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Depletion of Phosphatidylethanolamine—the Major Membrane Phospholipid of *Escherichia coli*—Depresses Posttranslocational Modification of Alkaline Phosphatase in the Periplasm

V. V. Golovastov, N. I. Mikhaleva, and M. A. Nesmeyanova*

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow Region, 142290 Russia; fax: (095) 956-3370; E-mail: aniram@ibpm.serpukhov.su

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Abstract—Unbalanced phospholipid composition due to depletion of the major phospholipid of *Escherichia coli*, phosphatidylethanolamine, was shown previously to significantly decrease the secretion and transcriptional expression of periplasmic alkaline phosphatase of this bacterium. The current work studies the effect of this unbalance on posttranslocational proteolytic modification of the enzyme, proceeding in the periplasm with the formation of its isoforms. It has been revealed that under phosphatidylethanolamine depletion the content of incompletely modified (intermediate) isoforms I and II increases. This increase does not depend on the level of enzyme synthesis and the mechanism of its regulation (expression of the chromosomal gene or the gene cloned in plasmid under the control of the endogenous promoter P_{PHO} or exogenous promoter P_{BAD}). Depression of posttranslocational modification of alkaline phosphatase in phosphatidylethanolamine-depleted cells is supposed to be a result of the lower efficiency of secretion of modifying proteinase (product of the *iap* gene) localized, like alkaline phosphatase, in the periplasm of *Escherichia coli*.

Key words: *Escherichia coli*, alkaline phosphatase, isoforms, secretion, phospholipids, bacteria, posttranslocational modification

Alkaline phosphatase (PhoA) is a typical secreted protein of *E. coli* localized in the periplasm. Biogenesis of this enzyme, coupled with secretion, passes several steps [1]. The enzyme is synthesized in the cytoplasm as a precursor (prePhoA) containing at the N-terminus a signal peptide. The prePhoA has to cross the cytoplasmic membrane to reach the place of its final localization. After the protein translocation the signal peptide is cleaved on the outer surface of the cytoplasmic membrane by leader peptidase. In the periplasm the protein undergoes further modification—the cleavage of N-terminal Arg by a modifying protease (product of the *iap* gene), simultaneously with dimerization of subunits and formation of an active macromolecule of the enzyme. During the last step of enzyme assembly in the periplasm three isoforms are formed: a dimer of identical subunits containing N-terminal Arg (isoform I), a heterodimer with only one subunit lacking Arg (isoform II), and a dimer with both subunits lacking Arg (isoform III) [1]. Under conditions of balanced PhoA synthesis and secretion, the completely processed isoform III is usually predominant in the periplasm of *E. coli*. The spectrum of alkaline phosphatase isoforms is known to depend on the conditions of culture growth and the level of the enzyme synthesis. In particular, all three isoforms are found under oversynthesis of the enzyme, encoded by the gene constituent of plasmids [2].

Our previous works showed that the strain AD93 with the membrane lacking the major phospholipid—zwitterionic phosphatidylethanolamine (PE)—and consisting solely of anionic phospholipids has a significantly lower efficiency of secretion of periplasmic proteins [3, 4], including alkaline phosphatase [4]. Moreover, it was also revealed that the unbalance of phospholipids due to PE depletion in cell membranes also affects the transcriptional expression of alkaline phosphatase [5]. However, the effect of PE on the final step of the enzyme biogenesis—posttranslocational modification—is still unclear, and the current work was aimed to solve this question. The main strategy of addressing this question was to analyze the spectrum of PhoA isoforms in PE-depleted *E. coli* cells in comparison with the cells containing this phospholipid. The results have shown that the absence of PE in membranes

Abbreviations: PhoA) alkaline phosphatase; PE) phosphatidylethanolamine; P_i) orthophosphate.

* To whom correspondence should be addressed.

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leads to a significant decrease in the rate of posttranslocational modification of PhoA and this decrease is supposed to be due to depression under these conditions of secretion of the modifying proteinase into the periplasm.

