental support to a previously suggested mechanism for the regulation of the polar head group composition in *E. coli* membranes.

INTRODUCTION

The regulation of the lipid composition in the plasma membrane of several prokaryotic organisms is a well-established phenomenon (see e.g., Rilfors et al., 1994; Morein et al., 1996; Andersson et al., 1996). *Escherichia coli* adjusts the ratio of saturated to unsaturated acyl chains in the membrane lipids when the growth temperature is regulated. The consequence is that the organism maintains the lipids in a lamellar liquid crystalline phase and avoids the formation of both a lamellar gel phase and reversed nonlamellar phases (Morein et al., 1996). In contrast, wild-type cells of *E. coli* have a nearly constant polar head group composition during a wide range of growth conditions. It has been proposed that this regulation occurs through the adjustment of the relative activities of the lipid synthesizing enzymes and not at the gene level (Sparrow et al., 1984; Dowhan, 1997). Therefore, most probably, the membrane lipids themselves are somehow involved in the triggering process of at least some of these enzymes.

Phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG) are the main membrane lipids synthesized by *E. coli*. Many important processes, e.g. electron transport, chemotaxis, solute transport, cell division, and membrane protein assembly, are compromised in the absence of PE. Further, the anionic phospholipids are required for initiation of DNA replication and for translocation of proteins across the inner membrane (Dowhan, 1997; Bogdanov and Dowhan, 1998; Mileykovskaya et al., 1998; van Klompenburg et al., 1998). PE constitutes 70–80 mol%, PG 20–25 mol%, and DPG 5 mol% or less, in wild-type cells of *E. coli* (Cronan and Rock, 1996; Morein et al., 1996; Dowhan, 1997). Four additional anionic phospholipids, including phosphatidic acid (PA), are intermediates in the lipid biosyntheses and constitute less than 1 mol% of the membrane lipids in wild-type cells.

It has been shown that the activity of isolated phosphatidylserine (PS) synthase (CDP-1,2-diacyl-*sn*-glycerol: L-serine *O*-phosphatidyltransferase, EC 2.7.8.8) is increased when it interacts with mixed micelles containing PG, DPG, or PA (Rilfors et al., 1999). Consistent results concerning the effect of PG and DPG have been obtained from in situ studies in which the fraction of these lipids in the *E. coli* inner membrane was manipulated (Sparrow et al., 1984; Saha et al., 1996). A simple model for the regulation of the balance between the fraction of PE, and the sum of the fractions of PG and DPG, has been presented based on these results (Shibuya, 1992; Matsumoto, 1997; Rilfors et al., 1999). The two enzymes PS synthase and phosphatidylglycerophosphate synthase catalyse the first step in the metabolic pathways leading to the syntheses of PE, and PG and DPG, respectively (Raetz and Dowhan, 1990). Both enzymes compete for the substrate CDP-diacylglycerol. PS synthase is a so-called amphitropic enzyme (Burn, 1988), i.e., it exists in an inactive form in the cytoplasm and in an

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active form in its membrane-bound state (Carman and Dowhan, 1979). One essential feature of the suggested regulatory mechanism is that PS synthase molecules are supposed to be more tightly bound to the membrane when the fraction of anionic lipids increases. The enzyme activity would, in this way, be directly linked to the membrane lipid composition, and the regulatory mechanism would yield a balanced composition of PE and anionic lipids.

In this work, we have studied the interaction between PS synthase and bilayers of different lipid composition. From hydrophobicity calculations on the known amino acid sequence of the enzyme, it was suggested that it can be categorized as a relatively hydrophilic protein, because no transmembrane peptide segments were predicted (Rilfors et al., 1999). However, the presence of a nonionic detergent is necessary during the purification of the PS synthase, indicating that the protein exposes hydrophobic regions toward the surrounding solution. Thus, it can be expected that the protein will be able to bind to a lipid bilayer via hydrophobic interactions. Furthermore, it should also be able to bind electrostatically to negatively charged membranes, because there are several basic amino acids located at the N- and C-termini of the protein (DeChavigny et al., 1991).

In the present study, we used a recently developed variant of the surface plasmon resonance (SPR) technique, referred to as coupled plasmon-waveguide resonance (CPWR) spectroscopy (Salamon et al., 1997a, 1998, 1999; Salamon and Tollin, 1999a,b; 2000), to investigate the mechanism of interaction between PS synthase and a lipid membrane. Briefly, this new technique combines a greatly enhanced sensitivity and spectral resolution as compared with conventional SPR (Salamon et al., 1997a,b,c; 1999; Salamon and Tollin, 1998; 1999a,b) with a unique ability to directly measure anisotropies in the refractive index and the optical absorption coefficient in a dielectric film adsorbed onto the surface of the coupled plasmon-waveguide resonator. As a consequence, the technique is especially well suited for studies of the anisotropic structural properties of ordered thin films including lipid and proteolipid membranes.

MATERIALS AND METHODS

Materials

PS synthase was prepared from the *E. coli* strain JA200/pPS3155-l as described by Rilfors et al. (1999). The purification and activity measurements of the enzyme were performed as reported previously (Rilfors et al., 1999), except that the glycerol concentration in the enzyme preparation was reduced to approximately 1.5% (w/v) by gel filtration through Sephadex G-25 M (PD-10 column, Amersham Pharmacia Biotech, Uppsala, Sweden). The enzyme was then concentrated through centrifugation in a Centricon YM-10 filter device (Millipore Corporation, Bedford, MA). The loss of protein during these procedures was about 20%, but the specific activity of the enzyme remained unaltered. The lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (POPG), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (monosodium salt) (POPA), were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Experiments were carried out using protein solutions in 10 mM Tris-HCl buffer, pH 7.3. The ionic strength was adjusted with KCl.

CPWR measurements

The details of the experimental procedures for CPWR measurement and data analysis have been described elsewhere (Salamon et al., 1997a,b,c; 1999; Salamon and Tollin, 1999a,b; 2000). Here we will provide a summary of these methods. The CPWR technique is an extension of the conventional plasmon resonance phenomenon. The latter can only be excited optically by tranverse magnetic polarized light, *p*, under total internal reflection conditions. This generates an outer surface-bound evanescent electromagnetic wave resulting from conduction electron oscillations in a thin metallic layer (for example, silver) deposited on a prism or grating, which is capable of probing the optical properties of a thin dielectric film layered onto this surface. The creation of such surface-bound electromagnetic waves (surface plasmons) is a resonance phenomenon occurring at either a specific incident angle, α_0, when a constant wavelength of light is used for excitation, or at a particular light wavelength, λ_0, when the measurement is performed at a constant value of the incident angle. The resonance can easily be detected by measuring the reflected light intensity, R, as a function of either one of the two above-mentioned variables. Such a measurement of R versus α (or λ) produces a resonance curve that is known as the SPR spectrum.

Conventional SPR technology was first used about 30 years ago (see Salamon et al., 1997b,c for reviews) and has since become a theoretically and experimentally well-established optical technique, which has been widely used in different fields. These include solid-state physics, where it has been used to characterize the structural properties of thin films, and physical chemistry, especially in the study of metal–electrolyte interfaces and Langmuir–Blodgett films (Salamon et al., 1997b,c). Over the last decade, these applications have been extended to include biosensors, pharmaceuticals, analytical chemistry, and membrane biochemistry and biophysics (Salamon et al., 1997b,c).

SPR has several advantages over other techniques for characterizing thin films (Salamon et al., 1997b,c; Salamon and Tollin, 1999a,b; 2000): 1) It is ideally suited to directly probe, in real time, both the dynamics and the structural changes occurring during molecular interactions within such films; 2) it has a superior sensitivity over these other techniques; 3) it is the only technique that allows the evaluation of the complete set of optical parameters, i.e., refractive index and extinction coefficient, in addition to the thickness of the thin film sample (see below); and 4) its simplicity and rapidity are particularly important for systems in which the amount and stability of the material are limiting factors, as is often the case in studies of biomembranes.

As has been described in detail elsewhere (Salamon et al., 1997b,c; Salmon and Tollin, 1999a,b; 2000), these abilities of SPR are a result of two main characteristics of the phenomenon. First, the evanescent electromagnetic field generated by plasmon excitation decays exponentially with penetration distance into an emergent medium adjacent to the outer metal surface, i.e., the depth of penetration into the emergent material extends only to a fraction of the light wavelength used to generate the plasmons. This makes the phenomenon highly sensitive to the optical properties of the metal–emergent medium interface, without any interference from the properties of the bulk volume of the emergent material. Second, the angular position and shape of the resonance curve are very sensitive to the optical properties of both the metal and the emergent medium. Because of the ability of SPR to probe only those properties that are perpendicular to the film plane, its usefulness is limited to isotropic systems (Salamon et al., 1999).

