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Effect of Membrane Phospholipid Composition and Charge of the Signal Peptide of *Escherichia coli* Alkaline Phosphatase on Efficiency of Its Secretion

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Abstract—The secretion of the *Escherichia coli* alkaline phosphatase with a different charge of signal peptide due to replacement of positively charged Lys(−20) has been studied depending on the phospholipid composition of the membranes and the activity of the translocational ATPase—protein SecA. Changing the signal peptide charge, along with a change in phospholipid composition, has been shown to reduce the efficiency of secretion. In the absence of phosphatidylethanolamine the membrane contains anionic phospholipids only, and the dependence of secretion on the signal peptide charge decreases. The dependence of secretion on membrane phospholipid composition and the signal peptide charge is also determined by the activity of SecA protein. If SecA is inactivated by sodium azide, then the dependence of secretion on anionic phospholipids increases; on the contrary, higher content of anionic phospholipids (in the absence of phosphatidylethanolamine) decreases the dependence of secretion on the SecA activity. The results suggest a direct interaction of positively charged signal peptide with negatively charged membrane phospholipids under initiation of secretion and also interdependent contribution of the signal peptide charge, anionic phospholipids, and translocational ATPase to secretion.

Key words: alkaline phosphatase, secretion, signal peptide, phospholipids, substitutions for amino acid residues, SecA, *Escherichia coli*

According to the theory of protein topogenesis [1], proteins destined to be secreted from the cytoplasm to cell compartments where they function contain specific so-called topogenic sequences of amino acid residues, which in the course of secretion interact with components of the secretory machinery. In bacteria this machinery includes cytoplasmic chaperones, cytoplasmic and membrane Sec proteins (translocational ATPase SecA, integral membrane heterotrimer SecYEG, and other proteins that all together form a "translocon"), and phospholipids [2]. However, the structural principles of interaction between these components and a secreted protein, as well as between the components themselves, are not quite clear. The most significant and well-studied topogenic sequence is a signal peptide (SP) localized in the N-terminal region of secreted protein precursors [3]. SP is necessary for recognition of a secreted protein by the components of the cell secretory machinery and for initiation of protein translocation across the cytoplasmic membrane. After the translocation is completed, SP is cleaved by a membrane-bound signal peptidase on the outer surface of the cytoplasmic membrane [4, 5]. The canonic SP has three specific regions: an N-terminal positively charged region, an extended hydrophobic region, and a more polar C-terminal region containing the site of recognition by signal peptidase [3]. Under secretion initiation, SP is supposed to interact not only with protein components of the secretory machinery but also with membrane phospholipids [6-9]. Indeed, SP contains the information for SP interaction with phospholipids. So, the hydrophobic region of SP promotes the forming of its α-helical conformation, which facilitates penetration of SP into the hydrophobic region of the cytoplasmic membrane lipid bilayer [10]. A decrease in the size or introduction of charged amino acid residues as well as residues breaking the secondary structure in the hydrophobic core results in a significant or complete inhibition of protein translocation across the membrane [10, 11]. Positively charged amino acid residues in the N-terminal region of the signal peptide are supposed to interact with negative-

Abbreviations: PhoA, prePhoA) mature alkaline phosphatase and its precursor, respectively; SP) signal peptide; PE) phosphatidylethanolamine; APL) anionic phospholipids; CL) cardiolipin; PG) phosphatidylglycerol; PA) phosphatidic acid.