MATERIALS AND METHODS

Bacterial strains and plasmids. Mutant strain *E. coli* AD93 (*pss93::kan recA srl::*Tn*10* nadB$^+$) lacking PE due to the absence of the *pss* gene was used in the work. The same strain carrying plasmid pDD72 with the *pss* gene and containing PE was used as the control [6]. These strains were also used for expression of the *phoA* gene under the control of the endogenous P_{PHO} promoter [7] constituent of plasmid pHI-7, or under the control of P_{BAD} promoter [8] constituent of plasmid pSAP-1 [5].

Medium and conditions of cultivation. Cells were grown on a mineral medium [9] containing 0.002% thiamine, 100 μg/ml tryptophan, 0.07% Bactophoc-MP, and 0.5 mM K_2HPO_4 at 30°C for the strain AD93/pDD72 and at 37°C for other strains under intensive aeration. Plasmids pHI-7 and pDD72 were maintained using ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml), respectively. Growth medium for the strains *E. coli* AD93 contained bivalent cations Mg^{2+} or Ca^{2+} in the final concentration of 50 mM [6], which were absolutely necessary for the culture growth.

Induction of the biosynthesis of alkaline phosphatase. Alkaline phosphatase is known to be a member of the pho regulon, and its biosynthesis occurs only under phosphorus starvation [10]. To induce the biosynthesis of PhoA encoded by the *phoA* gene under the control of the endogenous promoter, the strain *E. coli* AD93 was grown on the above media to the mid log or stationary growth phase, centrifuged at 5000 rpm for 10 min, twice washed with a mineral medium containing Mg^{2+} or Ca^{2+}, and incubated on the same medium without orthophosphate. To induce the alkaline phosphatase biosynthesis during the culture growth, a medium with low (0.01 mM) orthophosphate content was used, where the cells were incubated for 15 h. The synthesis of PhoA encoded by the *phoA* gene under the control of P_{BAD} promoter was induced by addition of 0.5% arabinose to the culture growing on the mineral medium to the mid log growth phase. Culture samples were taken at certain time intervals. Biosynthetic processes were stopped by adding merthiolate at the final concentration of 0.02%.

Spectrum of periplasmic alkaline phosphatase isoforms. The alkaline phosphatase isoforms were revealed by activity, using the treatment of gel with α-naphthylphosphate and Fast Red RR [11] after electrophoresis of periplasmic proteins under non-denaturing conditions. Periplasm was isolated according to Miura and Mitsushima [12].

Lipid analysis. Phospholipid composition of the studied strains was monitored by extracting lipids with the mixture of chloroform–methanol–water according to a published method [13]. Thin layer chromatography of phospholipids was carried out on plates with silica gel Kieselgel 60 (Merck, Germany) impregnated with 1.2% boric acid in 50% ethanol, in the system of chloroform–methanol–water–ammonium (60 : 37.5 : 3 : 1 v/v) [14]. Individual phospholipid spots were detected in iodine vapor, cut out, and extracted from the silica gel with chloroform–methanol–water (5 : 5 : 1 v/v).

Analytical methods. Periplasmic proteins were separated in 7.5% PAGE in denaturing conditions according to Davis [15]. The alkaline phosphatase activity was determined by the rate of *p*-nitrophenylphosphate hydrolysis [9]; the amount of the enzyme that hydrolyzed 1 μmol substrate in 1 min at 37°C was taken as the unit of the enzyme activity (U). PhoA activity was measured in cell culture (cells + culture medium), because the alkaline phosphatase encoded by the gene constituent of plasmid is partially secreted into the medium [2]. Lipid phosphorus was assayed as described [16].