Recently, this technology has been greatly enhanced by the development of the CPWR device (Salamon et al., 1997a; 1999). The important difference between CPWR and conventional SPR lies in the design of the optical resonator. The CPWR device is designed to couple surface plas-

mons excited in a thin metal film (silver film in the device used in this work), with waveguide modes excited in a dielectric film (e.g. SiO_2) deposited onto the outer surface of the silver film. Such a coupling adds several important new properties to those unique features of SPR spectroscopy summarized above: 1) An additional spectroscopic dimension, by generating resonance spectra using the electric fields of both tranverse magnetic, *p*, and transverse electric, *s*, polarized components of exciting light; 2) an increased sensitivity as a consequence of greatly decreasing the half-width of both *s*- and *p*-polarized resonances; and 3) the ability to adjust the resonance linewidth, and therefore the spectral sensitivity, by choosing the appropriate dielectric material as the overcoating. These properties allow evaluation of the thickness and optical parameters of thin films with even higher accuracy than is possible with SPR. What is even more important, such an evaluation can be performed using both *p*- and *s*-polarized resonances, thereby permitting mass and structural analysis of anisotropic molecular films. The technology has previously been tested with biomembrane systems of varying complexity, including measurements of single lipid bilayer structural anisotropy (Salamon et al., 1997a) and mass and structural changes occurring in anisotropic proteolipid systems containing cytochrome b_6f (Salamon et al., 1998). These results have been discussed in a broader theoretical context in a recent review article (Salamon et al., 1999).

SPR or CPWR spectra can be described by three parameters: α (or λ), the spectral width, and the resonance depth, which, in turn, depend on the refractive index, *n*, the extinction coefficient, *k*, and the thickness, *t*, of the sample. Thin film electromagnetic theory based on Maxwell's equations provides an analytical relationship between the spectral parameters and the sample properties (Salamon et al., 1997b,c), where the sample is modeled as a uniform layer (or layers) of anisotropic dielectric material. In the present case, in the absence of added protein, this layer includes a lipid bilayer membrane with water molecules bound to the head groups of the lipid molecules and to the SiO_2 surface (see Fig. 2 in Salamon et al., 1998 for more details). When protein is added to this system, these molecules are incorporated into the model as described in more detail below. This allows a unique evaluation of average values of *n*, *k*, and *t* by nonlinear least-squares fitting of a theoretical spectrum to the experimental one (Salamon et al., 1997b,c, 1999). As has been demonstrated previously (Salamon et al., 1997a), the high sensitivity of the CPWR device allows the determination of the above three parameters of a thin film (e.g., a membrane bilayer) with accuracies better than 0.001, 0.002, and 1 Å, respectively. In practical terms, this means that, in many cases, the limitation on accuracy will result not from the measuring technique itself, but from the ability to create a lipid membrane in a reproducible manner.

As noted above, the film parameters can be evaluated for both *p*- and *s*-polarizations, thereby allowing characterization of the molecular ordering in the film by determining the anisotropy in *n*. In addition, because the refractive index reflects a mass density (defined as mass per unit volume of deposited material), one can obtain the total deposited mass from the *t* and *n* parameters. Furthermore, preferential orientation of chromophores attached to the molecules within the sensing layer can be determined by measuring the anisotropy of *k*. In the experiments described in this work, the exciting light wavelength used is located away from the absorption maxima of both lipid and protein, and thus, *k* is determined mainly by scattered light due to imperfections in the proteolipid membrane structure.

Preparation of lipid membranes

In the present study, self-assembled solid-supported lipid membranes have been used (Salamon and Tollin, 1991; Salamon et al., 1994a,b, 1996a). The method of preparation uses the principles that govern the spontaneous formation of a freely suspended lipid bilayer membrane (a so-called black lipid membrane) (Mueller et al., 1962). This involves spreading of a small amount of lipid bilayer-forming solution (2–4 mL) across an orifice in a Teflon sheet that separates the thin dielectric film (SiO_2) from the aqueous phase. The hydrophilic surface of hydrated SiO_2 (Gee et al., 1990; Silberzan et al., 1991) attracts the polar groups of the lipid molecules, thus forming a lipid monolayer deposited on a layer of adsorbed water, with the hydrocarbon chains oriented toward the droplet of excess lipid. Subsequent to the first step of lipid membrane formation, the main body of the CPWR cell is filled with the appropriate aqueous solution. This initiates the second step, which involves a thinning process, i.e., formation of both the second monolayer and a plateau– border that anchors the bilayer film to the Teflon spacer, allowing the excess of lipid and solvent to move out of the orifice (Salamon et al., 1996b, 1997b,c). In the present experiments, the lipid films were formed on the SiO_2 surface from the following membrane-forming solutions: 8 mg/mL POPC in squalene/butanol (0.15:10, v/v); 10 mg/mL POPC-POPA (80:20, mol/mol) in squalene/butanol/methanol (0.15:9:1, v/v); 10 mg/mL POPC-POPA (60:40, mol/mol) in squalene/butanol/methanol (0.15:8.5:1.5, v/v); and 8 mg/ml POPC-POPG (75:25, mol/mol) in squalene/butanol/methanol (0.15:9.5:0.5, v/v).

RESULTS AND DISCUSSION

We have obtained new insights into the mechanism of the PS synthase interaction with lipid bilayer membranes by measuring protein binding to the membrane over a wide range of protein concentrations, bilayer compositions, and buffer ionic strengths. To address the role of electrostatic interactions, we introduced the anionic lipids POPA and POPG into the POPC membrane. In addition, experiments of protein binding have been performed at two ionic strengths: 15 mM (low) and 150 mM (high). By analyzing the CPWR spectra, we have directly monitored structural changes (mass density and molecular ordering) in the proteolipid membrane that occur during the binding process. The possible significance of these findings to the function of PS synthase in *E. coli* is discussed in the Conclusions section.

Lipid membrane structure

Figure 1 shows examples of CPWR spectra obtained with a lipid membrane formed from a solution containing POPC-POPA (80:20, mol/mol) at low ionic strength, using both *p*- and *s*-polarized components of exciting light. The optical parameters (i.e., *n* and *k*) obtained with *p*-polarization and *s*-polarization refer to the perpendicular and parallel directions with respect to the bilayer membrane surface, respectively (Salamon et al., 1997a; Salamon and Tollin, 1998). As a consequence, these parameters measure the anisotropic character of the lipid membrane structure. Inasmuch as the lipid does not directly absorb the exciting light (the wavelength of the laser excitation, 632.8 nm, is far removed from the absorption bands of POPC, POPG, and POPA, and PS synthase), a *k* value other than zero reflects a diminution of measured light intensity due only to scattering processes that result from imperfections in the lipid film. It is expected that, in anisotropic films, the two polarized components of exciting light will be scattered differently, thereby producing a scattering anisotropy; this effect will not be discussed further.

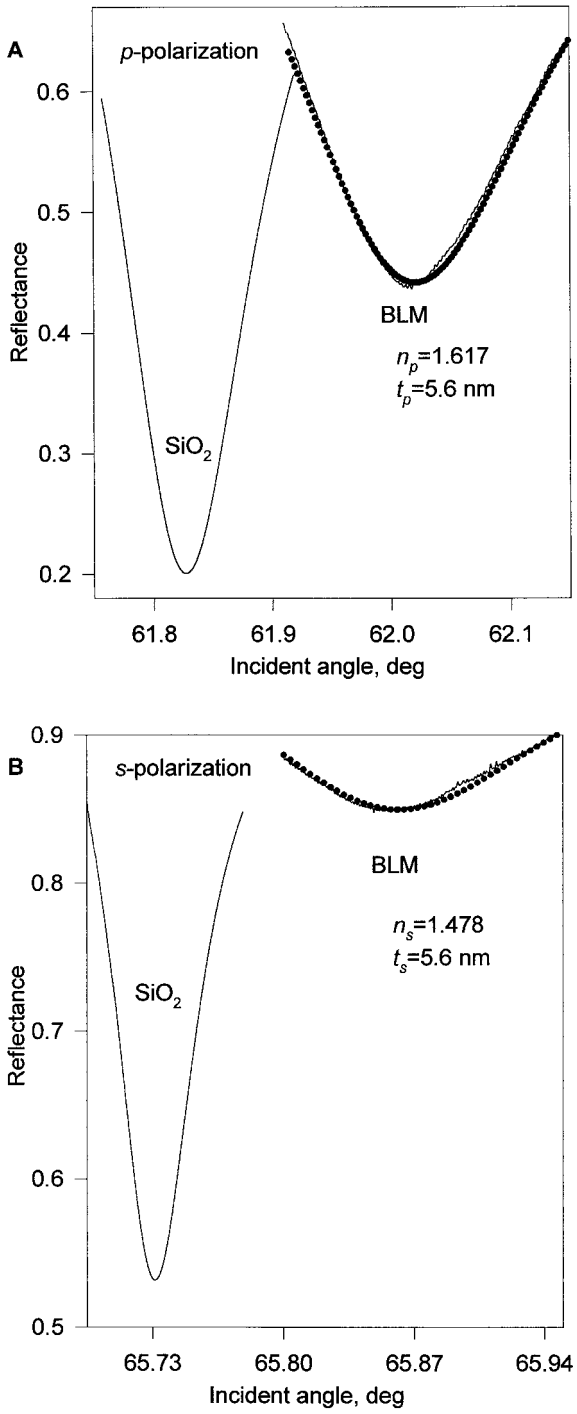


FIGURE 1 CPWR spectra obtained with bare SiO_2 and with a self-assembled solid-supported lipid membrane formed on the SiO_2 surface from a solution of 10 mg/mL lipid (POPC-POPA, 80:20, mol/mol) in squalene/butanol/methanol (0.15:9:1, v/v). The spectra were measured with 632.8 nm laser light, using both (*A*) *p*- and (*B*) *s*-polarization. Closed circles represent theoretical fits to the experimental data (*solid lines*). The buffer was 10 mM Tris-HCl, pH 7.3.

