Regulation of Signal Peptidase by Phospholipids in Membrane: Characterization of Phospholipid Bilayer Incorporated *Escherichia coli* Signal Peptidase

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ABSTRACT: Prokaryotic signal peptidases are membrane-bound enzymes. They cleave signal peptides from precursors of secretary proteins. To study the enzyme in its natural environment, which is phospholipid bilayers, we developed a method that allows us effectively to incorporate full-length *Escherichia coli* signal peptidase I into phospholipid vesicles. The membrane-bound signal peptidase showed high activity on a designed substrate. The autolysis site of the enzyme is separated from its catalytic site in vesicles by the lipid bilayer, resulting in a dramatic decrease of the autolysis rate. Phosphotidylethanolamine, which is the most abundant lipid in *Escherichia coli* inner membrane, is required to maintain activity of the membrane-incorporated signal peptidase. The maximal activity is achieved at about 55% phosphotidylethanolamine. Negatively charged lipids, which are also abundant in *Escherichia coli* inner membrane, enhances the activity of the enzyme too. Its mechanism, however, cannot be fully explained by its ability to increase the affinity of the substrate to the membrane. A reaction mechanism was developed based on the observation that cleavage only takes place when the enzyme and the substrate are bound to the same vesicle. Accordingly, a kinetic analysis is presented to explain some of the unique features of phospholipid vesicles incorporated signal peptidase, including the effect of lipid concentration and substrate-vesicle interaction.

Protein export in prokaryotic cells is a multistep reaction that involves many proteins and other factors (*1*, *2*, *3*). The cleavage of signal peptides from precursors of bacterial secreted proteins is the final step by which the proteins translocate across cytoplasmic membranes. After the signal peptide is cleaved, the mature part of the protein is released to the periplasmic space. The cleavage reaction is catalyzed by signal peptidase (SPase),[1] a membrane-bound protease (*4*, *5*). Amino acid sequences of signal peptidases of different prokaryotic cells share little similarity, except in the region of the active site. Among them, SPase I from *Escherichia coli* is the most extensively studied. *E. coli* signal peptidase consists of a single polypeptide chain with 344 residues. The enzyme is anchored to the cytoplasmic inner membrane by two putative transmembrane helices at the N-terminal end. The large C-terminal catalytic domain resides on the periplasmic side. The water-soluble catalytic domain itself was demonstrated to be active (*6*, *7*). The crystal structure of the catalytic domain in complex with an inhibitor was reported at 1.9A resolution (*8*). Recently, the structure of apo-form of the enzyme was also determined (*9*).

Gene deletion experiment shows that signal peptidase is an essential enzyme in *Escherichia coli* (*10*). This, together with its location and its unique Lys-Ser dyad catalytic mechanism, makes the enzyme an attractive antimicrobial drug target. Considerable progress has been made in the characterization of molecular and catalytic properties of the enzyme in recent years. It includes identification of the residues at active site (*11*−*14*), characterization of catalytic mechanism (*15*, *16*), analysis of kinetics (*12*, *17*, *18*), improvement on substrate design and assay methods (*7*, *17*), biophysical study of the enzyme-membrane interaction (*19*) as well as determination of crystal structure (*8*, *9*). Most of those studies were carried out in aqueous solution with detergent-dissolved SPase, either full length or membrane domain deleted. The kinetic properties of the enzyme in its biological environment (i.e., phospholipid membrane) are still obscure. The report by van Klombenburg et al concerning the binding of the SPase catalytic domain to membranes is one of the few studies that attempt to address this issue (*19*). They have shown that the N-terminal transmembrane helices deleted SPase is still capable of binding to lipid bilayers containing phosphotidylethanolamine (PE). The crystal structure of SPase shows that this domain has a large hydrophobic surface that has been proposed to be the membrane-association site (*8*).

The importance of studying the enzyme in a membrane environment is further demonstrated by the observation that both the active site of the enzyme and the cleavage site of the substrate are embedded in lipid bilayers (*8*, *20*). Therefore, it is to be expected that the chemical identity of phospholipids and the physical property of lipid bilayers can significantly affect the activity of the enzyme. To better understand the enzyme in a membrane environment, we reconstituted full length *E. coli* SPase I into phospholipid

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[1] Abbreviations: CL, cardiolipin; E. coli, *Escherichia coli*; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PLA2, phospholipase A_2; SPase, signal peptidase; CMC, critical micelle concentration.

