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Effect of Export-Specific Cytoplasmic Chaperone, Protein SecB, on Secretion of *Escherichia coli* Alkaline Phosphatase

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Abstract—The efficiency of secretion of *Escherichia coli* alkaline phosphatase depends on the presence in cells of a cytoplasmic chaperone—protein SecB. Secretion increases in the presence of this chaperone at 30°C, which is the most favorable for the interaction of SecB with the export-initiation domain found previously in the N-terminal region of the mature enzyme. This interaction most likely occurs in the region of the export domain, which is located close to the signal peptide and in complex with a translocational ATPase—protein SecA.

Key words: *Escherichia coli*, alkaline phosphatase, protein translocation, export domain, amino acid substitutions, chaperone SecB, protein SecA

Proteins of *Escherichia coli* destined to be secreted into the periplasm or outer membrane are synthesized as precursors containing an N-terminal signal sequence that is necessary for protein recognition by the secretory machinery and membrane translocation [1-3]. Protein membrane translocation via the Sec-dependent secretory pathway is catalyzed by a group of cytoplasmic and integral membrane Sec proteins. Most precursors interact in the cytoplasm with an export-specific chaperone, protein SecB, which prevents aggregation and untimely and incorrect folding of newly synthesized precursors and keeps them in a state competent for membrane translocation. The "precursor–SecB" complex then binds to the carboxyl terminus of a translocational ATPase, protein SecA, and targets with it to the integral membrane protein, the heterotrimer SecYEG. The latter, together with proteins SecD and SecF, forms the so-called "translocon". Here, SecB dissociates from the precursor. The latter, in stepwise fashion, is translocated across the cytoplasmic membrane due to conformational changes of protein SecA, a molecular motor promoting the translocation of the precursor. It is assumed that the mature portion of the precursor is involved in the interaction with the SecB and SecA [4, 5], but the specificity of such interaction is still obscure [3]. In the previous work, an export-initiation domain 14-16 amino acid residues in size was revealed in the N-terminal region of mature alkaline phosphatase (PhoA) based on statistical analysis of the known primary structures of N-terminal regions of secreted proteins and mutagenesis of PhoA [6]. The effect of substitutions for amino acid residues in different positions of this domain on protein secretion was studied in detail [6, 7], and it was assumed that the export domain is involved in PhoA secretion due to its interaction with cytoplasmic components of the secretory machinery.

It was shown earlier that the cytoplasmic chaperone SecB participates in the secretion of such proteins as PhoE [8], LamB, OmpA, and maltose-binding protein [9], but the secretion of some other proteins (β-lactamase and ribose-binding protein) is considered SecB-independent [10]. As for PhoA secretion, some researchers regard it as SecB-independent [9, 11], while others believe that it is SecB-independent only at 42°C [12]. Thus, the question about the involvement of SecB in the secretion of this enzyme is still open.

The goal of the current work was to study the effect of protein SecB on the efficiency of PhoA secretion. The effect of translocational ATPase protein SecA, which interacts with SecB as mentioned above, on PhoA secretion has also been studied. The main strategy was the analysis of the secretion efficiency of PhoA, both wild type and mutants with substitutions for amino acid residues in the export-initiation domain, in isogenic

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strains containing or lacking SecB due to mutations, as well as in the presence of active and inactivated SecA.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. The strain *E. coli* MC4100 (F^- Δ*lac U169 araD139 relA1 rpsL150 thi f1bB5301 ptsF25*) [13] and its derivative CK1953 (MC4100 *secB*::Tn5) [9] were used in the work. These strains were transformed by phagemid pPHOA13 carrying the native gene *phoA* or its mutant forms encoding PhoA with substitutions of two lysines for amino acid residues in positions +2+3, +5+6, +13+14 [6]. Cells were grown on a mineral medium [14] containing 0.07% peptone, 1 mM K_2HPO_4 for repression of alkaline phosphatase synthesis, and 0.5% glycerol as a carbon source [15]. The antibiotics chloramphenicol (25 μg/ml) and kanamycin (50 μg/ml) were added when required. Cells were grown to the mid-log phase at 37°C under aeration, centrifuged at 5000*g* for 10 min, twice washed with 0.14 M NaCl, and then placed into a medium of the same composition but without K_2HPO_4 for derepression of the enzyme synthesis at 30°C for 1 h.

Analysis of alkaline phosphatase secretion. The efficiency of secretion of wild type and mutant PhoAs was assessed using two approaches. One of them was the analysis of enzyme activity of cells producing PhoA, since it is known [16] that PhoA becomes enzymatically active only after translocation into the periplasm. The other was analysis of the dynamics of conversion of pulse-labeled PhoA precursor (prePhoA) into mature form (protein maturation) due to signal peptide cleavage, which also occurs after the protein translocation.