The anisotropy of the refractive index, A_n, can be defined as (Salamon et al., 1998)

$$A_n = (n_p^2 - n_s^2)/(n_{av}^2 + 2), \quad (1)$$

where n_{av} is an average value of the refractive index, given by

$$n_{av}^2 = 1/3(n_p^2 + 2n_s^2), \quad (2)$$

and n_p and n_s are the refractive indices measured in the perpendicular and parallel directions within the lipid membrane, respectively. There are, in general, two possible contributions to the refractive index anisotropy. One comes from the two-dimensional nature of the lipid membrane itself, and the other from the ordering of anisotropic molecules within the two-dimensional structure. As we have demonstrated elsewhere (Salamon et al., 1997a), the major source of the anisotropy in n lies in the anisotropic character of the lipid molecules comprising the bilayer structure. Therefore, A_n can be related to the molecular shape and the components of the mean polarizabilities parallel and perpendicular to the lipid membrane (Den Engelsen, 1976; De Jeu, 1978), and reflects the degree of molecular order within the membrane. The larger the value of A_n the higher is the molecular ordering in the lipid film.

A_n contains information about the so-called order parameters, S, defined by Saupe (1964), which characterize the ordering of molecules relative to the normal (the director) of the lipid bilayer. If the polarizability tensor of the molecule under study is known, it should, in principle, be possible to extract these order parameters from a determination of A_n. In this work, we will present only a qualitative description of the changes in molecular ordering in the lipid bilayer as the *E. coli* PS synthase interacts with it.

Table 1 gives the values of n and t, and the corresponding A_n values, obtained with four types of lipid membranes and both polarizations, in the absence of PS synthase. Although the membranes have been made using different lipid solvent compositions (see Materials and Methods), the results demonstrate some important facts regarding the anisotropic properties of the bilayers:

1. The value of n_p is consistently and significantly larger than that of n_s. This is a result of the ordering of the lipid molecules along the normal to the membrane (the director). As Eq. 1 indicates, the higher the order, the larger the difference between these two refractive indices. This observation is especially important in the context of conventional SPR measurements where only *p*-polarized excitation light can be used. Such measurements, therefore, cannot adequately describe the anisotropic nature of lipid and proteolipid membranes. In addition, there will also be an error in the calculation of the mass using conventional SPR, which is usually based on the value of the perpendicular refractive index, n_p (Salamon et al., 1999). For anisotropic systems, it is apparent that the

TABLE 1 Summary of the optical parameters for lipid bilayers formed from the solutions described in Materials and Methods

Lipid Bilayer	t (±0.1 nm)	n_p (±0.001)	n_s (±0.0002)	n_{av} (±0.001)	A_n (±0.002)
POPC	5.4	1.570	1.4800	1.511	0.060
POPC-POPG (75:25, mol/mol)	6.0	1.673	1.4590	1.533	0.154
POPC-POPA (80:20, mol/mol)	5.6	1.617	1.4780	1.526	0.100
POPC-POPA (60:40, mol/mol)	5.9	1.617	1.5160	1.550	0.072

t, thickness; n_p, n_s, refractive indices obtained with either p- or s-polarizations, n_{av}, average value of the refractive index; A_n, anisotropy of refractive index.

mass can only be accurately calculated from the n_{av} parameter. In fact, it is evident from Eqs. 1 and 2 that ordered systems may very well have the same value of n_{av}, despite having much different values of n_p and n_s. Conventional SPR would be unable to detect this.

2. As Table 1 indicates, the optical parameters of the lipid membrane are significantly changed by variations in the composition of the lipid mixture. The results demonstrate that addition of either POPG or POPA to POPC causes a considerable increase of both the bilayer thickness and its optical anisotropy. This effect is similar to that observed when long-chained hydrocarbons or cholesterol are incorporated in lipid bilayer membranes (Bloom et al., 1991; Davis et al., 1980; Nezil and Bloom, 1992; Salamon et al., 1996b; Thoma et al., 1996) and can be explained by an increase in the degree of ordering of the lipid molecules, which results in higher packing density and larger overall thickness. Furthermore, it has been shown that anionic lipids diminish lipid bilayer undulations (Evans and Needham, 1987), an effect that will also result in an increase in A_n.

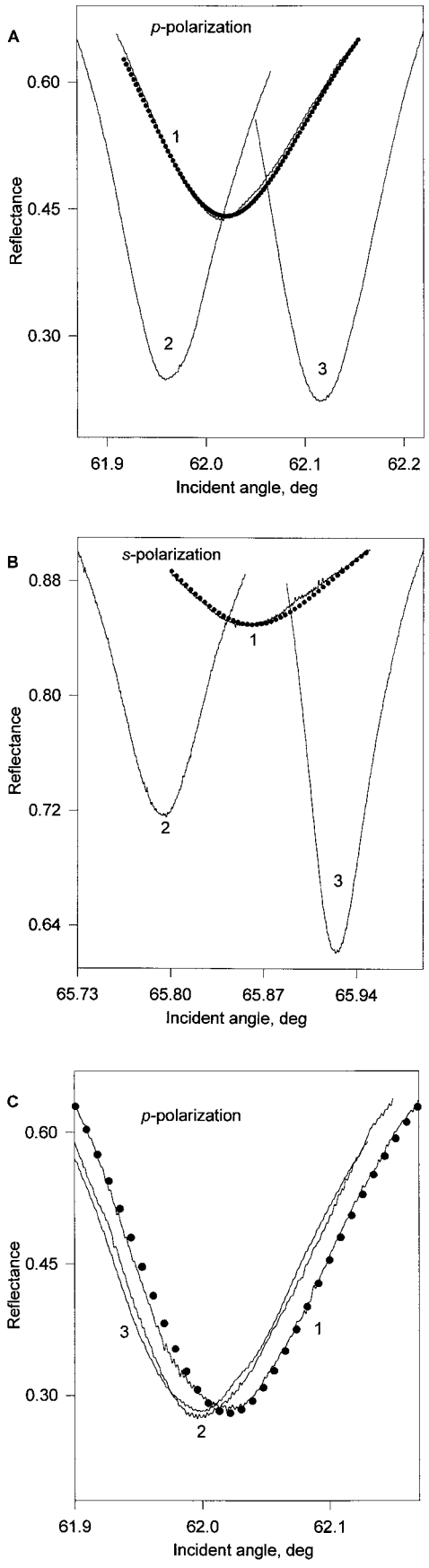
Biphasic interaction of PS synthase with POPC-POPA membranes

The binding of protein to a lipid membrane, and consequent structural alterations, can be followed by measuring the p- and s-polarized CPWR spectra obtained with preformed lipid bilayers upon addition of small aliquots of a concentrated solution of protein to the aqueous compartment of the sample cell. The results shown in Fig. 2, obtained with a bilayer consisting of POPC-POPA (80:20, mol/mol), clearly illustrate that the CPWR spectral changes involve both a shift of the resonance position and alterations in the curve shape due to changes in the resonance depth and width. As the protein concentration is increased using a low ionic strength buffer, we initially observe a shift of the resonance position toward smaller incident angles (defined as a negative shift, measured relative to the initial lipid bilayer resonance position; cf. Fig. 2, *A* and *B*, *curves 1 and 2*). This is followed by a shift in the opposite direction (defined as a positive shift), which ultimately saturates at high protein concentrations (Fig. 2, *A* and *B*, *curve 3*). This pattern of spectral changes occurs with all the lipid membranes used in this work at low buffer ionic strength.

An increase in the buffer ionic strength to 150 mM causes significant alterations to this general pattern of spectral changes (Fig. 2 *C*). The magnitude of the negative resonance spectral shift is decreased, and the positive resonance shift is no longer observed, probably because it is displaced to very high protein concentrations beyond the range used in this work. Furthermore, the spectral changes upon PS synthase binding to the lipid bilayer are considerably smaller at the high ionic strength, implying smaller structural perturbations in the membrane.