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ly charged (anionic) phospholipids (APL) of the membranes [6-9]. Some structural principles of such interaction have been revealed in our previous works [12, 13], as well as the principles of interaction of SP C-terminus with signal peptidase [14]. The interaction of SP with anionic phospholipids (their content in the *E. coli* membrane is 20-25% [15]) has been shown in experiments *in vivo* [13, 16] and *in vitro* [17-22]. However, the question about the character of such interaction *in vivo* is still open. It is unclear whether the electrostatic interaction between the N-terminal region of the SP and anionic phospholipids is direct, as predicted by molecular modeling [12] and some indirect data [13, 17], as well as by our recent experiments *in vitro* [23], or involves lipid-dependent components of the secretory machinery such as SecA protein [24]. A promising strategy for answering this question seems to be the analysis of efficiency of secretion *in vivo* of proteins with different SP charges across the membranes with different contents of anionic phospholipids. Such an attempt was made using the strain *E. coli* HDL11, in which the synthesis of anionic phospholipids was controlled within 4 to 20% [23], but it gave no answer to this question. The question whether the SP–membrane interaction involves the predominant *E. coli* membrane phospholipid, phosphatidylethanolamine (PE), is still open as well—nothing is known about its interaction with SP. Our previous papers have shown the efficiency of *E. coli* alkaline phosphatase (PhoA) secretion to depend on the PE content in the membrane [25, 26]. It was revealed that the effect of PE depends on the primary structure of an export-initiation domain in the N-terminal region of the mature protein [27]. The latter fact suggests an interaction of PE with this domain, which apparently facilitates the entering of the mature hydrophilic part of the protein into a "translocon" due to the known ability of this phospholipid to form non-bilayer structures in the membrane [28, 29]. It was also shown that the effect of PE on secretion depends on the activity of translocational ATPase—protein SecA.

The goal of the current work was to assess the possibility of interaction of SP with PE, the character of SP interaction with membrane anionic phospholipids *in vivo* by varying their content in membranes from 20 to 100%, and the contribution of protein SecA to these interactions. The main strategy to address this goal was the analysis of secretion of wild type and mutant PhoAs with altered SP charge in *E. coli* strains with and without PE, containing 20 and 100% anionic phospholipids, respectively.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strain *E. coli* AD93 (*pss93::kan recA srl::*Tn*10* nadB$^+$), PE-depleted due to a mutation in the *pss* gene, which is responsible for the synthesis of a precursor of this phospholipid, was used in the work. Plasmid pDD72 with a cloned *pss* gene recovers PE synthesis in the above strain [30]. Plasmid pSAP-1 contains the wild type *phoA* gene, plasmids pSAP-2 and pSAP-3 contain mutant *phoA* genes encoding PhoA with the replacement of Lys(–20) by Ala and Glu, respectively. All *phoA* genes are under the control of P_{BAD} promoter of the arabinose operon [23].

Cultivation conditions. Bacterial cells were grown on a mineral medium [31] containing 100 μg/ml tryptophan to the mid log phase: the strain *E. coli* AD93 with and without plasmid pDD72 at 30 and 37°C, respectively, under intensive aeration. Plasmids pSAP-1, pSAP-2, and pSAP-3 were maintained using ampicillin (100 μg/ml) and plasmid pDD72 – using chloramphenicol (25 μg/ml). Growth medium for the strain *E. coli* AD93 contained bivalent cations Mg^{2+} in the final concentration of 50 mM, which are absolutely necessary for cell growth [30]. PhoA synthesis was induced by addition of arabinose up to the final concentration 0.5% to a culture grown on a mineral medium until the mid log growth phase. The activity of protein SecA was inhibited by 2 mM sodium azide. Culture samples were taken at certain periods of time. Biosynthetic processes were stopped by addition of 0.02% Merthiolate.

Secretion of alkaline phosphatase. The protein secretion was analyzed by two methods: by phosphatase activity of the culture (since PhoA becomes enzymatically active only after translocation across the cytoplasmic membrane into the periplasm [32]); by the dynamics of conversion of pulse labeled alkaline phosphatase precursor (prePhoA) into a mature form (alkaline phosphatase maturation) due to SP cleavage after the protein translocation [33].

Maturation of alkaline phosphatase. *E. coli* cells grown to the mid log growth phase were incubated for 10 min with 0.5% arabinose to induce PhoA, pulse labeled for 60 sec with L-[^{35}S]methionine, and the incorporation was stopped by addition of unlabeled amino acid. Culture samples were taken at certain times; proteins were precipitated by 10% TCA, and prePhoA and PhoA were immunoprecipitated using rabbit antibodies against denatured alkaline phosphatase and separated by electrophoresis followed by autoradiography and densitometry.