Analysis of alkaline phosphatase maturation. *E. coli* cells were grown to the mid-log phase in complete mineral medium, then harvested by centrifugation, twice washed, and transferred into the same medium but without K_2HPO_4. In 30 min after induction of the enzyme, cells were labeled with [^{35}S]methionine (20 μCi/ml) for 30 sec; the label incorporation was stopped by addition of unlabeled methionine to the final concentration of 0.05%. Culture samples were taken in 0.1, 5.0, 15.0, and 30 min after dilution. Proteins were precipitated by 10% TCA, and PhoA and prePhoA were immunoprecipitated by rabbit IgG against alkaline phosphatase and separated by electrophoresis followed by radioautography. Protein content was assayed on a scanning laser densitometer (LKB, Sweden). The prePhoA/PhoA ratio was determined taking into account the difference in methionine content in precursor and in mature alkaline phosphatase.

SecB-dependence of PhoA secretion was studied using a mutant *E. coli* strain lacking the intact gene of SecB, and SecA-dependence was studied by inhibition of the activity of this protein with 2 mM sodium azide.

Analytical procedures. Proteins were separated by electrophoresis in 10% polyacrylamide gel in the presence of SDS [17]. PhoA activity was determined in *E. coli* culture producing wild type or mutant phosphatases by the rate of hydrolysis of *p*-nitrophenylphosphate [14] penetrating the periplasm of cells; the unit of enzyme activity (U) was taken as the enzyme amount hydrolyzing 1 μmol of substrate per min at 37°C. Specific activity was expressed in mU per mg cell protein. To reveal PhoA isoforms, periplasmic proteins were separated by electrophoresis in 7.5% polyacrylamide gel under non-denaturing conditions [18] followed by staining of isoforms by treating the gel with α-naphthylphosphate (Sigma, USA), and Fast Red Dye TR (Chemapol, Czechia) [19]. Protein content was assayed by the method of Lowry et al. [20].

RESULTS

Protein SecB affects the efficiency of alkaline phosphatase secretion. The study of the involvement of SecB in PhoA secretion in the current work has been based on the known data that SecB-dependence of protein secretion is determined by the rate of protein folding, which in turn may be determined by a factor such as temperature [21]. We have studied secretion of the alkaline phosphatase encoded by both the chromosomal gene *phoA* and the gene cloned in a plasmid. The main approach was to study the temperature dependence of PhoA secretion in strains containing or lacking protein SecB.

The temperature of cell cultivation was shown to actually determine the SecB-dependence of PhoA secretion. This dependence was the highest at 30°C, whereas no significant SecB-dependence was observed at other temperatures (Table 1). At 30°C, the level of PhoA activity in the strain containing SecB was twofold higher than in the strain lacking this protein. It increased both in case of PhoA encoded by the chromosomal gene and in case of the gene expressed from the plasmid (Fig. 1A). This increase was not associated with the change in the spectrum of enzyme isoforms, the ratio of which could affect the total PhoA activity since isoforms have different specific activities [22]. As Fig. 1B shows, the relative content of PhoA isoforms in cells containing or lacking SecB is actually the same. The effect of SecB exactly on secretion efficiency was also confirmed in pulse-chase experiments. Figure 1C shows that mature PhoA is predominant in SecB-containing cells 5 min after the chase. In SecB-lacking cells, in contrast, unprocessed precursor is found even 30 min after the label stops to be incorporated. Thus, the results obtained by the two approaches show that SecB affects the efficiency of PhoA secretion and SecB-dependence of the enzyme secretion is determined by the rate of the protein folding, which is probably the most favorable for the interaction between prePhoA and the

Table 1. Effect of temperature of *E. coli* cell cultivation on the dependence of alkaline phosphatase secretion on the presence of SecB in the cells

Cultivation temperature, °C	SecB-dependence	
	expression of chromosomal gene of alkaline phosphatase	expression of alkaline phosphatase gene cloned in plasmid
42	0.45	1.01
37	1.28	0.95
30	1.86	1.76
23	1.00	1.17

Note: SecB-dependence is presented as a ratio of the alkaline phosphatase activity (mU/mg) of cells containing SecB to the activity of cells lacking SecB. The average data of three independent experiments are given. The mean deviation is no more than 20%.

chaperone at 30°C. SecB-dependence of secretion of mutant PhoAs was studied also at 30°C.

