

Direct evidence for a coupling between synthesis and export of PhoS in *E. coli*

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The accumulation of pre-PhoS under conditions of PhoS overproduction has been previously described. It is now demonstrated that during the induction of PhoS, a delay in the completion of polypeptide chain elongation can be detected. This delay is related to the extent of jamming of export sites by pre-PhoS or by other exported proteins. These results suggest that a component required for completion of pre-PhoS polypeptide becomes limiting, being titrated by the excess of nascent chains bearing signal peptides. This component thus probably acts at an early step in the export pathway.

Protein synthesis *Protein export* *Periplasmic protein* *Signal sequence* *Signal recognition particle*
Pho regulon

1. INTRODUCTION

In recent years, the mechanism of protein secretion has been extensively studied in both eucaryotic and procaryotic systems. Similarities exist between protein translocation in the rough endoplasmic reticulum and early steps of protein export in *E. coli*.

In eucaryotic cells, a coupling has been evidenced between synthesis and export. This coupling appears to be mediated by a nucleoprotein complex, named SRP (signal recognition particle) [1] and a so-called 'docking protein' [2]. It has been proposed that the purpose of the translational pausing induced by these factors is to prevent the accumulation of presecretory proteins in the cytoplasm [3,4]. However, recent results have shown that SRP does not mediate a translational arrest of nascent secretory proteins in mammalian cell-free systems [5].

Results from various laboratories, mainly through genetic studies, have revealed the existence of

several components of a presumed export machinery (review [6,7]). We have reported [8] that hyperproduction of a periplasmic component, the phosphate-binding protein (PhoS), results in jamming of export sites which exist in *Escherichia coli* in limited numbers [9]. Under such conditions, we have demonstrated the accumulation of pre-PhoS within the cytoplasm in addition to membrane-associated pre-PhoS. The cytoplasmic precursor cannot be exported even when the export sites are cleared up. We have also demonstrated that maturation is not the rate-limiting step in the export pathway [10]. It would then make sense for *E. coli* cells to have a coupling mechanism between synthesis and export to prevent as much as possible the accumulation of presecretory proteins in the cytoplasm since these are lost for export.

To investigate this point, conditions of PhoS hyperproduction were again used to study the early steps of pre-PhoS synthesis and export. Here, it is demonstrated that the rate of completion of nascent pre-PhoS polypeptide chains depends upon the level of PhoS synthesis. The results suggest a coupling between translation and export. A com-

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ponent of the secretory machinery required for completion of nascent chains of export proteins is probably held back upon jamming of export sites.

2. MATERIALS AND METHODS

2.1. Chemicals

[³⁵S]Methionine (approx. 1200 Ci/mmol) was purchased from Amersham. ¹⁴C-labelled amino acids (1.75 Ci/g) were purchased from the CEA. All reagents used were of the best grade available.

2.2. Bacterial strains and plasmids

E. coli strains ANCC75 (leu, purE, trp, his, argG, strA, met, thi, phoS64), C600 (F⁻, leu, thr, thi, lacY) and plasmids pSN5182, pAJ202, pTD101 were as previously described [10-13].

2.3. Media

A Tris/glucose medium supplemented with required nutrients and phosphate as in [12] was used. Tetracycline (10 μg/ml) and ampicillin (100 μg/ml) were added to the medium for selection of transformants and for ensuring the presence of plasmids.

2.4. Derepression of *Pho* regulon and pulse labelling

Cells grown overnight in Tris/glucose high-phosphate medium [12] were harvested by filtration or centrifugation [8]. They were then resuspended in low-phosphate medium at $A_{600\text{ nm}} = 0.5$ and incubated at 37°C. At various times (0, 1, 2 h, ...) after transfer in phosphate-limiting medium, samples (10⁸ cells) were labelled for 2 min with [³⁵S]methionine (5 μCi/ml) or with ¹⁴C-amino acids (2 μCi/ml). Cells were removed by centrifugation and total proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. At various times after transfer in low-phosphate medium, cells (10⁹/ml) were removed and 100 μCi/ml [³⁵S]methionine was added. Samples pulse-labelled for 15 s were removed into trichloroacetic acid (15% final concentration), chloramphenicol (100 μg/ml) and immediately frozen in liquid nitrogen. Chases were carried out with unlabelled methionine (1% final concentration); 5 mM phosphate was added during the chase to repress further PhoS synthesis. Samples were removed at intervals and treated as described above.

