Lack of Effect of Leader Peptidase Overproduction on the Processing 
in vivo of Exported Proteins in Escherichia coli

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The kinetics of maturation of certain exported proteins were analysed in Escherichia coli strains that also concomitantly overproduce either a periplasmic protein or the leader peptidase. The results led to three conclusions. (a) Overproduction of leader peptidase has no effect on the rate of maturation of at least two exported proteins, one periplasmic (TEM β-lactamase), one outer membrane (PhoE); therefore, the quantity of leader peptidase is not rate-limiting for normal export. (b) Overproduction of PhoS reduces the rate of maturation of two other periplasmic proteins (β-lactamase and PhoA) and itself, presumably by competing for the rate-limiting component of the export apparatus. (c) Overproduction of leader peptidase in a strain overproducing PhoS has no effect on the retarded maturation of PhoS. Therefore even in these conditions, leader peptidase is not rate limiting.

INTRODUCTION

Periplasmic and outer membrane proteins of Escherichia coli destined for export are synthesized as precursor molecules containing an extra amino-terminal sequence of 15 to 30 residues. The amino-terminal extensions, termed signal sequences or leader sequences, are proteolytically removed during the export process. These proteins are synthesized on polysomes associated with the plasma membrane (Randall & Hardy, 1977; Smith et al., 1977; Varenne et al., 1978). Translocation of domains of nascent periplasmic proteins across the cytoplasmic membrane is not a vectorial process occurring during elongation (Randall, 1983) and cleavage of signal peptides is generally a late event (Josefsson & Randall, 1981) in the export pathway. In some cases, this cleavage occurs after completion of synthesis, and post-translational transfer across the inner membrane has been demonstrated (Josefsson & Randall, 1981; Koshland & Botstein, 1982). This situation has also been encountered with a membrane protein, the coat protein of phage M13 (Ito et al., 1980). An enzyme which can cleave precursor forms of many membrane and exported proteins, termed leader peptidase, has been isolated and purified from E. coli (Wolfe et al., 1982). The gene for this enzyme has been cloned on a high-copy-number plasmid (Date & Wickner, 1981), and its presence in cells leads to overproduction of leader peptidase.

We have recently reported (Pages et al., 1984) that hyperproduction of the phosphate-binding protein PhoS results in saturation of export sites which exist in limited number in E. coli (Ito et al., 1981). Consequently, pre-PhoS accumulates in both the inner membrane and the cytoplasm. Only the membrane-associated precursor form can be matured and exported. In this work, the effects of PhoS overproduction on the kinetics of maturation of other proteins were investigated. Kinetics of maturation were also investigated under conditions entailing overproduction of leader peptidase. This overproduction in vivo has been reported to result in a marked acceleration in the conversion of M13 procoat to coat protein (Zimmermann et al., 1982), whereas we observed no significant increase in rates of processing of precursor forms for periplasmic and outer membrane proteins.
Table 1. Bacterial strains and plasmids

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<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
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<tr>
<td>E. coli K12</td>
<td></td>
<td>D. Baty</td>
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<td>C600</td>
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<td>H. Shinagawa</td>
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<td>Tc&lt;sup&gt;+&lt;/sup&gt; phoS</td>
<td>H. Shinagawa</td>
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<tr>
<td>pBR322</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>T. Fraser</td>
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<tr>
<td>pTD101</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; lep</td>
<td>W. Wickner</td>
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<tr>
<td>pCC40</td>
<td>cai ori-pColA</td>
<td>D. Cavard</td>
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<tr>
<td>pAJ202</td>
<td>Tc&lt;sup&gt;+&lt;/sup&gt; phoS ori-pColA</td>
<td>This study</td>
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METHODS

Chemicals. [35S]Methionine (approximately 1200 Ci mmol<sup>-1</sup>; 44·4 TBq mmol<sup>-1</sup>) was purchased from Amersham. 14C-labelled amino acids (1·75 Ci g<sup>-1</sup>; 64·8 GBq g<sup>-1</sup>) were purchased from the Commissariat à l’Energie Atomique. T4 DNA ligase and T4 DNA polymerase were purchased from Boehringer. Restriction endonucleases were purchased from Bethesda Research Laboratories and from Boehringer. Routine conditions were used for DNA digestion and size estimation of DNA fragments on agarose gel electrophoresis (Crozel et al., 1983).

Bacterial strains and plasmids. These are listed in Table 1.

Media. A Tris/glucose medium, supplemented with required nutrients and phosphate as previously described (Morita et al., 1983), was used. Tetracycline (10 µg ml<sup>-1</sup>) and ampicillin (100 µg ml<sup>-1</sup>) were added to the medium for selection of transformants and for ensuring the maintenance of plasmids.

Preparation of plasmid DNA and cell transformation. The techniques described by Holmes & Quigley (1981) and by Humphreys et al. (1977) were used for plasmid preparation. Transformations were done as outlined by Lederberg & Cohen (1974).

Derepression of pho regulon and pulse-labelling. Cells grown overnight in Tris/glucose high-phosphate medium (Morita et al., 1983) were harvested by filtration and washed (Pages et al., 1984). They were then resuspended in low-phosphate medium at OD<sub>600</sub> 0·5 and incubated at 37 °C in a gyratory water bath. After 2 h (OD<sub>600</sub> 0·8) a sample (10<sup>9</sup> cells ml<sup>-1</sup>) was removed and 14C-labelled amino acids (10 µCi ml<sup>-1</sup>) were added. Samples (2 ml of culture at 10<sup>9</sup> cells ml<sup>-1</sup>) pulse-labelled for 15 s were removed into trichloroacetic acid (TCA: 15%, w/v, final concentration) plus chloramphenicol (100 µg ml<sup>-1</sup>) and immediately frozen in liquid nitrogen. Chases were done with unlabelled amino acid mixture (1% final concentration); 1 mM-phosphate was added during the chase to repress further PhoS synthesis. Samples were removed at intervals and treated as described above.