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vesicles. Using this reconstituted system, we characterized kinetics of the enzyme in lipid bilayers and studied the effects of various phospholipids on the activity of the enzyme.

MATERIALS AND METHODS

Materials E. coli PE and egg yolk PC were purchased from Sigma. Nitroxide -labeled PC was from Avanti Polar Lipid. Peptide substrates were synthesized by New England Peptides (17). *E coli* signal peptidase was purified as previously reported (*17*).

Membrane Incorporation of SPase Phospholipid vesicles were prepared using the method of polycarbonate membrane extrusion. The size of vesicles can be well controlled by this method. Lipids were dissolved in a 20-mL glass test tube in chloroform-methanol (4:1). Nitrogen gas was blown into the tube containing 0.5−1.0 mL of the lipid solution to evaporate the solvent. The tube was under a strong vortex that evenly dispensed lipids onto the wall of the tube. The residual organic solvent was removed by a vacuum pump. The dried lipid was re-suspended in 50 mM Tris-HCL buffer (pH 8.0). The suspension was vortex-mixed and then extruded through a 0.1-μm pore polycarbonate membrane with a mini-extrusion device (Avanti Polar Lipid) 20 times. The solution became clear after the extrution. The final concentration of the lipid was 40 mg/mL.

SPase stock solution (30 μM SPase in 50 mM Tris-HCL, pH 8.0 containing 0.5% Triton-100) was mixed with vesicle solution according to desired ratio. The mixture was subject to dialysis against 50 mM Tris-HCL buffer, pH 8.0 at 4 °C. Dialysis took 65−75 h with three solution changes to completely remove Triton-100 detergent.

SPase Activity Assay Two peptide substrates were used for assaying the activities of reconstituted SPase (*17*). Substrate 1 (S1) is $K_4-L_{10}-Y^{NO2}FSASALA-KIK^{abz}-NH_2$, and substrate 2 (S2) is $K_4-L_{10}-Y2FSASALA-KIK^{fluroescein}$. The two substrates have the same amino acid sequence but different fluorescence labeling groups. After the substrates are cleaved, $KIK^{abz}-NH_2$ of S1 is separated from fluorescence quenching group NO_2, which increases fluorescence intensity. S2 peptide was designed to measure SPase activity by fluorescence anisotropy method. Once the peptide that binds to phospholipid vesicle is hydrolyzed, the small fragment of the product, $KIK^{fluorscein}$, is released from vesicles into solution. This reduces the fluorescence anisotropy because $KIK^{fluorscein}$ tumbles much faster in solution than in vesicle. It was later found that fluorescence intensity increased about eight times after $KIK^{fluorscein}$ is released. So the fluorescence intensity can also be used to monitor the hydrolysis of S2. In a typical assay, the reaction was initiated by adding substrates to a cuvette containing buffer (50 mM Tris-HCL, pH 8.0) and phospholipid-vesicle-incorporated SPase. All reactions were recorded at 37 °C with constant stirring.

Fluorescence Experiments All fluorescence data were collected on a Perkin-Elmer LS50B fluorescence spectrophotometer. All measurements were taken at 37 °C. For S1, the exciting and emission wavelength were set at 360 and 412 nm, respectively. For S2, they were 495 and 522 nm, respectively. Fluorescence anisotropy (R) is calculated as

$$R = (I_{0/0} - I_{0/90}(I_{0/0}/I_{0/90}))/(I_{0/0} + 2 \cdot I_{0/90}(I_{0/0}/I_{0/90}))$$

where I is fluorescence intensity measured when the relative rotational degree between excitation and emission polarizers are set as indicated by the subscripts. When calculating S2 concentration based on R, it is noted that the fluorescence intensity changes before and after reaction. In this case, the measured R is no longer the simple population weighted linear combination of R_s and R_p (R_s is the fluorescence anisotropy of S2 in vesicle-bound form, R_p is that of product). Instead, fluorescence intensity of each species should also be included in the calculation (*21*)

$$R = ([P]F_pR_p + [S]F_sR_s)/([P]F_p + [S]F_s)$$

it can be shown that

$$[S] = \{(F_p)[S_o](R_p - [R])\}/\{[F_p](R_p - R) + F_s(R - R_s)\} \quad (1)$$

where F_p and F_s are fluorescence intensity of substrate and product respectively; $[S_o]$ is the initial concentration of the substrate in the reaction mixture. All data were processed with the software of PSI Plot 6.0 (Poly Software International, Salt Lake City, UT).