Spectrum of the alkaline phosphatase isoforms. Alkaline phosphatase isoforms were revealed by their activity in gel after electrophoresis of periplasmic proteins under non-denaturing conditions, using α-naphthyl phosphate and Fast Red RR [34]. The periplasm was isolated according to Miura and Mitsushima [35].

Lipid analysis. Lipids were extracted according to Aims et al. [36]. Thin-layer chromatography of the phospholipids was done on Kieselgel 60 plates (Merck, Germany) in the system chloroform–methanol–water–ammonium hydroxide (60 : 37.5 : 3 : 1 v/v) [37].

Individual phospholipids were detected by iodine vapor, cut out, and extracted from silica gel with chloroform–methanol–water (5 : 5 : 1 v/v), and the content of lipid phosphorus was measured as described previously [38].

Analytical methods. Protein electrophoresis was performed in 10% polyacrylamide gel in the presence of SDS [39]. Periplasmic proteins were separated under nondenaturing conditions in 7.5% polyacrylamide gel according to Davis [40]. Immunoblotting was carried out using an electrophoretic transfer of proteins from the gel to nitrocellulose BA 85 (Schleicher and Schull, Germany) followed by PhoA treatment with rabbit antibodies against denatured alkaline phosphatase and protein A conjugate with horseradish peroxidase (Bio-Rad, USA) as described previously [34]. Alkaline phosphatase activity was determined by the rate of *p*-nitrophenylphosphate hydrolysis [31], taking the amount of the enzyme hydrolyzing 1 μmol of substrate per 1 min at 37°C as a unit of enzyme activity (U). PhoA activity was determined in cell culture (cells + culture medium), because alkaline phosphatase encoded by the gene constituent of plasmid is partially secreted into the medium [41]. Protein was assayed by the Lowry method [42].

RESULTS

Dependence of PhoA secretion on the signal peptide charge is determined by membrane phospholipid composition. The secretion of the wild type *E. coli* alkaline phosphatase and its mutant forms with an altered SP charge depending on membrane phospholipid composition has been studied in cells of a mutant strain *E. coli* AD93 lacking PE, as well as in cells of the same strain containing PE due to the presence of plasmid pDD72. The strain AD93 lacks the pss gene encoding the synthesis of PE precursor [30] and as a result its membranes contain anionic phospholipids only. If the strain AD93 has plasmid pDD72 with a functional copy of the *pss* gene, then its phospholipid composition recovers and corresponds to that of wild type cells with the PE content of about 80% (Table 1). The secretion of PhoAs with different SP charges has been studied. The replacement of Lys in position –20 in the N-terminal region of SP by uncharged Ala or negatively charged Glu decreases the efficiency of PhoA secretion tested by its activity, both in PE-containing and PE-lacking cells (Fig. 1). However, in the absence of PE, the dependence of PhoA secretion efficiency on the signal peptide charge was less profound than in the presence of PE (Fig. 1). At the same time, secretion of the wild type protein decreases in the absence of PE actually to the same extent as with a change in the SP charge, indicating the importance of both phospholipid membrane composition and the SP charge for secretion. The lower activity of wild type and mutant alkaline phosphatases in the absence of PE is not associated with any significant changes in the protein expression, which was assessed by PhoA immunoblotting in protein-equated culture samples (Fig. 2A, a). However, it is known that isoforms of PhoA are formed in the periplasm during post-translocational proteolytic modification of the enzyme (the cleavage of N-terminal Arg by periplasmic proteinase) followed by dimerization of subunits [43], which differ in their specific activities [44]. The change in the spectrum of isoforms in the absence of PE were shown earlier [45]