2.5. Immunoprecipitation and SDS-PAGE procedures

Trichloroacetic acid precipitates from pulse-chased samples were washed twice with 90% acetone, dried and solubilized for 5 min at 96°C in a buffer containing 160 mM Tris-HCl, 4 mM EDTA, 0.8 M sucrose, 0.8% methionine, 3.6% SDS, 60 mM dithiothreitol, 1% β-mercaptoethanol, pH 8.8. Just before immunoprecipitation, a 15-fold excess of 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 5 mM EDTA, 0.05% methionine, 1% Triton X-100 was added. Specific antiserum directed against PhoS was then used as in [8]. SDS-PAGE, fluorography and densitometer scanning of fluorogram were performed as described [12].

3. RESULTS

3.1. Level of pre-PhoS accumulation after transfer in phosphate-limiting medium

Overproduction of PhoS results in jamming of export sites and in accumulation of pre-PhoS in both the inner membrane and cytoplasm [8,12]. At each hour after transfer in phosphate-limited medium, samples were labelled with [³⁵S]methionine (fig.1A) or with ¹⁴C-amino acids. By using elongation factor EF-Tu as an internal standard [15], variations in cellular pre-PhoS contents could be followed during the induction of PhoS synthesis (fig.1B). First, upon decrease of P_i (or a phosphate metabolite) concentration, in agreement with the known PhoS regulation [12], the differential rate of pre-PhoS synthesis increased to a maximum reached at 3 h ($A_{600\text{ nm}} = 1.1$) after the transfer (fig.1). This maximum is probably related to the saturation of export sites [8]. Then, the pre-PhoS/EF-Tu ratio decreased to an almost steady-state value. To investigate the origin of this decrease, the rate of completion of pre-PhoS polypeptide was measured at intervals after transfer in phosphate-limiting medium.

3.2. Time-dependent lag after transfer in phosphate-limited medium for completion of synthesis of pre-PhoS polypeptide chains

The pre-PhoS polypeptide chain contains only 3 methionine residues which are all located within the signal sequence [16,17]. This feature allows specific labelling of the amino-terminal part of

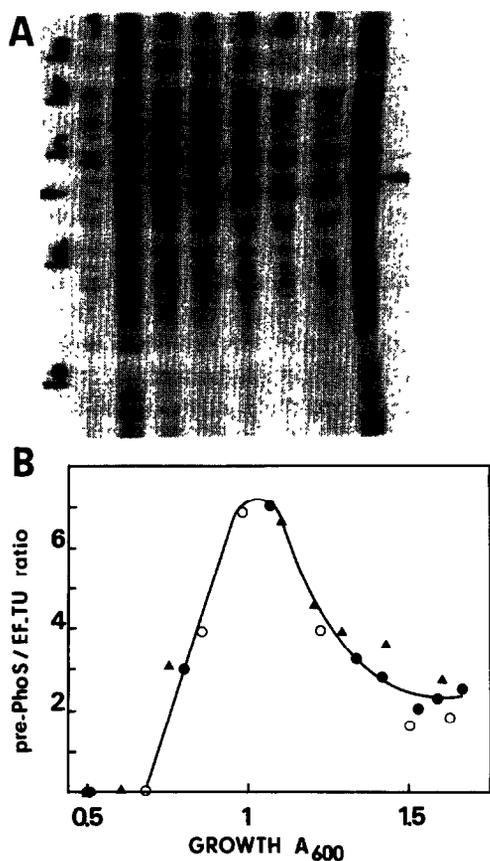


Fig.1. Level of pre-PhoS accumulation during overproduction. Cells (ANCC75 pSN 5182) were grown overnight in Tris-glucose high-phosphate medium, then transferred into low-phosphate medium [8,12]. At each hour after transfer in phosphate-limiting medium, samples (10^8 cells) were pulse-labelled for 2 min with [35 S]methionine. Proteins were analyzed by SDS-PAGE and fluorography [8]. Arrow indicates the migration of pre-PhoS. Relative molecular mass standards (in kDa): (a) 94, (b) 67, (c) 46, (d) 30, (e) 20. Lanes: 1, zero time; 2, 1 h; 3, 2 h; 4, 3 h; 5, 4 h; 6, 5 h; 7, 6 h; 8, 7 h. (B) Fluorograms were analyzed by densitometer scanning. The relative synthesis of pre-PhoS is expressed with reference to the 43 kDa band which corresponds to EF-Tu [15]. The pre-PhoS/EF-Tu ratio has been corrected for the respective methionine content of the two proteins. The results of 3 independent experiments carried out with [35 S]methionine (\blacktriangle) or [14 C]-amino acid mixtures (\circ, \bullet) are presented.