RESULTS AND DISCUSSION

Incorporation of Signal Peptidase into Phospholipid Bilayers The methods most commonly used to reconstitute membrane protein into lipid bilayer involve removal of the detergent from the mixture of detergent−lipid−protein to allow bilayers to form around protein. The detergent is usually removed by dialysis. This method failed to give us reproducible results for SPase in our hands. It was later found that a quantitatively reproducible activity of the enzyme could be achieved by directly mixing SPase dissolved in 0.5% Triton-100 with preformed phospholipid vesicles. Unlike lipid−detergent mixed micelles that have high a detergent-to-lipid ratio, the detergent concentration was kept at a very low level to that of the lipids in this experiment. Consequently, the lipid bilayer structure is not disrupted. The key to this method, we believe, is that the enzyme can only adapt one orientation in membranes, which allows the catalytic domain to reside on the outside of vesicle. The variation of activity of SPase reconstituted by this method is less than 25% between experiments. The small amount of Triton-100 detergent that probably facilitated insertion of the protein into lipid bilayers was then removed by dialysis. This procedure is similar to the method of spontaneous incorporation of membrane proteins into the performed lipid bilayers (*22*). Figure 1 shows that SPase reconstituted by this method can cleave both S1 and S2. The activity was low initially after the enzyme was mixed with the vesicle. It increased about three times in 20−30 min and then stabilized (data not shown). Because critical micelle concentration(CMC) of Triton-100 is very low, it took a much longer time for the dialysis (>70 h) to completely remove Triton-100 detergent from the solution. To minimize effect of Triton-100, all activity assays were performed with the samples dialyzed for at least 60 h. No activity loss was observed during the dialysis period.

Figure 2 shows the results of an experiment designed to test whether SPase is indeed incorporated into lipid bilayers. In the experiment, substrate was first incubated with phos-

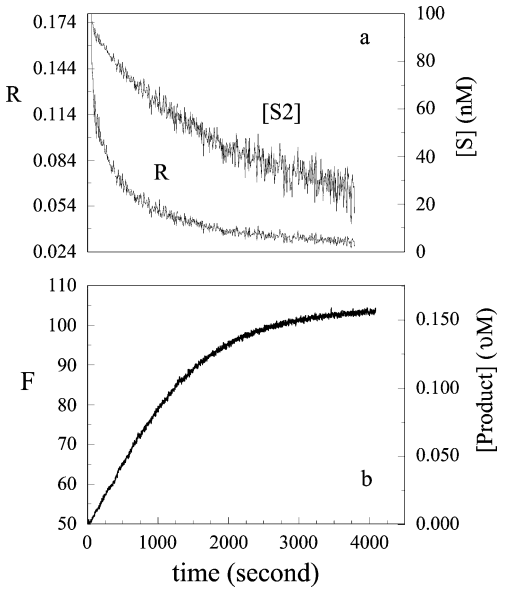

FIGURE 1: Cleavage of S1 and S2 by vesicle-incorporated SPase (a) Hydrolysis of S2 was measured by the fluorescence anisotropy method. The anisotropy is shown on the graph as R. The trace shows change of concentration of S2 is calculated according to eq 1 (see Materials and Methods). Because the product and the substrate have different fluorescence intensity, the change of substrate concentration ([S]) does not coincident with the change of the anisotropy (see Materials and Methods) (b) Hydrolysis of S1 is measured by fluorescence intensity. The product concentration was calculated according to the equation $[P] = (F - F_s) \cdot [S_o]/(F_p - F_s)$. K_m for S1 is about 10 times lower than that for S2 in Triton-100 detergent solution (data not shown).