The effect of SecB on alkaline phosphatase secretion is determined by the primary structure of the export-initiation domain. The effect of SecB on secretion of mutant PhoAs (K(+2+3), K(+5+6), K(+13+14)), where amino acid residues were replaced by two lysine residues in positions +2+3, +5+6, +13+14, respectively, has been studied. As we established previously [6, 7], the efficiency of PhoA secretion depends on the primary structure of the export-initiation domain in the mature portion of polypeptide chain of the enzyme. Amino acid substitutions close to the signal peptide (positions +2+3) were shown to have the greatest effect on PhoA secretion in the strain *E. coli* E15. Both the level of activity of this mutant protein and the rate of its maturation were the lowest. This peculiarity was confirmed also in the current work, where secretion of mutant proteins has been studied in other strains by the analysis of their PhoA activity (Fig. 2). Moreover, the secretion of all mutant PhoAs with amino acid replacements in the export-initiation domain, except for one PhoA, decreased in the absence of SecB (similar to the secretion of wild type PhoA). The level of PhoA activity, both wild type and mutant, decreased for ~40% in the absence of SecB. The level of activity of mutant PhoAs with substitutions for amino acids close to the signal peptide (in positions +2,+3) in the absence of SecB actually was not reduced. Thus, SecB-dependence of this mutant form of PhoA was lower compared with those of other mutant forms and wild type PhoA (Table 2). The efficiency of secretion of both wild type and mutant PhoAs also decreased under SecA inactivation by sodium azide (data not presented). In these conditions, SecB-dependence of the wild type protein secretion sig-

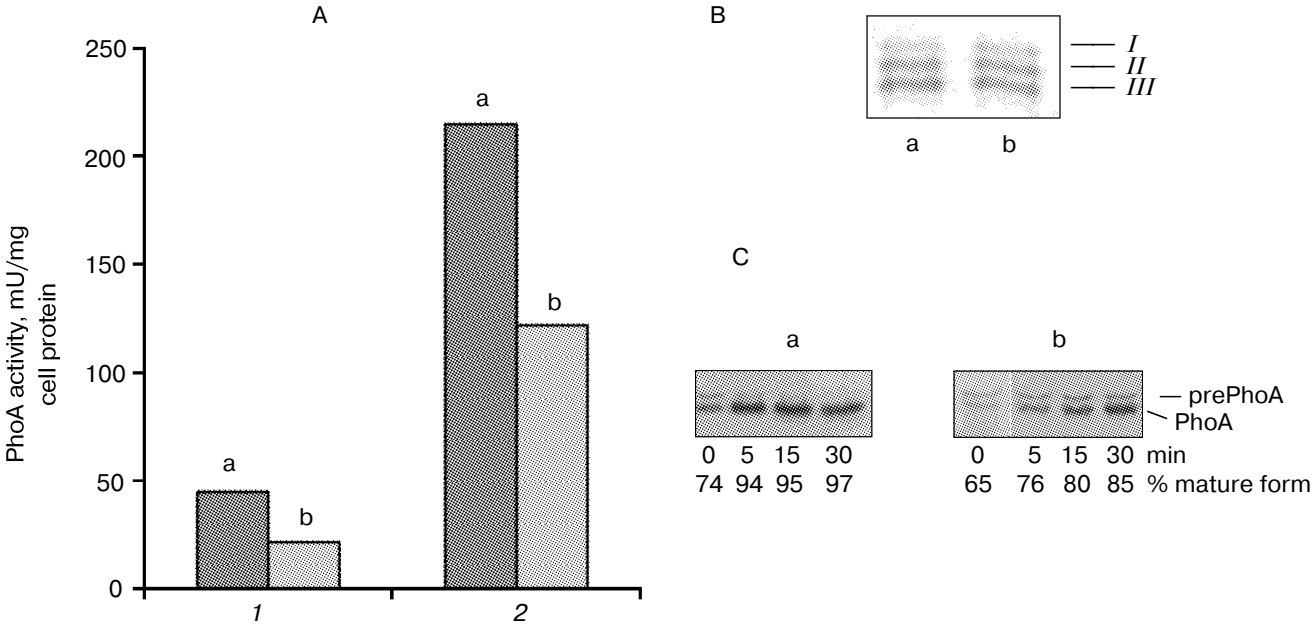

Fig. 1. Effect of SecB on PhoA secretion in *E. coli* cells. A) Activity of PhoA encoded by the chromosomal *phoA* gene (*1*) and the *phoA* gene cloned in plasmid (*2*) in cells with (a) and without (b) SecB. B) Spectrum of isoforms (*I*, *II*, and *III*) of PhoA secreted in cells with (a) and without (b) SecB. C) The dynamics of alkaline phosphatase maturation in cells with (a) and without (b) SecB. Radioautograph of PhoA and prePhoA immunoprecipitates after their electrophoretic separation is presented. Numbers are the relative content of mature PhoA in the dynamics of prePhoA maturation.