These rather complex spectral changes, which occur both in the position and in the width and depth of the resonance spectrum, indicate a corresponding complexity in the mass and structural alterations occurring in the proteolipid membrane as a result of PS synthase binding to the lipid bilayer. To characterize these processes further, it is useful to examine the resonance position shift as a function of added protein, because this parameter provides the best indication of total proteolipid mass changes. Figures 3 and 4 show such plots for the low and high ionic strength experiments, respectively. Deconvolution of the experimental curves presented in these two figures (Fig. 3, *dashed lines*) clearly indicates the presence of either two binding phases (at low ionic strength, as shown in Fig. 3), or one binding phase (at high ionic strength, as shown in Fig. 4). Note that the first phase is, itself, biphasic, i.e., it consists of resonance shifts in both the negative and the positive directions and can be deconvoluted into two hyperbolic curves (Figs. 3 and 4), whereas the second phase, occurring at higher protein concentrations, can be fit by a single sigmoidal function (Fig. 3). The latter deconvolution implies that cooperativity is involved, i.e., the second phase does not occur until the first phase is complete. Furthermore, the protein binds more tightly in the first phase (lower dissociation constants) than in the second phase. The first phase occurs at somewhat lower protein concentrations at low ionic strengths (Figs. 3 and 4), indicating that the binding constants for this phase are affected by electrostatic forces. However, strong hydrophobic interactions are also involved in the first binding phase, as will be discussed in detail below. In contrast, the second binding phase, occurring at higher protein concentrations, is predominantly sensitive only to the electrostatic interactions between the protein and the membrane.

The observation that PS synthase is strongly bound to a lipid bilayer by hydrophobic forces in the first binding



phase is not surprising in view of the fact that the protein is stabilized and activated in the presence of detergent micelles (Rilfors et al., 1999). This clearly indicates that the protein prefers conformations that expose hydrophobic surfaces. However, as will be discussed further below, electrostatic interactions do play a role in the initial binding process, probably by increasing the local concentration of the protein at the membrane surface and by facilitating the insertion process into the membrane interior.

To obtain further insights into the processes involved in PS synthase binding, we must first describe the possible causes of the CPWR spectral shifts. Positive and negative shifts of the resonance position are the result of an increase or a decrease, respectively, in the value of the refractive index of the membrane (assuming $\Delta k = 0$). There are two possible physical mechanisms that can generate changes in the refractive index. 1) Changes in mass density (packing density) of the molecules in the film, i.e., the changes in the surface area occupied per molecule: in the present system, this can be accomplished by transferring lipid molecules between the bilayer and the plateau–Gibbs border, resulting in a thinning or a thickening of the membrane. 2) Alterations in the molecular ordering in the bilayer: in general, the degree of long-range molecular order that occurs in lipid and proteolipid membranes is correlated with the molecular packing density. Thus, the higher the packing density, the larger is the degree of molecular ordering, i.e., the higher is the anisotropy, and vice versa. To completely characterize the structural changes that occur in anisotropic systems, the contributions of the mass density and the molecular ordering have to be separated, at least conceptually. This generally constitutes a major experimental problem, because, to accomplish this, it is necessary to separately measure the polarized components of the refractive index. The ability of the CPWR technique to do this makes it a powerful tool in membrane studies.

We have carried out the analysis of the experimental data obtained at low protein concentration by applying the following model, which allows us to calculate and analyze the changes in mass density and molecular ordering occurring in the proteolipid system. The CPWR results (Figs. 2 and 3) clearly show that both the *p*- and *s*-polarized spectra initially

FIGURE 2 (*A* and *B*) CPWR spectra obtained with the lipid membrane described in Fig. 1 (experimental spectrum, *curve 1*; theoretical fit, *closed circles*), with both (*A*) *p*-polarization and (*B*) *s*-polarization. Curves 2 (0.135 μM PS synthase) and 3 (3.27 μM PS synthase) were obtained after addition of the indicated amounts of protein solution to the sample compartment of the CPWR cell, which contained buffer solution at low ionic strength (15 mM). (*C*) CPWR spectra obtained with *p*-polarization with the lipid membrane and under conditions described in (*A*) and (*B*) using high ionic strength (150 mM) buffer solution. Curve 1 (*solid line*, experimental; ●, theoretical fit) was measured in the absence of protein, curve 2 was obtained at low protein concentration (0.225 μM PS synthase), and curve 3 at high protein concentration (1.45 μM PS synthase).

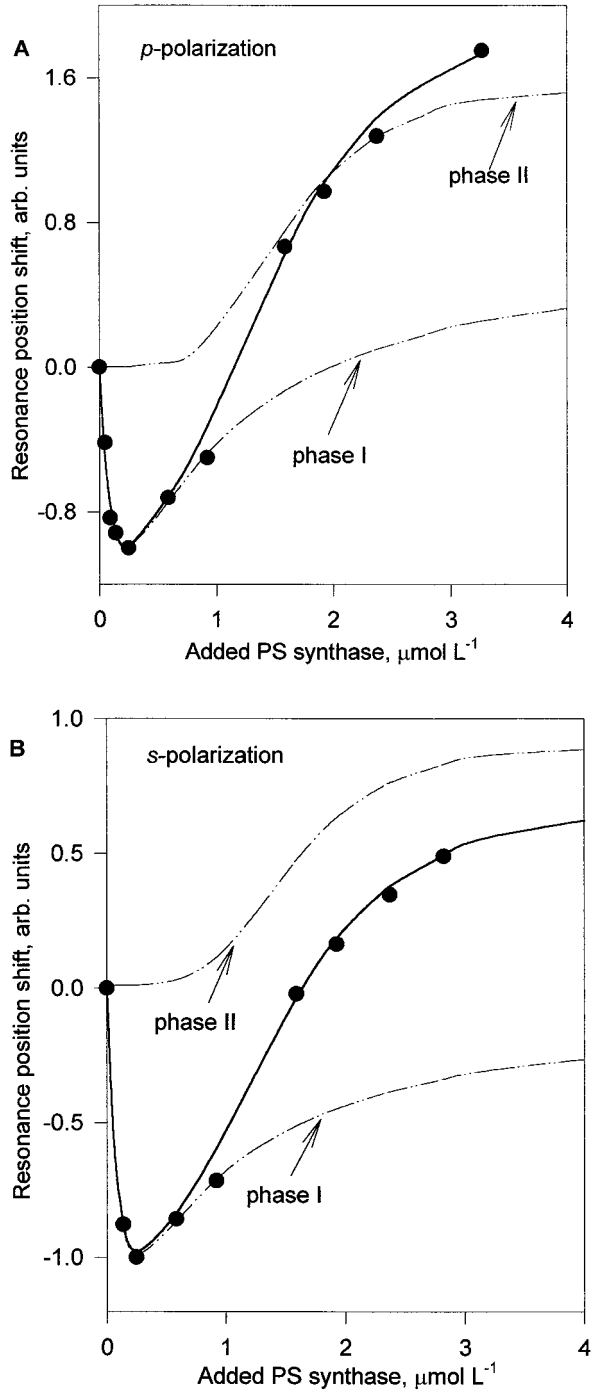


FIGURE 3 Dependence of the relative position of the CPWR resonance minimum (●) on the PS synthase concentration in the sample compartment of the cell, obtained with the lipid membrane described in Fig. 1 at low ionic strength, using either (*A*) *p*- or (*B*) *s*-polarization. A negative value represents a shift toward smaller incident angles, whereas positive values correspond to shifts toward higher angles, as compared with the resonance position obtained for the lipid membrane alone. Dashed curves show nonlinear least-squares fits to either two hyperbolic functions with opposite signs (phase I) or to a sigmoidal function (phase II). The sum of these theoretical curves results in the solid curve passing through the experimental data points.

shift toward smaller values of incident angle, which argues that both refractive indices decrease in value during the first phase of the PS synthase–lipid membrane interaction process. This strongly indicates that the binding of protein molecules to the membrane produces a large decrease in the lipid packing density in the membrane. This decrease is large enough to overcome the effect of the addition of adsorbed protein mass to the membrane, which would result in a resonance shift in the opposite direction, i.e., an increase in refractive index. To cause such a large perturbation in the lipid membrane, the PS synthase molecules most likely insert at least partially into the lipid bilayer. Such an inference is consistent with the presence of two long, hydrophobic sequences in the PS synthase molecule (DeChavigny et al., 1991). Thus, we assume that, during the first phase of the protein–lipid membrane interaction, the protein molecules penetrate into the bilayer hydrophobic core, producing a proteolipid membrane characterized by much different optical parameters than those of the initial lipid membrane. These new parameter values can be obtained by fitting theoretical resonance spectra to the experimental curves (Salamon et al., 1997b,c). This procedure will be described below.

As the results in Figs. 2 and 3 demonstrate, a second process involving positive spectral shifts occurs during the lipid membrane–PS synthase interaction. Although this begins at low protein concentrations, it becomes dominant at higher protein concentrations. Such positive spectral shifts are obtained with both polarized components of the exciting light and clearly indicate an increase in the n_{av} value. This can be attributed to the adsorbed mass of PS synthase on the lipid membrane. Because, as noted above, this process is strongly influenced by the ionic strength, we assume that the adsorbed protein binds electrostatically to the surface of the proteolipid membrane created during the first phase of the binding process, thereby forming a separate layer.