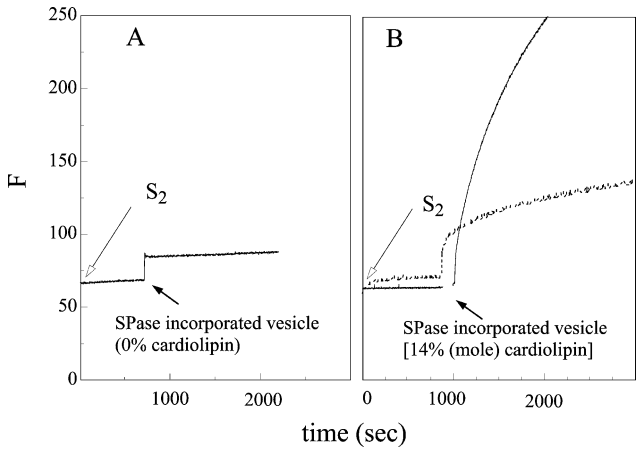

FIGURE 2: Membrane-incorporated SPase can only hydrolyze substrates in the same vesicles. The increase of fluorescence intensity resulting from the hydrolysis of S2 was used for activity assay. At time 0, the substrate (S2) was added to the solution that contained empty vesicles (50% PE + 50% PC). After the incubation period that allowed S2 to bind to empty vesicles, the vesicle that had been incorporated with SPase (SPase vesicle) was added at the time indicated to start the hydrolysis reaction. Lipid composition of the vesicle incorporated with SPase is 50% PE + 50% PC (a) and 50% PE + 36% PC + 14% CL (b), respectively. The dotted line in (b) is the result of SPase that had been incorporated in vesicles with lipid composition of 50% PE + 36% PC + 5% CL. The final concentration in the reaction mixture is [S2] = 100 nM and [E] = 10 nM. The small fluorescence intensity jump upon adding SPase vesicle in (a) is caused by turbidity of added vesicles.

pholipid vesicles in which no SPase had been incorporated (empty vesicle) to allow the substrate binding. Then, SPase which had been reconstituted by the procedure described above was added, and substrate hydrolysis was subsequently

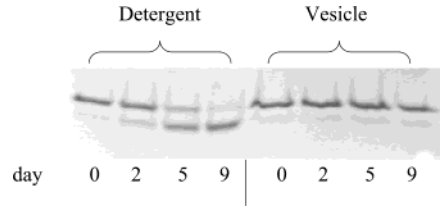

FIGURE 3: Autolysis of SPase in detergent and in membrane. SPase (25 nM) dissolved in detergent (0.5% Triton-100) or incorporated in vesicle(50% PC + 50% PE, [lipid] = 450 uM) was incubated at room temperature. Samples were subjected to SDS-PAGE at indicated time. The upper bands are full length SPase, and the lower bands are autolyzed SPase. The small amount of autolysis product found since day 0 was produced during purification and storage.

recorded. The result shows that the rate of substrate hydrolysis in this experiment is negligible (Figure 2a), suggesting that the enzyme cannot access substrates that bind to empty vesicles. This in turn indicates that when SPase molecules have been inserted into lipid bilayers, they cannot move freely from one vesicle to another. So, the observed substrate hydrolysis in the experiments that the substrate was directly added to the solution which only contained SPase-incorporated vesicles (Figure 1) is apparently catalyzed by the enzyme that binds to the same vesicle. This is consistent with the results that the reaction rate depends on the concentration ratio of the lipid to the enzyme and the ratio of the lipid to the substrate. However, the results changed when negatively charged lipids were included in the vesicles used for SPase reconstitution. Negatively charged lipids restored the reaction rate (Figure 2b). The reaction rate in this experiment depends on the charge difference between the empty vesicle and the SPase-incorporated vesicle. In this case, electrostatic attractive force drives positively charged substrates from the empty vesicles that have neutral charge to SPase vesicles that have net negative charge. It is also possible that the intervesicle reaction pathway contributed to the reaction in this experiment. The results of these two experiments suggest that the enzyme has been successfully incorporated into the lipid bilayer of the vesicle. The results also lead to the conclusion that when there is no charge difference among vesicles, the reconstituted enzyme can only hydrolyze the substrates that bind to the same vesicle. The hydrolysis is an intra-vesicle reaction. This conclusion is important for kinetic analysis of the reaction.