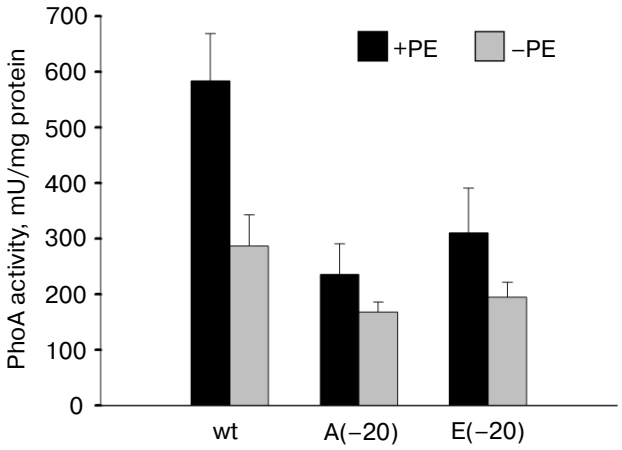

Fig. 1. Effect of membrane phospholipid composition on secretion of wild type and mutant alkaline phosphatases in the strains *E. coli* AD93 (–PE) and AD93/pDD72 (+PE). Here and in other experiments PhoA secretion was determined 1 h after the induction of the enzyme synthesis.

Table 1. Phospholipid composition of *E. coli* AD93 cells

Strain *E. coli*	Content of phospholipids, mole %			
	PE	PG	CL	PA
AD93/pSAP-1	0.0	62 ± 4	32 ± 3	6.3 ± 0.7
AD93/pDD72/pSAP-1	79 ± 5	15 ± 2	4.0 ± 0.5	2.7 ± 0.3