pre-PhoS with [35 S]methionine and study of early steps of synthesis. Thus, at intervals after transfer in phosphate-limiting medium, we could determine

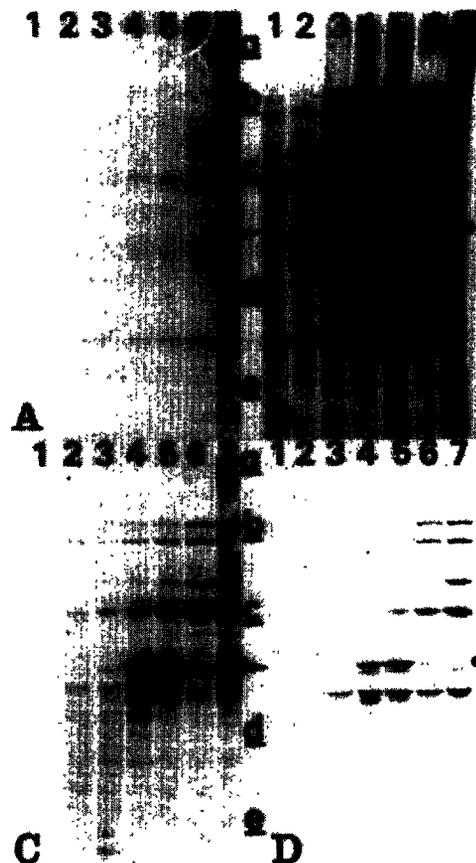


Fig.2. Kinetics of completion of nascent pre-PhoS polypeptide chains during overproduction. At 0 (A), 1.5 (B), 3 (C) and 5 h (D) after transfer in low-phosphate medium, cells of ANCC75 pSN5182 were pulse-labelled for 15 s with [35 S]methionine. Then, an excess of unlabelled methionine was added and aliquots subsequently removed during the chase. Samples were analyzed by SDS-PAGE and fluorography. Lanes: 1 (pulse), 2 (15 s chase), 3 (30 s chase), 4 (45 s chase), 5 (60 s chase), 6 (120 s chase), 7 (300 s chase). The migration of pre-PhoS is indicated either by small arrows or by dots. Relative molecular mass standards a-e, as in fig.1.

the time required to complete the polypeptide chain of pre-PhoS at 37°C. At 1.5 h ($A_{600 \text{ nm}} = 0.8$) before reaching maximal production of pre-PhoS (see fig.1), the completed precursor form was detected after a 30 s chase (fig.2B). When the maximal production was reached at 3 h ($A_{600 \text{ nm}} = 1.1$), the appearance of pre-PhoS was delayed to 45 s (fig.2C) during the chase. The same delay was observed at 5 h ($A_{600 \text{ nm}} = 1.4$). Similar pulse-

chase experiments were also carried out at 25°C (not shown). Early in the induction, pre-PhoS was detected at 1 min chase; this time was increased to 2 min at longer times after induction. The results suggest that a component of the machinery required for synthesis of exported proteins, being titrated by an excess of nascent PhoS upon saturation of export sites, becomes limiting. This hypothesis was tested by competition experiments.