Membrane-Incorporated SPase is Protected from Autolysis

It was reported that detergent dissolved-full length *E. coli* SPase could be autoproteolyzed between Ala 40 and Ala 41. This site is located between transmembrane helix 1 and 2. It is on the cytoplasmic side. The autolysis reaction is a bimolecular reaction (*17*, *23*, *24*). Because the catalytic site and the autolysis site are located on opposite sides of the membrane, we expected that insertion of SPase into the membrane would prevent the autolysis. To test this, we compared the autolysis rate of our membrane-incorporated SPase with that of the detergent-dissolved one. The result presented in Figure 3 shows that the autolysis rate of membrane-incorporated SPase is dramatically reduced. In fact, little autolysis was observed for membrane-incorporated SPase after 9 days at room temperature. Meanwhile, more than 90% SPase in detergent had been autolyzed. This cannot be attributed to the activity difference between the two systems, because under the conditions of Figure 3, the activity of Triton-100

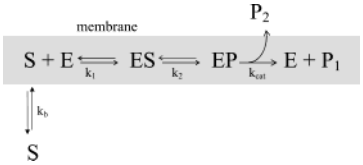

FIGURE 4: Reaction scheme of membrane-incorporate SPase. The substrate first binds to membrane (k_b). This is a fast step. It then diffuses in the lipid bilayer laterally to encounter E (k_1). This is the rate-limited step. Then the Michaelis−Menten complex is formed and the hydrolysis is catalyzed. S, substrate; E, enzyme; P, product; $P_1$and P_2, small and large fragments of cleavage products, respectively.

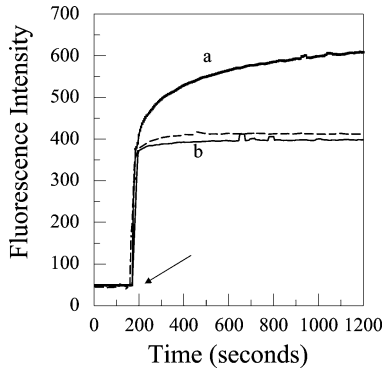

FIGURE 5: Binding of SPase substrate to membranes S_1 was added to solution containing empty vesicle (binding, trace b) or SPase incorporated vesicle (hydrolysis, trace a) at indicated time. [S] = 0.65 uM, [lipid] = 78 uM and [E] = 40 nM. The fluorescence intensity of the substrate in buffer was 11. The lipid composition of the vesicle was 42% PE + 42% PC + 12% PG (mole). Dotted line is the binding of S1 to vesicles containing 0% PG.

dissolved SPase measured by the initial rate of S1 hydrolysis is less than two times higher than that of reconstituted SPase (1.1 [nM]/s vs 0.7 [nM]/s), while the difference of the autolysis rates between the two systems is more than 40 times. This result confirms that SPase has indeed been inserted in phospholipid bilayer. The transmembrane topology of the polypeptide chain is accurately arranged.

Reaction Kinetics As demonstrated previously, the cleavage reaction only takes place for the enzymes and the substrates residing in the same vesicles. The intervesicle reaction pathways, such as vesicle fusion or substrate hopping between vesicles, are negligible. This greatly simplifies the kinetic analysis of the reaction.

The reaction scheme can be drawn as Figure 4. The first step of the reaction is the binding of the substrate to the membrane. Quantitative determination of this binding is essential for kinetic analysis of the reaction. A dramatically increased fluorescence intensity of S1 upon binding to membranes provides a sensitive method to study the binding. A similar fluorescence enhancement was also observed for the substrate−detergent interaction (*17*). Compared with hydrolysis reaction, the binding is very fast. It finished almost instantly upon mixing, as shown in Figure 5. The rate constant of the binding is too fast to be reliably obtained by the manual mixing method used in this study. The fast binding ensures that observed reaction rate is not limited by this step. The affinity of the binding can be determined by fluorescence titration experiments. It showed that almost all substrates have bound to vesicles in the experimental conditions of Figure 5, because adding more vesicle to the solution causes no further fluorescence increase.