RESULTS

The effect of PE on the spectrum of alkaline phosphatase isoforms in *E. coli* cells with different levels of the enzyme synthesis under the control of the endogenous P_{PHO} promoter. The spectrum of isoforms of PhoA encoded by the chromosomal *phoA* gene and the same gene cloned in plasmid pHI-7 has been analyzed depending on the PE content. The strain *E. coli* AD93 completely lacks PE due to the absence of the *pss* gene responsible for the synthesis of the enzyme phosphatidylserine synthase, which is involved in biosynthesis of a metabolic precursor of PE [6]. In the same strain but carrying plasmid pDD72 with the *pss* gene, the phospholipid content corresponds to that of the wild type cells with the PE content up to 80%, which was confirmed in the current work (table). The strain AD93/pDD72 was used as the control. In the control strain with the chromosomal level of PhoA synthesis, isoform III is predominant both under cell growth and without cell growth (Fig. 1A). Cells of the PE-depleted mutant strain with a 3-10-fold lower activity of the enzyme were shown to contain PhoA as three isoforms. Accumulation of incompletely modified isoforms I and II in PE-depleted cells, in spite of the low level of the enzyme synthesis corresponding to the chromosomal level, suggests an impairment of posttranslocational modification of PhoA under these conditions.

The analysis of the isoform spectrum of PhoA encoded by the gene constituent of plasmid pHI-7 supports this assumption (Fig. 1B). The cells of the control strain AD93/pDD72 contain PhoA encoded by the gene constituent of plasmid pHI-7 as three isoforms (I, II, III), which is typical for the overproduced enzyme [2]. In

Phospholipid composition of *E. coli* AD93 strain and its derivatives

E. coli strain	Cation, 50 mM	Phospholipid content, mole %			
		CL	PG	PA	PE
AD93	Mg^{2+}	41.7 ± 3.7	55.3 ± 3.9	3.0 ± 0.2	—
	Ca^{2+}	15.8 ± 1.9	81.3 ± 4.2	2.9 ± 0.3	—
AD93/pHI-7	Mg^{2+}	47.2 ± 3.5	47.6 ± 2.8	5.1 ± 0.7	—
	Ca^{2+}	14.6 ± 1.9	80.9 ± 4.7	4.5 ± 0.6	—
AD93/pSAP-1	Mg^{2+}	32.1 ± 3.0	61.6 ± 3.6	6.3 ± 0.7	—
	Ca^{2+}	16.5 ± 2.0	79.5 ± 4.7	4.0 ± 0.6	—
AD93/pDD72	—	2.8 ± 0.3	19.1 ± 1.8	1.6 ± 0.2	76.5 ± 4.8
AD93/pDD72/pHI-7	—	2.1 ± 0.2	18.8 ± 2.0	0.4 ± 0.2	78.7 ± 5.1
AD93/pDD72/pSAP-1	—	4.0 ± 0.5	14.8 ± 1.7	2.7 ± 0.3	78.6 ± 4.5

Note: CL, cardiolipin; PG, phosphatidylglycerol; PA, phosphatidic acid; PE, phosphatidylethanolamine.

PE-depleted cells, the level of the enzyme activity is much lower (70 and 220 mU/mg protein during growth on the medium with Ca^{2+} or Mg^{2+}, respectively) than in the control cells (1695 mU/mg protein). As shown previously [5], this is a result of impairment of PhoA expression and secretion under these conditions. However, these cells also contain three PhoA isoforms. Thus, the accumulation of isoforms I and II in PE-depleted cells growing in the presence of Ca^{2+} or Mg^{2+} on the background of insignificant PhoA activity demonstrates the impairment of posttranslocational modification of the enzyme in these cells. A significant increase in the relative content of isoform I, observed under the induction of synthesis of the alkaline phosphatase encoded in plasmid pHI-7 during the culture growth, is probably due to the action of orthophosphate present in the medium.