These binding interactions can be quantified as follows. As noted above, the first phase of PS synthase binding, occurring at low protein concentrations, contains two opposing effects. One of these involves structural alterations in the lipid bilayer, which cause large negative shifts of the position of the resonance curve and some changes in its shape, and the other involves accumulation of protein mass within the membrane, resulting in smaller resonance shifts in the opposite direction. Therefore, assuming hyperbolic concentration dependencies, the first phase was deconvoluted into two binding curves occurring in opposite directions. In the second binding phase, which occurs at higher protein concentrations, weaker PS synthase binding results from electrostatic interactions with a new proteolipid membrane structure generated in the first binding phase. The second phase was therefore fit with a sigmoidal curve. The solid line in Fig. 3 represents the sum of these three curves, and is seen to be in good agreement with the experimental points.

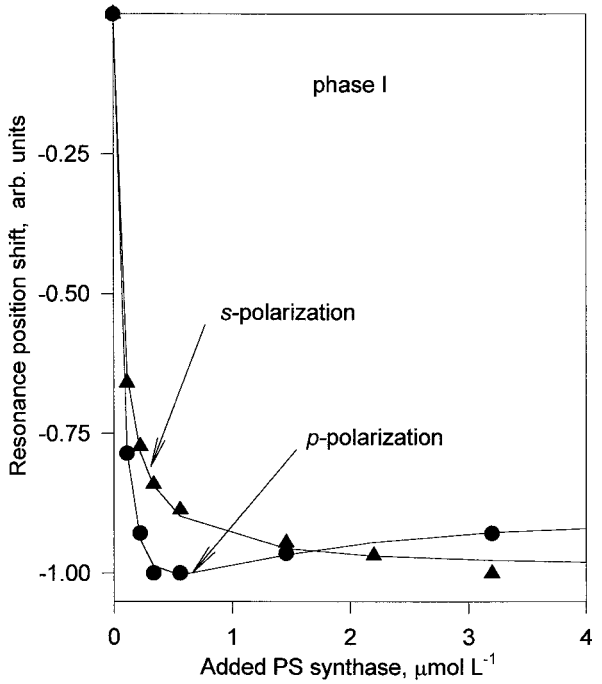


FIGURE 4 Dependence of the position of the CPWR resonance minimum on the PS synthase concentration in the sample compartment of the cell, obtained with the lipid membrane described in Fig. 1 at high ionic strength, using either *p*- (●) or *s*-polarization (▲). Solid curves show nonlinear least-squares fits to two hyperbolic functions of opposite sign (corresponding to phase I).

These deconvolutions allow some important insights to be obtained. First, the parameters of the two hyperbola describing the first phase indicate that the negative shift, which is caused by changes in the ordering and packing density of the lipid molecules in the bilayer, saturates at a lower protein concentration (the apparent dissociation constant, $K_D = 0.1–0.2\ \mu M$) than the subsequent positive shift due to PS synthase mass accumulation ($K_D = 0.5–0.8\ \mu M$). Second, these two dissociation-constant values are not significantly changed at high ionic strength, implying that the hydrophobic interactions are stronger than the electrostatic ones during this first binding phase. In contrast, the K_D value of PS synthase during the second binding phase is 1.5–2.0 μM, and the binding is predominantly controlled by electrostatic forces because it is strongly affected by the salt concentration.

Additional insights into the binding of the enzyme to the lipid bilayer can be obtained by fitting theoretical CPWR spectra to the experimental data (Salamon et al., 1997b,c). These analyses are described separately for the two binding phases.

Binding phase I

The structural characteristics and the mass of the proteolipid membrane generated during the first binding phase can be calculated from the parameters obtained by the fitting procedure for both *p*- and *s*-polarized components of exciting light. These are given in Figs. 5 and 6. Note that, during this phase, the thickness of the proteolipid membrane remains

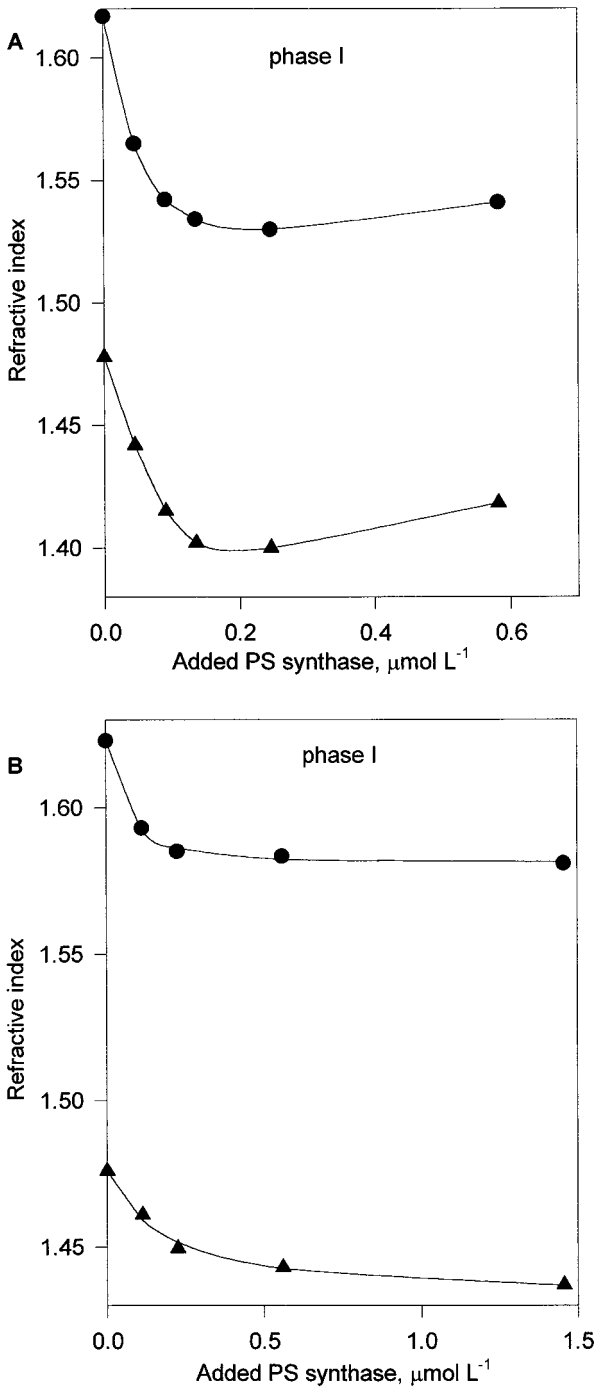


FIGURE 5 Refractive index of the proteolipid film as a function of PS synthase concentration in the sample compartment of the cell, obtained with the lipid membrane described in Fig. 1 at (*A*) low and (*B*) high ionic strength for phase I, using either *p*- (●) or *s*- (▲) polarized light. Solid lines in both panels represent nonlinear least squares fits to two hyperbolic functions of opposite sign.

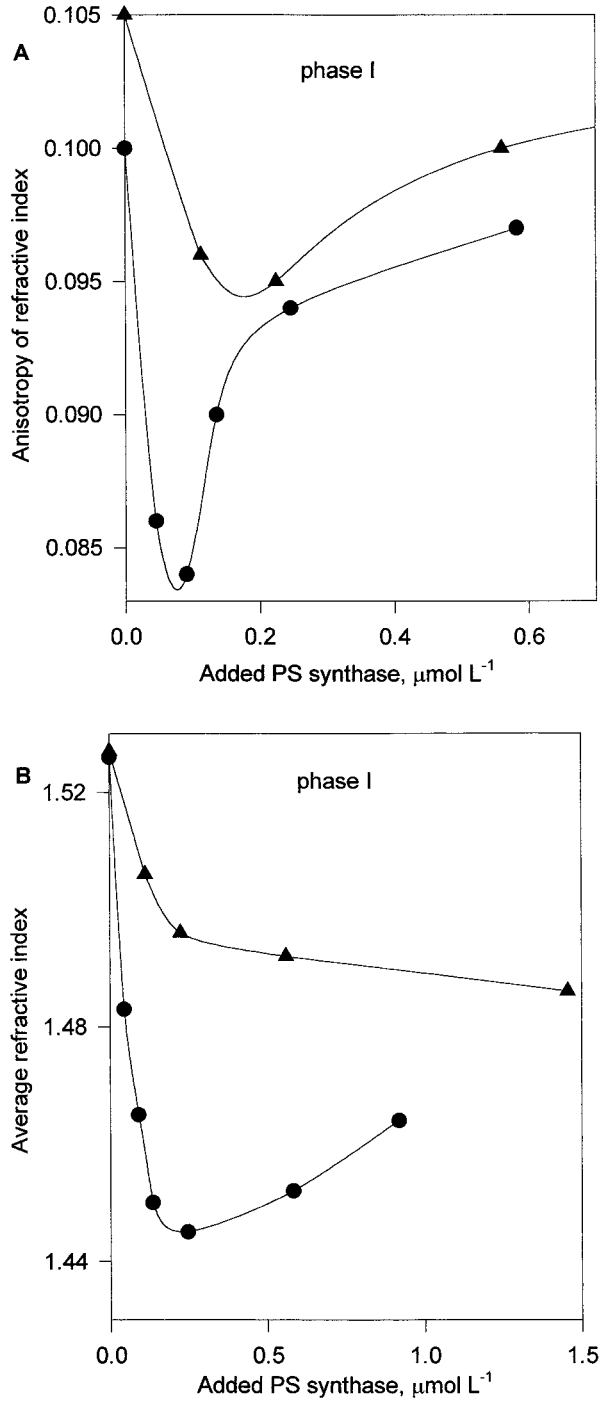


FIGURE 6 (*A*) Anisotropy of refractive index and (*B*) average value of refractive index as a function of the PS synthase concentration, obtained for phase I with the lipid membrane described in Fig. 1, in low (●) or high (▲) ionic strength buffer solution. Solid lines in both panels represent nonlinear least squares fits to two hyperbolic functions of opposite sign.