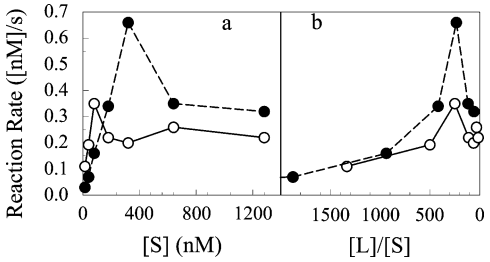

FIGURE 6: SPase activity as a function of substrate concentrations. SPase was incorporated into vesicles with [lipid]/[E] = 15 000 (filled circle) or 4000 (open circle), respectively. (a) Initial reaction rates vs substrate concentrations. (b) Initial reaction rates vs [lipid]/[substrate]. [E] = 5 nM for all the reactions.

The reaction scheme shown in Figure 4 indicates that each vesicle is a closed system. The reaction rate depends on the concentrations of the substrate and the enzyme of each vesicle. The observed reaction rate is the population-weighted sum over all vesicles. Assuming that interaction is random, the distribution of substrate and enzyme over vesicles follows Poisson distribution. Because the substrate-to-vesicle ratio is high (more than 150 in most experiments), variation of substrate numbers among vesicles generates little effect on initial rate of reaction. However, a potential problem is inhomogeneity of enzyme number among vesicles. An extreme situation is that in a reaction mixture, some phospholipid vesicles do not contain any enzyme (empty vesicle). Binding of substrates to those vesicles would be futile for the hydrolysis reaction. To minimize this problem, enzyme-to-vesicle ratio is kept at more than 10 in each reconstitution experiment. Statistically, the chances of having an empty vesicle are less than 97% at this ratio.[2] It is rather obvious that, for membrane-bound enzymes, the observed reaction rate depends not only on apparent enzyme concentration in reaction mixture but also on the ratio of lipid to enzyme ([L]/[E]), because this ratio decides the effective concentration of the enzyme in vesicles. As expected, the reaction rate of membrane-incorporated SPase increases linearly with the increase of substrate concentration. However, this relation collapsed when the molar ratio of lipid to substrate is down to about 100, regardless of the concentration of SPase in vesicles (Figure 6). It appears that at this ratio, the vesicle structure is disrupted by high concentration of the substrate that has strong affinity to the phospholipids. We did observe fiber-shaped precipitation in the solution below this lipid/substrate ratio. This phenomena prevented us from obtaining the kinetics parameters by commonly used methods, because the enzyme could not be saturated by the substrates.

The kinetics of membrane-bound SPase could be analyzed by interfacial catalysis theory that was developed based on phospholipase A_2 (PLA2), a membrane-bound enzyme(*25*). In addition to basic assumptions of Michaelis−Menten kinetics, the interfacial theory also considered the features that uniquely affect the membrane-bound enzymes, such as vesicle size, number of enzyme per vesicle, and binding rates of enzyme and substrate to vesicles and others. The reaction progress curve is as follows:

[2] This is one of the classical probability problems. The probability (P) for every vesicle to have at least one enzyme can be calculated as $P = V^{-E}\sum_{i=0}^{v}\binom{v}{i}(-1)^i(V-i)^E$, where E is enzyme concentration and V is vesicle concentration.

$$k_i t = -\ln(1\text{-}P_t/P_m) + (k_i N_s/\upsilon_o - 1)P_t/P_m \qquad (2)$$

In this equation, P_t is the amount of product at a given time, and P_m is the maximal amount of the product, N_s is the amount of the substrate available for reaction, k_i is the kinetic parameter related to K_m and k_{cat}, and υ_o is the initial reaction rate. The equation is the interfacial form of integrated Michaelis−Menten equation (*25*).