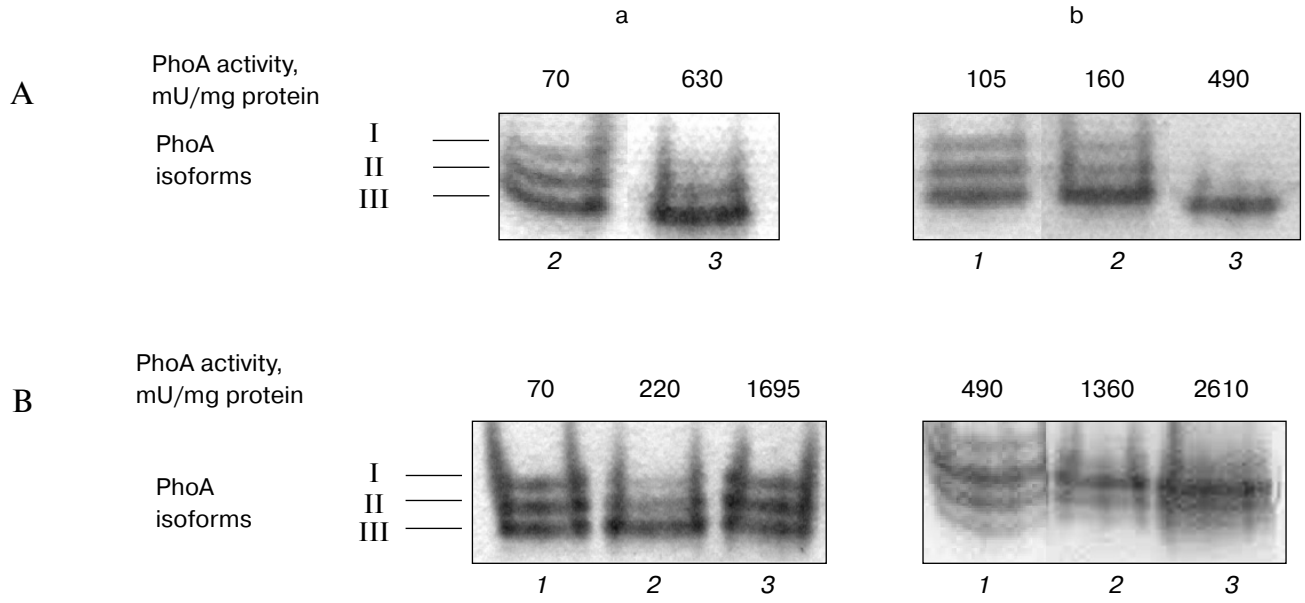

Fig. 1. Spectrum of isoforms of PhoA encoded by the chromosomal gene (A) and the gene cloned in plasmid pHI-7 (B) under the control of the endogenous P_{PHO} promoter in PE-depleted cells of the mutant strain *E. coli* AD93 grown in the presence of Ca^{2+} (*1*) and Mg^{2+} (*2*) cations and in PE-containing cells of the control strain AD93/pDD72 (*3*). The enzyme synthesis was induced under complete phosphorus starvation for 1 h (a) and during the culture growth at low orthophosphate content for 15 h (b). Periplasmic proteins were separated by electrophoresis in 7.5% PAGE under non-denaturing conditions followed by visualization of the PhoA activity by treatment of the gel with α-naphthylphosphate and Fast Red RR. PhoA isoforms are indicated with Roman numerals (I, II, III).

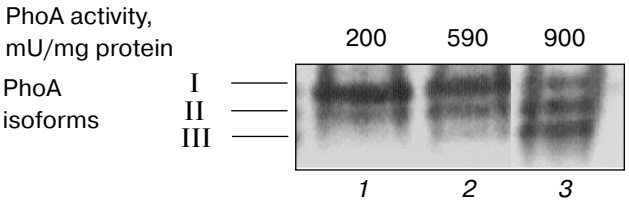

Fig. 2. Spectrum of isoforms of PhoA encoded by the gene cloned in plasmid pSAP-1 under the control of arabinose promoter in PE-depleted cells of the mutant strain *E. coli* AD93 grown in the presence of Ca^{2+} (*1*) or Mg^{2+} (*2*) and in PE-containing cells of the control strain AD93/pDD72 (*3*). The enzyme synthesis was induced by addition of 0.5% arabinose to the cells grown to the mid-log phase on a medium with orthophosphate.