3.3. The lag in completion of synthesis of pre-PhoS polypeptide is related to the level of PhoS production

If a component of the export machinery is titrated as mentioned above, increasing the amount of exported protein should lead to a lag in completion of synthesis of pre-PhoS polypeptide. This was checked by using 3 isogenic strains carrying either no plasmid (C600), or pAJ202 encoding PhoS or

pAJ202 and pTD101 encoding PhoS and β -lactamase. At 3 h after transfer in low-phosphate medium, the cells were pulse-labelled with [³⁵S]methionine and chased (fig.3). The maximal level of pre-PhoS was observed at 30 s and rapidly decreased due to the fast processing [10] in C600. It was detected at 45 and 60 s, respectively, when PhoS and PhoS plus β -lactamase were overproduced. It thus appears that there is a correlation between the extent of jamming of export sites and the lag in completion of pre-PhoS polypeptide chain. Since PhoS and β -lactamase exert synergistic effects, this suggests the existence of a shared component in the export machinery.

4. DISCUSSION

The existence of the cytoplasmic precursor pool previously demonstrated in our laboratory has been interpreted as the result of an abortive attempt to export PhoS under conditions of hyperproduction [8]. Results on the trypsin accessibility of pre-PhoS (Anba et al., submitted) suggest that the membrane-associated pre-PhoS has not been translocated across the membrane. This explains why leader peptidase has no access to pre-PhoS signal peptide. Moreover, we have recently observed that overproduction of this peptidase cannot relieve the jamming of export sites resulting from PhoS hyperproduction [10]. Since maturation is not the limiting step in the export pathway, we deduce from these results that overproduced nascent polypeptide chains might hold back some component required in the export mechanism. This competition should disrupt the normal protein traffic at an early step in the export process, probably before translocation across the inner membrane takes place. This hypothesis has been checked in this work. The delay in completion of the pre-PhoS polypeptide chain, which is only observed when a threshold pre-PhoS/EF-Tu ratio has been reached, suggests that a component of the export machinery is indeed held back, i.e. titrated by overproduced nascent pre-PhoS polypeptide chains. This component can also be held back by nascent β -lactamase; with regard to the nature of this component, we have no evidence.

The existence of a delay in completion of pre-PhoS polypeptide elongation is fully consistent with the increase in apparent half-life of the pre-

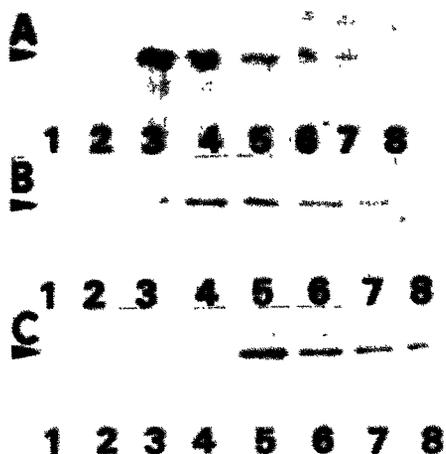


Fig.3. Delay in completion of pre-PhoS polypeptide chains is related to the level of PhoS production. At 3 h after transfer in phosphate-limiting medium, cells from strain C600 (A), C600 pAJ202 (B) and 600 pAJ202, pTD101 (C) were pulse-labelled for 15 s with [³⁵S]methionine (lane 1). Then an excess of unlabelled methionine was added and other samples were removed during the chase at 15 s (lane 2), 30 s (lane 3), 45 s (lane 4), 60 s (lane 5), 120 s (lane 6), 300 s (lane 7) and 600 s (lane 8). Immunoprecipitation by the antiserum directed against PhoS was carried out and immunoprecipitates were analyzed in SDS-PAGE and fluorography. Panel A corresponds to an overexposed film (obtained with the normal producer strain). Arrows indicate the migration of pre-PhoS.

cursor form under conditions of overproduction [10].

We interpret this result as meaning that the true rate of maturation is not altered but rather that the rate of completion of the polypeptide chain is delayed, thus inducing an apparent extension of the half-life of pre-PhoS.

All results presented here are consistent with the hypotheses of a coupling between synthesis and export first proposed by Hall et al. [18] and supported by further evidence from Ferro-Novick et al. [19] and Kumamoto and Beckwith [20].

Our results also demonstrate that regardless of the type of processing, post-translational for pre- β -lactamase or co-translational for pre-PhoS, overproduction of nascent chains results in both cases in a delay in completion of polypeptide synthesis. This delay results in more than a doubling (42 to 100 s) in the apparent half-life for pre- β -lactamase [10].

To conclude, regardless of the temporal relationship between synthesis and translocation across the membrane, there appears to be a coupling between these two steps during export, although one does not yet understand how.

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