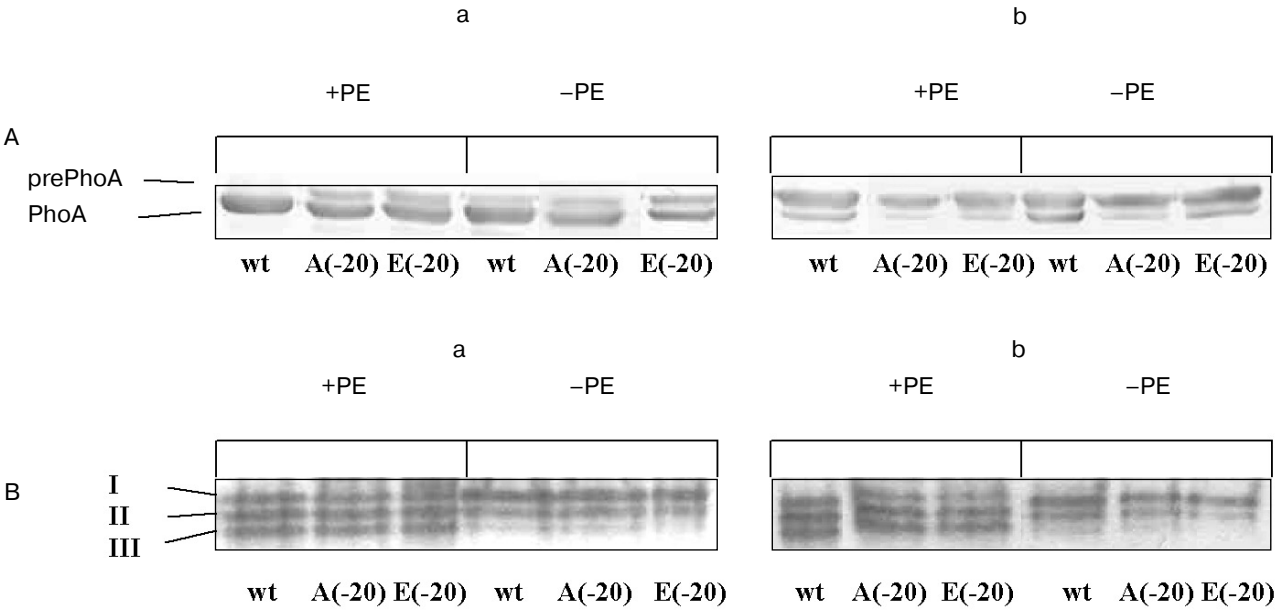

Fig. 2. Immunoblotting (A) and isoform spectrum (B) of wild type and mutant alkaline phosphatases in the strain *E. coli* AD93 (–PE) and AD93/pDD72 (+PE) in the absence (a) and presence (b) of 2 mM sodium azide (azide was added at the moment of the enzyme induction). Roman numerals indicate alkaline phosphatase isoforms.

to be associated with the decrease of secretion of a modifying proteinase localized in the periplasm, and the revealed changes in the spectrum of isoforms (Fig. 2B, a) could affect the total phosphatase activity of the culture. Therefore, a more accurate estimation of secretion efficiency was made by the dynamics of maturation of pulse labeled prePhoA (Fig. 3A), which confirmed, as a whole, the revealed dependence of PhoA secretion on the signal peptide charge and membrane phospholipid composition. Figure 3A (a) shows that the maturation of mutant proteins in PE-containing cells is much slower than the maturation of wild type protein. Whereas the content of mature wild type PhoA is 100% in 1 min after labeling is stopped, the content of mature mutant proteins is 66 and 52%. In the absence of PE, this feature remains but is expressed much weaker (79, 68, and 41%, respectively) (Fig. 3A, b). Moreover, whereas in the absence of PE the wild type protein and protein E(–20) with positively and negatively charged SP, respectively, mature slower than in the presence of PE, the mutant protein A(–20) with uncharged SP matures in the absence of PE with nearly the same rate as in its presence (Fig. 3A). Thus, the replacement of a positively charged SP by a negatively charged one makes the secretion less dependent on the phospholipid membrane composition, while the change in the phospholipid membrane composition makes the secretion efficiency less dependent on the signal peptide charge. These results suggest a direct interaction of a positively charged region of the signal peptide with negatively charged membrane anionic phospholipids *in vivo*. However, it should be noted that secretion of wild type PhoA having positively charged SP decreases under PE depletion much more than secretion of mutant PhoAs, indicating also the possibility of interaction of SP with this phospholipid. More direct evidence of the interaction of a secreted protein with phospholipids at the initial steps of its translocation across the membranes were obtained using molecular cross-linking in the only work using a eukaryotic protein [46].

Dependence of secretion on membrane phospholipid composition and signal peptide charge is determined by the activity of translocational ATPase—protein SecA. To assess the contribution of protein SecA, the most important protein of the cell secretory machinery, to the interaction of the PhoA signal peptide with membrane phospholipids and the character of this interaction, the efficiency of secretion of wild type alkaline phosphatase and its mutant forms with a changed SP charge in the absence and presence of sodium azide was studied. Sodium azide is known [47] to be an inhibitor of many ATPases, and SecA is the most azide-sensitive ATPase of *E. coli*. As one might expect, inactivation of SecA by sodium azide significantly decreased the efficiency of PhoA secretion, tested both by its activity (Table 2) and by pulse labeling of the protein (Fig. 3B). No significant effect of sodium azide on the expression and isoform spectrum of PhoA was revealed under these conditions (Fig. 2). The degree of secretion inhibition under SecA inactivation is determined by membrane phospholipid composition and the prePhoA signal peptide charge. Under SecA inactivation,