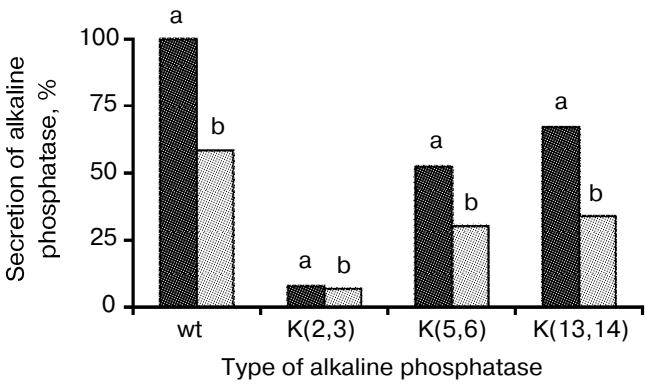

Fig. 2. Efficiency of PhoA secretion depending on the primary structure of export-initiation domain in the presence (a) or absence (b) of SecB in the cells (activity of wild type alkaline phosphatase in cells containing SecB is taken to be 100%).

nificantly decreased (~2-fold), while it remained unchanged for all mutant PhoAs. It is interesting that the inactivation of SecA enhanced this dependence in mutant forms as substitutions for amino acid residues receded from the signal peptide (Table 2).

DISCUSSION

The theory of protein topogenesis [23] suggests that the information about secretion of a protein is contained in the primary structure of its specific, so-called "topogenic", sequences. This information is realized via the interaction of topogenic sequences with components of cell secretory machinery (in procaryotes, this is mainly the family of Sec proteins), which catalyze protein translocation across membranes and their sorting in cell compartments. However, the best studied of all predicted topogenic sequences is the additional N-terminal sequence, the so-called signal peptide [24], which is obligatory for recognition of the nascent polypeptide chain of secreted protein by the secretory machinery and for initiation of protein translocation across membranes, and which is cleaved by signal peptidase after the translocation is completed. Much less is known about the topogenic sequences of mature polypeptide chains of secreted proteins. Our previous works revealed some structural principles of the interaction of N-terminal region of PhoA signal peptide with membrane anionic phospholipids [25] and its C-terminal region with signal peptidase [26]. Besides, the export-initiation domain was first found in the N-terminal region of PhoA mature polypeptide chain [6, 7]. This domain 14-16 amino acid residues in size is necessary for efficient PhoA secretion, probably due to the interaction of this domain with cytoplasmic factors of secretion.

Table 2. Effect of SecB presence and SecA activity on secretion of *E. coli* alkaline phosphatase depending on the structure of its export-initiation domain

Type of alkaline phosphatase	SecB-dependence	
	active SecA	inactivated SecA
wt	1.76	0.67
K (+2, +3)	1.04	1.03
K (+5, +6)	1.60	1.44
K (+13, +14)	1.87	3.05

Note: SecB-dependence is calculated as a ratio of alkaline phosphatase activity (mU/mg) of cells containing SecB to the activity of cells lacking SecB; wt, wild type protein. The average data of five independent experiments are given. The mean deviation is no more than 30%.

The current work has first established the dependence of PhoA secretion on a cytoplasmic chaperone, protein SecB, previously assumed [27] but not reliably proved. This dependence was revealed only at 30°C, which supported the original model [22] showing that SecB-dependence of protein secretion is determined by the rate of protein folding. Moreover, the current work has shown that SecB-dependence of PhoA secretion is determined by the primary structure of the export-initiation domain. Substitutions for amino acid residues close to the signal peptide (in positions +2,+3) suppress protein secretion most significantly. Meanwhile, the dependence of secretion of the mutant PhoA with such substitutions on the presence of SecB in cells and the activity of SecA differs from that in wild type and other mutant PhoAs. The results suggest the interaction of export-specific chaperone SecB with the export-initiation domain of mature PhoA close to the signal peptide, probably in concert with SecA. Now there are two opposite points of view for the interaction of preproteins with chaperone SecB: specific interaction with a small region of polypeptide chain having definite primary structure [28], and without specificity for sequence or structural content [29]. The data obtained here support the first alternative.

Thus, the results of the current study have shown for the first time that: 1) the efficiency of secretion of the *E. coli* alkaline phosphatase depends on SecB at 30°C, which is the most favorable for prePhoA interaction with this protein, and 2) SecB interacts with the export-initiation domain, most probably close to the signal peptide and in complex with the translocational ATPase protein SecA.

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