constant and is equal to the value obtained with the lipid bilayer (POPC-POPA, 80:20, mol/mol) in the absence of PS synthase ($t = 5.6$ nm for both these cases). This is consistent with the conclusion stated above that the protein penetrates into the interior of the membrane, rather than forming a separate layer on the membrane surface. In contrast, both refractive index values vary with increasing protein concentration, as shown in Fig. 5 for a membrane containing POPC-POPA (80:20, mol/mol) at low and high ionic strengths. The characteristic feature of this variation at low ionic strength involves an initial decrease of both refractive indices followed by a smaller increase; at high ionic strength only the A_n parameter exhibits the increase (compare Fig. 6, *A* and *B*). These changes can be adequately described by the sum of two hyperbolic functions (Figs. 5 and 6, *solid curves*), as was done with the resonance position shifts shown in Figs. 3 and 4, again indicating that the experimental observations result from two separate processes. This is especially clear in Fig. 6, *A* and *B*, which show the changes in the A_n and n_{av} values of the proteolipid membrane as a function of protein concentration. As discussed above, for uniaxial anisotropic systems, such as a proteolipid membrane, the values of A_n are proportional to the order parameter *S,* which describes the long-range molecular order of the membrane, whereas n_{av} describes the mass density (molecular packing density) of the system (Salamon et al., 1999; Salamon and Tollin 1999 a,b; 2000). Thus, Fig. 6 demonstrates that alterations occur in both molecular order and lipid packing density during the first phase of the lipid membrane–PS synthase interaction.

To further characterize the changes in molecular ordering, one has to take into consideration that the binding of PS synthase molecules during the second phase results in an increase in the A_n value with increasing protein concentration (see section Binding phase II). This result demonstrates that the protein molecules are ordered on the membrane surface and have different polarizabilities along their parallel and perpendicular molecular axes, i.e., the protein molecule deviates from a spherical shape. It is reasonable to expect that these properties of PS synthase also contribute to the results observed during the first phase, and are associated with the reversal of the initial decline in the A_n value with increasing protein concentration (Fig. 6 *A*).

The following picture of binding phase I emerges from these considerations. Interaction between the lipid bilayer and PS synthase results in what is probably a local perturbation of the ordering of the lipid molecules, leading to an initial decrease in the overall A_n value (Fig. 6 *A*). Such a local perturbation will require more space for individual lipid molecules resulting in a lower molecular packing density. This is achieved by moving lipids into the plateau–Gibbs border region. This is presumably what is monitored by the initially decreasing n_{av} value (Fig. 6 *B*). However, at low ionic strength, these decreases in the molecular order and mass density of the proteolipid membrane are eventually overcome by the incorporation of increasing numbers of PS synthase molecules as the protein concentration is raised.

As Fig. 6 shows, an increase in the ionic strength causes a significant decrease in the magnitude of the changes in both the A_n and n_{av} values; interestingly, the n_{av} parameter continuously decreases. At high ionic strength, the physicochemical properties of the membrane change due to screening of the charges of the lipid molecules. This, in turn, might decrease the ease of insertion of PS molecules into the bilayer. Such an effect can also account for the changes in the A_n and n_{av} parameters obtained at low ionic strength with a lipid membrane containing a larger fraction of the anionic lipid POPA (POPC-POPA, 60:40, mol/mol). In this case, the magnitude of the changes in both the parameters are increased compared to the membrane containing 20 mol% POPA, along with a decrease in the protein concentration at which these effects occur (Fig. 7). This could result from increased electrostatic repulsion between lipid head groups, which would increase the ease of penetration of PS synthase molecules into the bilayer interior. The decrease in protein concentrations required to produce these effects may also reflect an electrostatic attraction between the enzyme and the membrane surface, leading to a higher local protein concentration at the membrane surface.

Binding phase II

We assume that the second binding phase results in a cooperative binding of the protein to the membrane surface (cf. sigmoidal curves in Fig. 3), as a consequence of predominantly electrostatic interactions between the proteolipid membrane produced during the first phase and the added PS synthase molecules. The fitting of the theoretical resonance curves to the experimental spectra yielding the optical parameters of the protein layer have been carried out using the optical parameters obtained from the first phase (Fig. 5 *A*) extrapolated into the PS synthase concentration range of the second phase (Fig. 8 *A*). From the values of the refractive indices measured with both *s*- and *p*-polarized components of the exciting light, one can calculate the n_{av} values (Fig. 8 *B*, *curve 1*). Using the Lorentz–Lorenz relationship (Salamon et al., 1997b,c, 1999), the adsorbed mass of the protein extrapolated to infinite concentration can be calculated. The surface area occupied by one PS synthase molecule at saturation is ~2500 Å^2/molecule; this would correspond to a diameter of 56 Å, assuming a cylindrical shape. This value, taken together with the thickness of the protein layer (t = 43 Å), characterizes the dimensions of the protein molecule on the membrane surface. The surface area occupied by one PS synthase molecule, and the thickness of the protein layer, compares favorably to the calculated diameter of ~50 Å for PS synthase, assuming that the protein is a sphere with the specific volume of 0.75 cm^3/g. Additional molecular properties can be evaluated from the fact that, although both refractive indices increase over the range of PS synthase concentrations, larger changes are apparent in the *p*-polarized refractive index value (Fig. 8 *A*). This is a clear experimental indication that the PS synthase molecules are forming an ordered layer on the membrane surface, resulting in a significant degree of anisotropy of the refractive index (Fig. 8 *B*, *curve 2*).

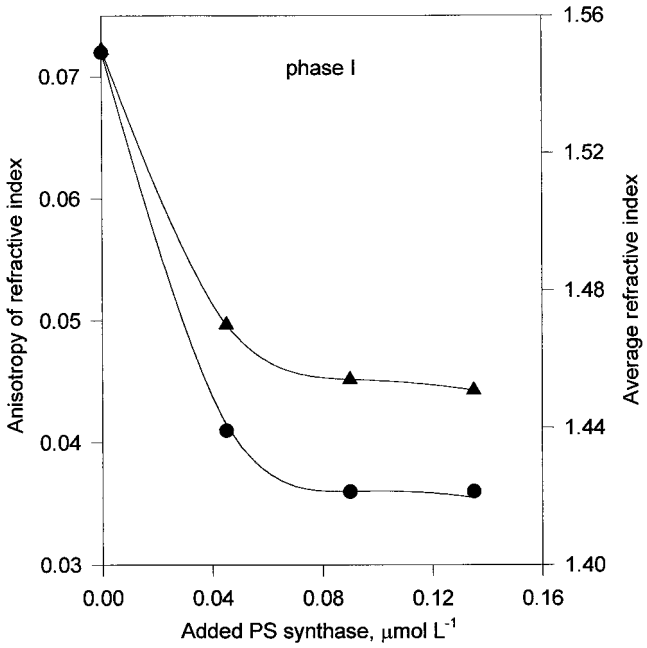


FIGURE 7 Anisotropy of refractive index (●) and average value of refractive index (▲) as a function of PS synthase concentration, obtained for phase I with a lipid membrane containing POPC-POPA (60:40, mol/mol) at low ionic strength. Solid lines represent nonlinear least squares fits to two hyperbolic functions of opposite sign.

Interaction of PS synthase with POPC and POPC-POPG membranes

The binding of PS synthase to a pure POPC lipid bilayer at low ionic strength was also studied. The general dependence of the shift of the resonance position on the enzyme concentration (data not shown) is similar to these obtained with the POPC-POPA (80:20 and 60:40, mol/mol) bilayers (Fig. 3 *A*). However, the negative shift is not as large, and the negative minimum occurs at a significantly higher enzyme concentration (approximately 1.0 μM). When PS synthase interacts with a POPC-POPG (75:25, mol/mol) bilayer at low ionic strength, the dependence of the shift of the resonance position on the enzyme concentration (data not shown) is essentially identical to the one obtained with the POPC-POPA (80:20, mol/mol) bilayer. The negative minimum again occurs at an enzyme concentration of approximately 0.2 μM (cf. Fig. 3 *A*). The only significant difference is that the negative shift is less pronounced and is of comparable magnitude as the shift obtained with the pure POPC bilayer. These results clearly demonstrate the influence of anionic lipids on the initial phase of the PS synthase–membrane interaction.