The reaction pathway of membrane-incorporated SPase summarized in Figure 4 is very similar to that of PLA2. In fact, the reaction conditions satisfied all constraints for the "scooting model" of interfacial catalysis. They include the following: (1) both enzyme and substrate are tightly bound to membranes, they will not leave the vesicle where they have initially resided during reaction cycles; (2) inter-vesicle reaction is negligible; (3) enzyme-to-vesicle ratio is large enough to minimize in-homogeneity problem; and (4) the vesicle size is well controlled (see Materials and Methods). The major difference between the two enzyme is that for SPase, the first step of the reaction is the binding of substrate to membrane. For PLA2, it is the enzyme (*25*). However, the fast and tight binding of the substrate of SPase to the membrane eliminated any impact of this step to the reaction rate as long as the ratio of substrate to vesicle is high enough to ensure that variation among vesicles is small.

Although the reaction of membrane-inserted SPase meets all the conditions of interfacial catalysis, the fitting of the reaction progress curve to eq 2 yielded a poor result. The fitting of the data to the classical integrated Michaelis−Menten equation also failed. This indicates that the reaction may not follow steady-state assumption of Michaelis−Menten kinetics. One possibility is that the concentration of the substrate is well below its K_m. In this situation, the reaction is pseudo first order in substrate. However, fitting the reaction progress curve to the first-order kinetics did not yield a satisfied result either. It turns out that the progress curve could be best fitted to a simple 2nd-order reaction equation, as shown in Figure 7. The departure of the reaction kinetics from Michaelis−Menten kinetics to second-order kinetics can be explained by diffusion-limited reaction and product inhibition. Product inhibition reduces the amount of SPase available for catalysis. In this situation, concentration of enzyme is no longer a constant during reaction. Meanwhile, if reaction rate is limited by formation of [ES] complex that was controlled by diffusion, the reaction becomes a true 2nd-order one

$$\upsilon = k[S][E] = k([S_o] - [P])([E_o] - [EP]) \qquad (3)$$

here, $[S_o]$ and $[E_o]$ are initial concentration of substrate and enzyme respectively, and [EP] is the concentration of the enzyme inhibited byproduct. Equation 3 shows that under this condition, the reaction obeys the 2nd-order kinetics. If the product only binds to the free enzyme, we have

$$[EP] = \{[E_o] + [P] + K_d - (([E_o] + [P] + K_d)^2 - 4[E_o][P])^{0.5}\}/2 \qquad (4)$$

in this equation, K_d is the dissociation constant of [EP]. There is evidence to support this explanation. It is known that SPase can be inhibited by the product of S1 (*17*). The apparent K_d between enzyme and product is about 10 μM in Triton-100

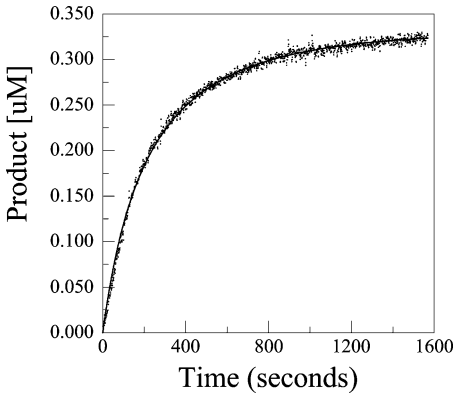

FIGURE 7: Reaction progress curve of membrane-incorporated SPase. See Materials and Methods about reaction condition. [E] = 9.5 nM, [S1] = 330 nM, and [lipid] = 180 uM. The experimental data (dotted) was fitted by a secondary-order reaction rate equation ($A + B ==> X$) with apparent enzyme concentration equals 520 nM. The solid line is the fitting result. The final form of the equation is $x = ([A][B] - [A][B]\exp(z))/([B] - [A]\exp(z))$, where [*A*] and [*B*] are the initial concentrations of reactants, which are the substrate and the enzyme in this reaction, respectively, x is the concentration of product, $z = ([A] - [B]) \cdot k \cdot t$, k is the reaction rate constant, and t is the time. The best fitting yields $k = 0.01([s][nM])^{-1}$

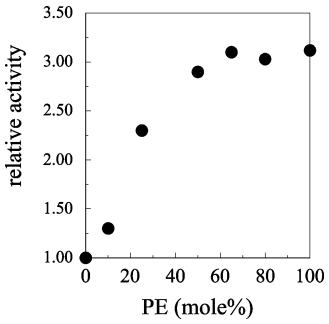

FIGURE 8: Activity of SPase as a function of PE concentration. SPase is reconstituted into PC vesicles containing various concentrations of PE. The reaction rates are normalized to that of 0% PE to generate relative activities. Reaction condition: [E] = 9.5 nM, [S1] = 330 nM, and [lipid] = 180 uM, pH 8.0 and 37°C.