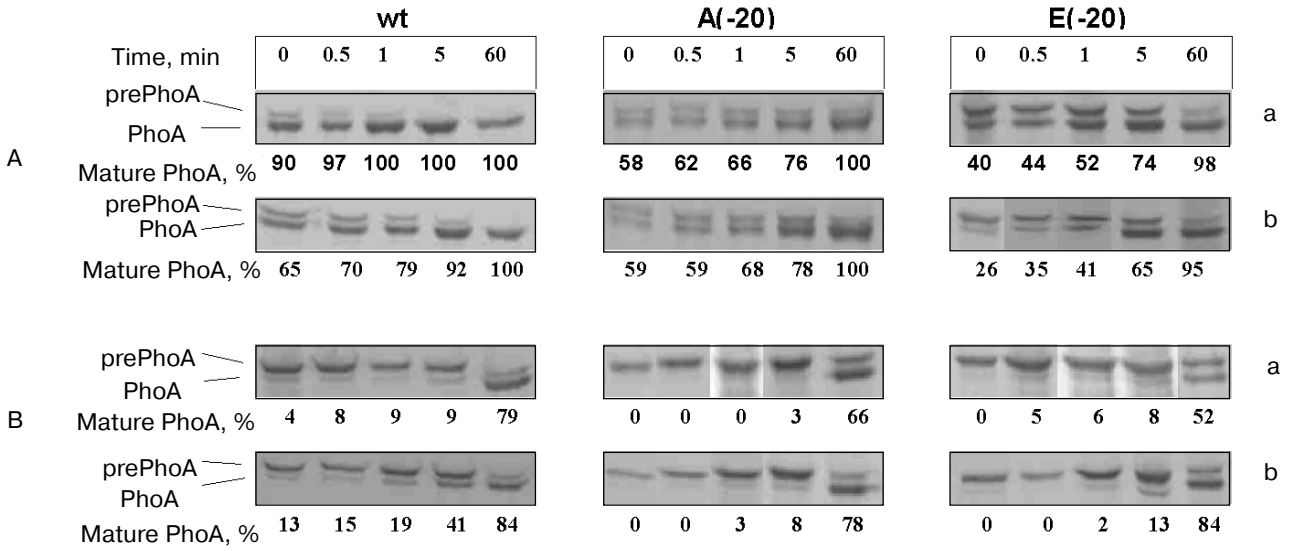

Fig. 3. Dynamics of conversion of precursors of wild type alkaline phosphatase (wt) and its mutant forms A(−20) and E(−20) into a mature protein *in vivo* in cells of the strain *E. coli* AD93 containing (a) or completely lacking (b) PE in the absence (A) and presence (B) of 2 mM sodium azide. After induction of PhoA with 0.5% arabinose for 10 min, cells were labeled with L-[^{35}S]methionine (50 μCi/ml) for 60 sec, and incorporation was stopped by addition of unlabeled amino acid. Culture aliquots were taken at indicated times, proteins were precipitated by 10% TCA, PhoA and prePhoA were immunoprecipitated with antibodies against denatured alkaline phosphatase and analyzed by electrophoresis followed by autoradiography and densitometry to assess the relative content of mature protein.

the secretion of wild type PhoA decreases much stronger in the presence of PE than in its absence. The secretion of mutant protein E(−20) with the replacement of Lys by Glu shows approximately the same or somewhat lesser dependence on membrane phospholipid composition as in the wild type protein, whereas the efficiency of secretion of protein A(−20) with uncharged SP actually does not depend on the PE content (Table 2 and Fig. 3B). Thus, under SecA inactivation the secretion of proteins with positively charged SP is more efficient (particularly at the initial steps of translocation), if the membranes contain anionic phospholipids only.

Comparison of the dependence of secretion efficiency on PE content and SecA activity (Table 3) shows an interesting peculiarity. On one hand, the dependence of secretion on anionic phospholipids increases under SecA inactivation. On the other hand, the dependence of secretion on SecA proportionally increases with the decrease in the content of anionic phospholipids. Secretion of the mutant protein with uncharged SP shows no such cross dependence on APL and SecA (Table 3). The results suggest an interdependent contribution of the translocational ATPase (protein SecA), phospholipid membrane composition, and the signal peptide charge to the efficiency of

Table 2. Effect of SecA inactivation on secretion of wild type and mutant alkaline phosphatases depending on PE content

PE content	Conditions of PhoA secretion	Activity of PhoA, mU/mg protein*		
		wt	A(−20)	E(−20)
+ PE	− azide	580 ± 80	240 ± 60	310 ± 80
	+ azide	80 ± 17	23 ± 10	14 ± 3
— PE	− azide	290 ± 60	170 ± 20	190 ± 30
	+ azide	80 ± 30	14 ± 5	29 ± 7

* Activity of cell culture 1 h after the induction of PhoA synthesis.