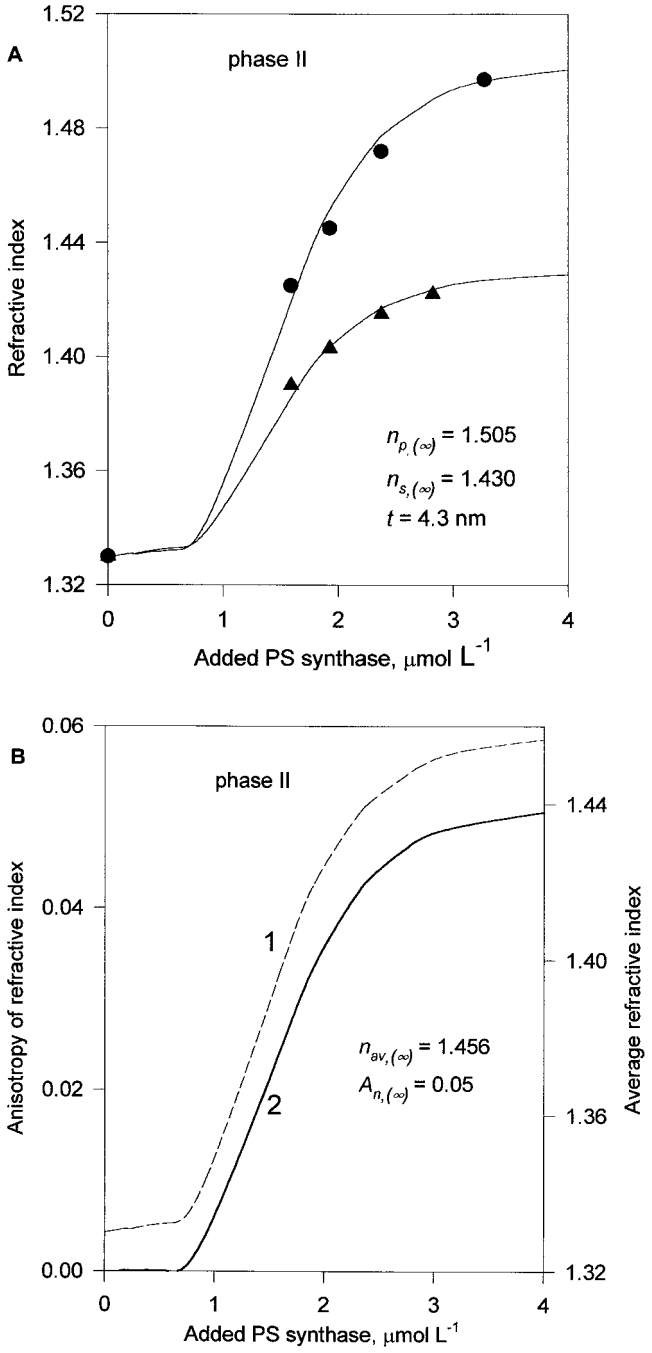


FIGURE 8 (*A*) Refractive index values measured with either *p*- (●) or *s*- (▲) polarization, obtained as a function of PS synthase concentration for phase II with the lipid membrane of Fig. 1. Nonlinear least squares data fitting to sigmoidal curves are shown by solid lines. (*B*) Anisotropy of refractive index (*curve 2*) and average value of refractive index (*curve 1*), obtained as a function of PS synthase concentration for phase II with the lipid membrane of Fig. 1. These curves have been calculated from the refractive index values obtained from the sigmoidal curves in (*A*). Parameter values extrapolated to infinite protein concentration are given in both panels of the figure. Thickness of protein film is given in (*A*).

CONCLUSIONS

At low concentrations, PS synthase hydrophobically inserts into the lipid bilayer and causes a local decrease of the ordering of the lipid molecules and a lower lipid-packing density. For a POPC bilayer, this effect is most pronounced at protein concentrations around 1.0 μM. This insertion process is facilitated by negatively charged lipids and by low ionic strength, indicating that an electrostatic interaction is also involved. At higher protein concentrations, the interaction process is dominated by electrostatic binding of enzyme molecules to the proteolipid membrane formed during the first binding phase. When the bilayer contains 25 mol% POPG, or 20 mol% POPA, the perturbation of the ordering of the lipid molecules is most prominent at PS synthase concentrations ~0.1–0.2 μM, and the enzyme causes a greater perturbation of the POPC-POPA bilayer as compared to the POPC bilayer. The protein concentration at which the electrostatic binding of enzyme molecules dominates the interaction process is also decreased when POPG or POPA are incorporated into the POPC bilayer. When the POPA content is increased to 40 mol%, the perturbation of the molecular ordering is further enhanced, and it reaches its full effect at enzyme concentrations around 0.06 μM.

The concentration of PS synthase in an *E. coli* cell can be calculated to be approximately 1.7 μM, assuming that there are 800 enzyme molecules per cell (Larson and Dowhan, 1976) and that the cell is a cylinder that is 2.0 μm in length and has a diameter of 0.75 μm. As shown here, electrostatic binding of the enzyme to the membrane occurs at this concentration; furthermore, a larger fraction of the enzyme molecules present in a cell should be bound to the membrane when its content of anionic lipids is increased. Because the enzymatic reaction is carried out when the enzyme is bound to the membrane (Carman and Dowhan, 1979), the synthesis of PE (proceeding via PS by means of the enzyme PS decarboxylase) will increase under these conditions, and this response balances the elevated levels of anionic lipids. The effect of the anionic lipids present in *E. coli* on the synthesis of PE or PS has been investigated previously: elevated levels of PA in *E. coli* membranes result in elevated levels of PE (Ganong et al., 1980); the rate of PE synthesis is about 3-fold higher with *E. coli* membranes enriched in PG as compared to membranes enriched in PE (Sparrow et al., 1984); the rate of PE synthesis decreases 10-fold when the fraction of anionic lipids in the membranes is reduced to less than 20% of the level in wild-type *E. coli* cells (Saha et al., 1996); and finally, when PS synthase interacts with mixed micelles, the enzyme can be activated 10-fold when the fraction of PG is increased 7-fold in the micelles (Rilfors et al., 1999). Potential binding sites on PS synthase are the N- and C-terminal ends of the protein; there are 5 basic amino acid residues among the 19 N-terminal ones, and 10 basic residues among the 22 C-terminal ones (DeChavigny et al., 1991). An electrostatic

binding of PS synthase to the membrane may also explain the result obtained from in vitro studies that the enzyme changes from a ribosomal association to a membrane association in the presence of intact *E. coli* membranes supplemented with the anionic lipids CDP-diacylglycerol, PS, PG, or DPG (Louie et al., 1986).

It was found in the present study that hydrophobic interactions between PS synthase and a lipid bilayer dominate at low protein concentrations. Such interactions are very reasonable, considering the fact that the protein has two large hydrophobic domains (approximately residues 125–165 and 235–285) (DeChavigny et al., 1991). The nearly 1000-fold activation of PS synthase observed when lipid vesicles are transformed to mixed micelles (Rilfors et al., 1999) may partly be explained by a hydrophobic interaction between the enzyme and lipid aggregates. Thus, the catalytic activity of the enzyme may be dependent upon its ability to penetrate into the hydrophobic core of a lipid aggregate.

In conclusion, the results reported herein lend further support to the previously presented model for the maintenance of the polar head group composition in *E. coli* membranes (Shibuya, 1992; Matsumoto, 1997; Rilfors et al., 1999). The assumption that PS synthase binds more strongly to a lipid bilayer when the fraction of anionic lipids increases is experimentally substantiated. The activity of PS synthase is related to the fraction of anionic phospholipids in the membrane, and this enzyme thus seems to be one of the lipid synthases that is directly involved in maintaining the polar head group composition at a nearly constant value.

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REFERENCES

Andersson, A.-S., L. Rilfors, M. Bergqvist, S. Persson, and G. Lindblom. 1996. New aspects on membrane lipid regulation in *Acholeplasma laidlawii* A and phase equilibria of monoacyldiglucosyldiacylglycerol. *Biochemistry.* 35:11119–11130.

Bloom, M., E. Evans, and O. G. Mouritsen. 1991. Physical properties of the fluid lipid-bilayer component of cell membranes: a perspective. *Q. Rev. Biophys.* 24:293–397.

Bogdanov, M., and W. Dowhan. 1998. Phospholipid-assisted protein folding: phosphatidylethanolamine is required at a late step of the conformational maturation of the polytopic membrane protein lactose permease. *EMBO J.* 17:5255–5264.

Burn, P. 1988. Amphitropic proteins: a new class of membrane proteins. *Trends Biochem. Sci.* 13:79–83.

Carman, G. M., and W. Dowhan. 1979. Phosphatidylserine synthase from *Escherichia coli*. The role of Triton X-100 in catalysis. *J. Biol. Chem.* 254:8391–8397.

Cronan, J. E., Jr., and C. O. Rock. 1996. Biosynthesis of membrane lipids. In: *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd ed., Vol. 1. F. C. Neidhardt (ed.). American Society for Microbiology, Washington, DC. 612–636.

Davis, J. H., M. Bloom, K. W. Butler, and I. C. P. Smith. 1980. The temperature dependence of molecular order and the influence of cholesterol in *Acholeplasma laidlawii* membranes. *Biochim. Biophys. Acta.* 597:477–491.