micelles. Meanwhile, due to the high viscosity of lipid bilayers, the lateral diffusion rates of integral membrane proteins are very low. Diffusion as the rate-limited step is often observed for membrane-bound enzyme, particularly if the substrate is also a membrane bound protein (*26*). In *E. Coli* cells, whether signal peptide directly interacts with SPase after it emerges from the translocation channel is still not clear. If this is the case, The substrate does not need to be diffused in lipid bilayers to encounter the enzyme.

Effect of phosphotidylethanolamine (PE) PE is the most abundant phospholipid in *E. coli* inner membrane. It has been demonstrated that the catalytic domain of SPase can only bind to lipid bilayers that contained PE (*19*). Crystal structure shows that this domain has a large exposed hydrophobic surface that is responsible for the membrane-binding. However, it is not clear whether PE is required for catalytic activity of the enzyme. To find this out, we measured activity of SPase incorporated in vesicles containing different amounts of PE. Figure 8 is the plot of SPase activity as a function of molar percentage of PE in the phospholipid membrane. It shows that although SPase can hydrolyze its substrate in the membrane that contained only phosphotidylcholin(PC), the activity is enhanced by PE. The reaction

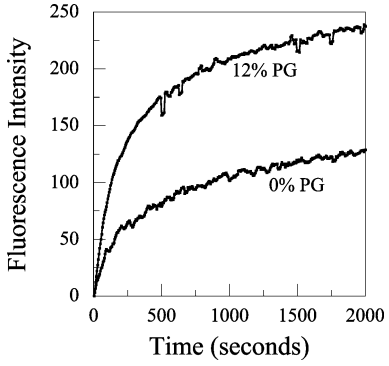

FIGURE 9: Reactions of SPase in vesicles containing 12% (mole) (a) and 0% (b) PG. [E] = 20 nM, [S1] = 650 nM and [lipid] = 178 μM. Phospholipid composition is [(50% PE) + (38% PC) + (12% PG)] (a) and [(50% PE) + (50% PC)] (b) (all are mole percentage).

rate rises linearly with the increase of PE. It reached maximal level at about 60% PE. This is roughly the percentage of PE in *E. coli* inner membrane. At this PE concentration, SPase activity is about 3 times higher than that of 0% PE.

Negatively Charged Lipids Enhance Reaction Rate Negative charged lipids, mainly phosphatidylglycerol (PG) and cardiolipin (CL), are also abundant in the *E. coli* inner membrane. It is interesting to know how negatively charged lipids affect the activity of SPase. The role of negatively charged lipids in the interaction of membrane and signal peptides that are rich in positively charged residues has been well studied (*27*−*29*). In general, including negatively charged lipids in vesicles increases the affinity of the signal peptide to the membrane. In the other aspect, negatively charged lipids showed little effect on the interaction of membrane domain deleted SPase with membrane (*19*), though its effect on full length SPase has not been reported.

Fluorescence titration experiment indicates that PG increases the affinity of S1 that has five Arg residues to vesicles. The binding affinity could not be quantitatively determined, because fluorescence data at high substrate/lipid ratio could not be reliably recorded (see kinetic section). Accordingly, PG increased the reaction rate of membrane-incorporated SPase, as shown in Figure 9. The mechanism of this reaction rate enhancement, however, is not as readily to be completely understood. It first appears that the higher activity results from the higher affinity of the substrate to negative charged membrane. However, fluorescence titration experiments showed for both vesicles, most substrate have bound to membrane in the experimental condition (Figure 5). The difference of substrate concentration between the vesicles with and without PG is too small to fully account for the 2-fold difference in initial reaction rate shown in Figure 9. Several alternative mechanisms can be offered to explain the effect of negatively charged lipids, such as conformation and orientation, as well as diffusion rate of substrate in membrane. Obviously, further studies are needed to understand this phenomena.

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