Table 3. Dependence of PhoA secretion (1 h after enzyme induction) on phospholipid membrane composition, SecA activity, and signal peptide primary structure

Dependence of secretion	Conditions of PhoA secretion	Dependence index*		
		wt	A(−20)	E(−20)
APL-dependence	− azide	0.5	0.7	0.6
	+ azide	1.0	0.6	2.0
SecA-dependence	+ PE	7.3	10.4	22.1
	− PE	3.6	12.1	6.6

* APL and SecA dependences are presented as a ratio of PhoA activity in the culture of cells without/with PE (APL-dependence); cells grown in the absence/presence of sodium azide (SecA dependence). Average values of six independent experiments are given.

protein secretion under its initiation and a partial interchangeability of SecA and anionic phospholipids in providing PhoA secretion.

DISCUSSION

Considerable progress has been made in understanding the molecular bases of protein topogenesis [2, 9, 48]. The nature of intragenic information that determines protein secretion has been established [1, 2] and the functions of numerous protein factors of secretion, which catalyze this process, have been revealed [2, 48]. Currently it is considered quite proved that phospholipids are also an integral part of the secretory machinery of a bacterial cell [9, 49, 50]. However, in contrast to the protein components of the secretory machinery, the role of individual phospholipids in this process is still far from complete understanding. The mutants in the biosynthesis of specific classes of phospholipids are good tools for the study of phospholipid involvement in different membrane-bound processes, including protein translocation across the membranes. Using the strain *E. coli* AD93, which is unable to synthesize the major phospholipid of *E. coli*—PE, it has been shown that PE promotes the folding of a polytopic membrane protein—lactose permease [51]. The absence of this phospholipid disturbs not only secretion but also expression of proteins, which is regulated by two-component systems of signal transduction having membrane-bound sensors [26, 52]. In *E. coli*, PE is the main phospholipid able to form a non-bilayer structure in the membrane [28, 29], which was supposed [6, 7] and then substantiated *in vitro* [53] and *in vivo* [25, 26] to facilitate the protein translocation across the membrane. It was also shown that PE interacts with the N-terminal region of the export-initiation domain of mature alkaline phosphatase, which is assumed to promote the entering of the hydrophilic part of a protein into the membrane "translocon" [27]. The current work demonstrates that the absence of PE in the membrane decreases the efficiency of PhoA secretion (both wild type and mutant with a changed SP charge), showing the main function of this phospholipid in the secretory process—formation of a non-bilayer structure in the membrane. Besides, the work has first shown that the phospholipid membrane composition determines the dependence of secretion efficiency both on the charge of the SP N-terminal region and on the activity of translocational ATPase—protein SecA. The data obtained are in line with the suggestion of an electrostatic interaction between SP and APL [6, 12, 13], which is important for wild type proteins with canonic signal peptides interacting with wild type membranes. The signal peptide of prePhoA has all attributes of the canonic SP and contains a positively charged N-terminal region with Lys(−20). Using amber-suppressor mutagenesis that allowed the replacement of this positively charged residue by other amino acid residues [13, 54], it was shown that only the replacement of Lys(−20) by uncharged or negatively charged residues hinder prePhoA translocation across the cytoplasmic membrane. The same data were obtained *in vivo* with lipoprotein of the outer membranes where the SP charge was also changed by way of amino acid replacement [55]. Both cases showed that positively charged residues in the SP N-terminus are not critical for translocation but appreciably increase its efficiency. Molecular modeling and stereochemical analysis of the interaction of SP N-terminus with anionic phospholipids [12] by the example of prePhoA showed that the formation of their complex due to hydrogen bonds promotes insertion of its components into the lipid bilayer at a lower energy expense, as compared with the insertion of the above components sepa-