Effect of PE on the isoform spectrum of alkaline phosphatase encoded by the *phoA* gene cloned in plasmid under the control of arabinose P_{BAD} promoter. The spectrum of alkaline phosphatase isoforms has been studied in PE-depleted cells of *E. coli* AD93 and in the control strain. Both strains contained the plasmid pSAP-1 with the *phoA* gene under the control of the arabinose promoter. The enzyme synthesis in these strains occurs under cell growth on a medium with orthophosphate and is induced by arabinose. The cells of the control strain with the wild type phospholipid composition have three isoforms, typical of the enhanced enzyme synthesis, with approximately the same relative contents (Fig. 2). The spectrum of PhoA isoforms in PE-depleted cells growing in the presence of Ca^{2+} or Mg^{2+} is represented by isoforms I and II (the former is predominant). However, the relative content of isoform I is somewhat higher in cells growing in the presence of Ca^{2+} than in the presence of Mg^{2+}. Isoform III is actually absent or present in the lowest quantity in these cells. Thus, the depletion of PE in cells of *E. coli* AD93/pSAP-1 results also in a significantly higher relative content of incompletely modified PhoA isoforms I and II and, consequently, in a disturbance of the rate of posttranslocational modification of PhoA.

Effect of Ca^{2+} and Mg^{2+} on the spectrum of alkaline phosphatase isoforms. To exclude the effect of high concentrations of Ca^{2+} or Mg^{2+} contained in the growth medium of the PE-depleted mutant strain AD93 on the spectrum of PhoA isoforms, the latter was studied in cells of the control strain AD93/pDD72 grown in the presence of the above cations (Fig. 3). One can see that Mg^{2+} affected neither the activity nor the isoform spectrum of PhoA encoded by the gene controlled both by the endogenous and exogenous promoters. PhoA was present in the cells of the control strain as three isoforms independent of the presence in the medium of Mg^{2+} and orthophosphate (P_i) as well. As regards the effect of Ca^{2+}, the PhoA activity in cells grown in their presence decreased about 2-fold as compared with that in cells grown in the presence of Mg^{2+} or without cations. Also, the isoform spectrum of PhoA encoded by the gene in pSAP-1 also changed, particularly in cells grown in the presence of orthophosphoate (Fig. 3, b and c). The content of unmodified isoform I in these cells increased, and isoform III was almost completely absent. A possible reason for differences between the isoform spectra of the enzyme in cells grown in the presence of Ca^{2+} and Mg^{2+}, particularly in the presence of orthophosphate, may be associated with the inhibiting effect of orthophosphate on the activity of modifying proteinase, since its effect on the isoform spectrum was revealed previously *in vivo* [17, 18]. Obviously, Ca^{2+} itself, judging from Fig. 3, slightly decreased the rate of modification, which along with the inhibiting effect of orthophosphate makes this latter effect most expressed. As a whole, however, one can conclude that cations have no significant effect on the isoform spectrum of PhoA and, thus, its change in a PE-depleted strain results mainly from the absence of this phospholipid.