DeChavigny, A., P. N. Heacock, and W. Dowhan. 1991. Sequence and inactivation of the *pss* gene of *Escherichia coli*. Phosphatidylethanolamine may not be essential for cell viability. *J. Biol. Chem.* 266: 5323–5332.

De Jeu, W. H. 1978. Physical properties of liquid crystalline materials. *In* Liquid Crystal Monographs, Vol. 1. G. Gray (ed.). Gordon and Breach, Amsterdam. 34–48.

Den Engelsen, D. 1976. Optical anisotropy in ordered systems of lipids. *Surf. Sci.* 56:272–280.

Dowhan, W. 1997. Molecular basis for membrane phospholipid diversity: why are there so many lipids? *Annu. Rev. Biochem.* 66:199–232.

Evans, E., and D. Needham. 1987. Physical properties of surfactant bilayer membranes: thermal transitions, elasticity, rigidity, cohesion, and colloidal interactions. *J. Phys. Chem.* 91:4219–4228.

Ganong, B. R., J. M. Leonard, and C. R. H. Raetz. 1980. Phosphatidic acid accumulation in the membranes of *Escherichia coli* mutants defective in CDP-diacylglyceride synthetase. *J. Biol. Chem.* 255:1623–1629.

Gee, M. L., T. W. Healy, and L. R. White. 1990. Hydrophobicity effects in the condensation of water films on quartz. *J. Colloid. Interface Sci. USA.* 83:6258–6262.

Larson, T. J., and W. Dowhan. 1976. Ribosomal-associated phosphatidylserine synthetase from *Escherichia coli*: Purification by substrate-specific elution from phosphocellulose using cytidine 5′-diphospho-1,2-diacyl-*sn*-glycerol. *Biochemistry.* 15:5212–5218.

Louie, K., Y.-C. Chen, and W. Dowhan. 1986. Substrate-induced membrane association of phosphatidylserine synthase from *Escherichia coli. J. Bacteriol.* 165:805–812.

Matsumoto, K. 1997. Phosphatidylserine synthase from bacteria. *Biochim. Biophys. Acta.* 1348:214–227.

Mileykovskaya, E., Q. Sun, W. Margolin, and W. Dowhan. 1998. Localization and function of early cell division proteins in filamentous *Escherichia coli* cells lacking phosphatidylethanolamine. *J. Bacteriol.* 180: 4252–4257.

Morein, S., A.-S. Andersson, L. Rilfors, and G. Lindblom. 1996. Wild-type *Escherichia coli* cells regulate the membrane lipid composition in a "window" between gel and non-lamellar structures. *J. Biol. Chem.* 271: 6801–6809.

Mueller, P., D. O. Rudin, H. T. Tien, and W. C. Wescott. 1962. Reconstitution of cell membrane structure in vitro and its transformation into an excitable system. *Nature.* 194:979–980.

Nezil, F. A., and M. Bloom. 1992. Combined influence of cholesterol and synthetic amphiphilic peptides upon bilayer thickness in model membranes. *Biophys. J.* 61:1176–1183.

Raetz, C. H. R., and W. Dowhan. 1990. Biosynthesis and function of phospholipids in *Escherichia coli. J. Biol. Chem.* 265:1235–1238.

Rilfors, L., J. B. Hauksson, and G. Lindblom. 1994. Regulation and phase equilibria of membrane lipids from *Bacillus megaterium* and *Acholeplasma laidlawii* strain A containing methyl-branched acyl chains. *Biochemistry.* 33:6110–6120.

Rilfors, L., A. Niemi, S. Haraldsson, K. Edwards, A.-S. Andersson, and W. Dowhan. 1999. Reconstituted phosphatidylserine synthase from *Escherichia coli* is activated by anionic phospholipids and micelle-forming amphiphiles. *Biochim. Biophys. Acta.* 1438:281–294.

Saha, S. K., S. Nishijima, H. Matsuzaki, I. Shibuya, K. Matsumoto. 1996. A regulatory mechanism for the balanced synthesis of membrane phospholipid species in *Escherichia coli. Biosci. Biotech. Biochem.* 60: 111–116.

Salamon, Z., M. F. Brown, and G. Tollin. 1999. Surface plasmon resonance spectroscopy applied to membrane protein systems. *Trends Biochem. Sci.* 24:213–219.

Salamon, Z., D. Huang, W. A. Cramer, and G. Tollin. 1998. Coupled plasmon-waveguide resonance spectroscopy studies of the cytochrome

b6f/plastocyanin system in supported lipid bilayer membranes. *Biophys. J.* 75:1874–1885.

Salamon, Z., H. A. Macleod, and G. Tollin. 1997a. Coupled plasmon-waveguide resonators: a new spectroscopic tool for probing proteolipid film structure and properties. *Biophys. J.* 73:2791–2797.

Salamon, Z., H. A. Macleod, and G. Tollin. 1997b. Surface plasmon resonance spectroscopy as a tool for investigating the biochemical and biophysical properties of membrane protein systems. I. Theoretical principles. *Biochim. Biophys. Acta.* 1331:117–129.

Salamon, Z., H. A. Macleod, and G. Tollin. 1997c. Surface plasmon resonance spectroscopy as a tool for investigating the biochemical and biophysical properties of membrane protein systems. II. Applications to biological systems. *Biochim. Biophys. Acta.* 1331:131–152.

Salamon, Z., R. A. Schmidt, G. Tollin, and H. A. Macleod. 1996a. Reusable biocompatible interface for immobilization of material on a solid support. *United States Patent* 5,521,702.

Salamon, Z., and G. Tollin. 1991. Interfacial electrochemistry of cytochrome *c* at a lipid bilayer modified electrode: effect of incorporation of negative charges into the bilayer on cyclic voltammetric parameters. *Bioelectrochem. Bioenerg.* 26:321–334.

Salamon, Z., and G. Tollin. 1998. Surface plasmon resonance spectroscopy: a new biophysical tool for probing membrane structure and function. *In* Biomembrane Structures. P. I. Haris and D. Chapman (eds.). IOS Press, Amsterdam. 186–204.

Salamon, Z., and G. Tollin. 1999a. Surface plasmon resonance: theoretical principles. *In* Encyclopedia of Spectroscopy and Spectrometry. Vol. 3. J. C. Lindon, G. E. Tranter, and J. L. Holmes (eds.). Academic Press, New York. 2311–2319.

Salamon, Z., and G. Tollin. 1999b. Surface plasmon resonance: applications. *In* Encyclopedia of Spectroscopy and Spectrometry. Vol. 3. J. C. Lindon, G. E. Tranter, and J. L. Holmes (eds.). Academic Press, New York. 2294–2302.

Salamon, Z., and G. Tollin. 2000. Surface plasmon resonance (SPR) spectroscopy in peptide and protein analysis: instrumentation and applications. *In* Encyclopedia of Analytical Chemistry. John Wiley and Sons. In press.

Salamon, Z., Y. Wang, M. F. Brown, H. A. Macleod, and G. Tollin. 1994b. Conformational changes in rhodopsin probed by surface plasmon resonance spectroscopy. *Biochemistry.* 33:13706–13711.

Salamon, Z., Y. Wang, J. L. Soulages, M. F. Brown, and G. Tollin. 1996b. Surface plasmon resonance spectroscopy studies of membrane proteins: transducin binding and activation by rhodopsin monitored in thin membrane films. *Biophys. J.* 71:283–294.

Salamon, Z., Y. Wang, G. Tollin, and H. A. Macleod. 1994a. Assembly and molecular organization of self-assembled lipid bilayers on solid substrates monitored by surface plasmon resonance spectroscopy. *Biochim. Biophys. Acta.* 1195:267–275.

Saupe, A. 1964. Kernresonanzen in kristallinen Flüssigkeiten und kristallinflüssigen Lösungen. *Teil I. Z. Naturforschung.* 19A:161–171.

Shibuya, I. 1992. Metabolic regulations and biological functions of phospholipids in *Escherichia coli. Prog. Lipid. Res.* 31:245–299.

Silberzan, P., L. Leger, D. Ausserre, and J. J. Benattar. 1991. Silanation of silica surfaces. A new method of constructing pure or mixed monolayers. *Langmuir.* 7:1647–1651.

Sparrow, C. P., B. R. Ganong, and C. R. H. Raetz. 1984. *Escherichia coli* membrane vesicles with elevated phosphatidic levels. A detergent-free system for in vitro phospholipid synthesis. *Biochim. Biophys. Acta.* 796:373–383.

Thoma, M., M. Schwendler, H. Baltes, C. A. Helm, T. Pfohl, H. Riegler, and H. Mohwald. 1996. Ellipsometry and x-ray reflectivity studies on monolayers of phosphatidylethanolamine and phosphatidylcholine in contact with *n*-dodecane, *n*-hexadecane and bicyclohexyl. *Langmuir.* 12:1722–1728.

van Klompenburg, W., M. Paetzel, J. M. de Jong, R. E. Dalbey, R. A. Demel, G. von Heijne, and B. de Kruijff. 1998. Phosphatidylethanolamine mediates insertion of the catalytic domain of leader peptidase in membranes. *FEBS Lett.* 431:75–79.