rately. At the same time, Lys(–20) in SP is able to form an additional ion pair with a negatively charged phosphate group of anionic phospholipid molecule [13], stabilizing this complex. This suggests the positive charge of the SP N-terminus to enhance the efficiency of protein translocation just because of stabilization of the "signal peptide–anionic phospholipids" complex under the initiation of protein translocation (the stage of SP insertion into the lipid bilayer), improving the kinetic parameters of the process. However, the question about the character of such interaction is still open—whether it is a direct interaction between the positively charged signal peptide N-terminus and anionic phospholipids, or it involves the protein components of the secretory machinery [24, 56]. The results of the current work have first shown that SP charge has less effect on the dependence of secretion with a change in membrane phospholipid composition, in particular, when the membranes contain anionic phospholipids only. We consider this as evidence of a direct electrostatic interaction between the positively charged SP N-terminus and negatively charged phospholipids under secretion initiation. PE is also needed for this interaction, because the secretion of the wild type protein is more affected under PE depletion than the secretion of the mutant protein, particularly of the protein with uncharged SP. The most direct evidence of intermolecular interaction are being obtained currently using the technique of molecular cross-linking by introduction of amino acids with photoactivated chemical groups by the method of amber-suppressor mutagenesis [46]. Note that the cited paper is the only work, which in this way shows the interaction of a secreted (eukaryotic) protein at the initial steps of its membrane translocation not only with proteins but also with phospholipids. However, such technology actually cannot be used for demonstration of the interaction under study due to the presence of a modified amino acid N-formyl-methionine at the N-terminus of the bacterial preproteins.

One more important result of the current work is the fact that the dependence of secretion on phospholipid composition is determined by the activity of translocational ATPase—protein SecA. This protein is a molecular motor, which as a result of an ATP-dependent change in its conformation coupled with ATPase activity regulated by anionic phospholipids [24] promotes the translocation of a mature hydrophilic part of a protein across the translocon formed by integral membrane proteins SecYEG and a number of additional proteins, pushing the secreted protein through a protein pore [2, 48, 57]. As concerns SP, its insertion into the membrane resulting in preprotein "anchoring" in the membrane before its hydrophilic mature part enters the translocon, according to the modern conceptions [2, 9, 13], is initiated first of all by SP–phospholipid interaction, and the role of protein SecA in this process has not yet been determined anyhow. Probably, the results of the current work will stimulate the study of this role. The current work has shown that under SecA inactivation the secretion proceeds somewhat more efficiently in the absence of PE, when the membranes comprise anionic phospholipids only, whereas in the presence of PE, i.e., at the lower APL content, the dependence of secretion on the activity of translocational ATPase is much more profound. The results of the work suggest a possibility of interaction of positively charged SP with anionic phospholipids and protein SecA simultaneously or in a yet unknown succession—with one and then with the other. The results also show a possible interrelation between the contribution into secretion of APL and SecA. The results also indicate the suppression of wild type PhoA secretion decreasing due to inactivation of SecA by anionic phospholipids. These results are in accordance with the data [56], which show that the higher concentration of SecA in the cytosol may compensate for the defect in protein translocation caused by deficiency of anionic phospholipids. However, it should be noted that anionic phospholipids have never restored secretion efficiency to the level observed in the presence of intact SecA. This points to the more important role of the protein SecA in secretion than the role of APL in Sec-dependent secretory pathway, by which the protein under study is translocated across the cytoplasmic membrane. It cannot be excluded, however, that the interdependent involvement of anionic phospholipids and/or SecA in protein translocation may reflect the existence of a different level of evolutional development of secretory pathways in bacteria. Recently it has been shown [58] that Sec-dependent secretion is much less determined by the content of anionic phospholipids in the membrane than Sec-independent (or TAT-dependent) secretion.

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