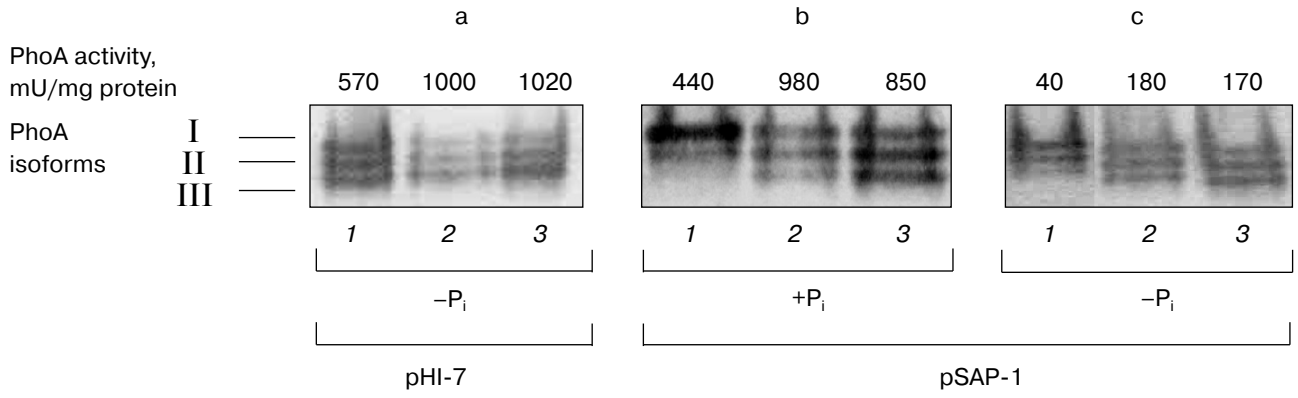

Fig. 3. Spectrum of isoforms of PhoA in cells of the control strains *E. coli* AD93/pDD72/pHI-7 (a) and *E. coli* AD93/pDD72/pSAP-1 (b, c) grown on a complete mineral medium to stationary or mid-log phase, respectively, in the presence of Ca^{2+} (*1*) or Mg^{2+} (*2*) and in the absence of cations (*3*). The enzyme synthesis was induced by complete phosphorus starvation (a) and 0.5% arabinose in the presence (b) and absence (c) of orthophosphate for 1 h.

DISCUSSION

The current work shows that the absence in the membranes of *E. coli* AD93 cells of the predominant phospholipid (PE) results in the impairment of posttranslocational modification of the alkaline phosphatase. This shows in a change of the spectrum of its isoforms due to the increase in the relative content of incompletely modified isoforms I and II. This effect was revealed in the cells with PhoA synthesis controlled by both the chromosomal gene and the gene constituent of plasmids under the control of the endogenous P_{PHO} and arabinose P_{BAD} promoters. The spectrum of PhoA isoforms is known to depend on cell growth conditions [17, 18] and changes most distinctly under overproduction of the enzyme encoded by the gene constituent of plasmids [2]. In the latter case isoforms I and II are accumulated. This may be a result of insufficiency of periplasmic proteinase (the *iap* gene product), splitting Arg, for complete processing of the greater quantity of mature polypeptide chains entering the periplasm in the course of protein secretion. The results obtained in this work demonstrate that the relative content of isoforms under PE depletion changes not only under the enzyme overproduction but also in cells with its chromosomal level. It might be assumed that the occurrence of isoforms I and II in PE-depleted cells results either from the lower activity of modifying proteinase in the absence of PE, which might be an effector of this enzyme, or from the inhibiting effect of cations whose presence in the medium in high concentrations is vitally important for the growth of a PE-depleted strain. However, the modifying proteinase is known to be a periplasmic protein, whereas PE is localized in another cell compartment—membranes. Besides, special experiments showed that the cations present in the medium have no significant effect on the isoform spectrum and consequently on protease activity by themselves. The change in the spectrum of enzyme isoforms under PE depletion may be caused, however, by the decrease in the modifying proteinase content. It is known that the alkaline phosphatase becomes active only after its translocation across the cytoplasmic membrane into the periplasm, where it finds necessary conditions for active enzyme formation [19]. One of these conditions is proteolytic modification of mature polypeptide chain of PhoA via the N-terminal Arg cleavage by the modifying proteinase, which, like the alkaline phosphatase, is a secreted protein localized in the periplasm [1]. Our previous research into the PhoA secretion in PE-depleted cells showed a decrease of the secretion efficiency of this protein [4, 5]. The impairment of posttranslocational modification of PhoA revealed in the current work is evidence of impairment in PE-depleted cells of secretion of not only PhoA but also the PhoA-modifying proteinase, which results in a disturbance of the rate of modification. This paper also presents results demonstrating possible inhibiting effect of orthophosphate on the modifying proteinase activity, which intensifies in the presence of Ca^{2+}.

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