Immunoprecipitation and SDS-PAGE. TCA precipitates from pulse-chased samples were washed twice with 90% (v/v) acetone, dried and solubilized by incubation for 5 min at 96 °C in a buffer containing 160 mM-Tris/HCl, 4 mM-EDTA, 0·8 mM-sucrose, 0·8% (w/v) methionine, 3·6% (w/v) SDS, 60 mM-dithiothreitol and 1% (v/v) 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% (w/v) methionine and 1% (v/v) Triton X-100 was added. Specific antisera directed respectively against PhoA, PhoE, PhoS, β-lactamase and leader peptidase were then used as previously described (Pages et al., 1984). SDS-PAGE, fluorography and densitometer scanning of the fluorograms were done as described by Pages & Lazdunski (1982). The antisera directed against PhoA protein, PhoE protein, β-lactamase and leader peptidase were generous gifts from Drs H. Shinagawa, J. Tommassen, G. Cesarini and W. Wickner, respectively. The antiserum directed against PhoA has been previously described (Pages & Lazdunski, 1981).

RESULTS

Effect of overproduction of leader peptidase on the kinetics of processing of pre-β-lactamase

TEM β-lactamase is a periplasmic protein. It is produced by membrane-bound polysomes in a precursor form containing a signal sequence of 23 amino acid residues (Baty et al., 1981; Sutcliffe, 1978). The high-copy number plasmid pBR322 that was used for cloning the leader peptidase gene (lep) (Date & Wickner, 1981) carries the β-lactamase gene (bla), which made it possible to investigate the effect of overproduction of leader peptidase on the processing of pre-β-lactamase. This precursor can be conveniently pulse-labelled since its processing is post-translational (Koshland & Botstein, 1982). The kinetics of processing were compared in isogenic strains with normal and increased levels of leader peptidase (Fig. 1a). Densitometer scanning of
Fig. 1. Effect of overproduction of leader peptidase on kinetics of processing of pre-β-lactamase and of pre-PhoE. Cells were grown overnight in Tris/glucose high-phosphate medium, then transferred to low-phosphate medium (these conditions were chosen to permit comparison of the results from this experiment with those presented in Fig. 4). (a) After 2 h growth at 37 °C in phosphate-limiting medium, one sample was labelled for 5 min with 14C-labelled amino acids (1 µCi ml⁻¹) and used to compare, after immunoprecipitation, the level of leader peptidase in cells of strains C600(pBR322) (lane 1) and C600(pTD101) (lane 2). (b, c) One sample was removed after a 15 s pulse (lane 1) with 14C-labelled amino acids (10 µCi ml⁻¹) and an excess of unlabelled amino acids was then added with 1 mm-phosphate. Samples were subsequently 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), 10 min (lane 7) and 30 min (lane 8). Immunoprecipitation by antiserum against β-lactamase was carried out and the immunoprecipitates were analysed by SDS-PAGE. (b) C600(pBR322), (c) C600(pTD101); the arrows indicate the positions of mature and precursor forms of β-lactamase. (d) The half-life of the pre-β-lactamase was determined by densitometer scanning of fluorograms from three independent experiments [O, C600(pBR322); •, C600(pTD101)]. (e) The rate of processing of pre-PhoE was determined by the same method (but using antiserum against PhoE protein for the immunoprecipitation) in strains C600(pBR322) (O) and C600(pTD101) (•).

fluorograms indicated that the half-life of the precursor form was 42 s (Fig. 1 b–d). Overproduction of leader peptidase in strain C600(pTD101) did not result in faster processing since no change in kinetics was observed.

In phosphate-limiting medium, synthesis of PhoE and PhoA proteins is derepressed (Tommassen et al., 1982). PhoE is an outer membrane porin (Lugtenberg & Alphen, 1983) and thus it was of interest to also test the effect of high levels of leader peptidase on pre-PhoE processing. Again no effect on the kinetics was observed (Fig. 1 e).
We recently described a system in which precursor forms of exported protein accumulated, due to the saturation of export sites (Pages et al., 1984). We have also investigated the effect of overproduction of leader peptidase in this system. However, this required the construction of a strain carrying two plasmids allowing overproduction of both an inducible periplasmic protein, PhoS (for saturation of export sites), and leader peptidase.

Construction of plasmid pAJ202

Since plasmid pTD101 encoding leader peptidase had the pColE1 origin of replication, a compatible plasmid with a different origin had to be used. The plasmid pCC40 (or mini-ColA), previously constructed in our laboratory, fulfilled this requirement (Crozel et al., 1983). From this plasmid and from pSN5182 (Morita et al., 1983) which encodes PhoS, a new plasmid with the pColA origin was constructed (Fig. 2). The PstI–NdeI restriction fragment from pSN5182 containing the pColE1 ori region was replaced by the large PstI–NdeI fragment from pCC40 which contains the pColA ori region (Crozel et al., 1983). After ligation, transformants were selected for tetracycline resistance and for overproduction of PhoS in low-phosphate medium.

Hyperproduction of PhoS protein induces a delay in maturation of precursor forms of periplasmic proteins

By using cells carrying a multicopy plasmid containing the phoS gene, overproduction of the phosphate-binding protein (PhoS) can be obtained under phosphate limitation (Morita et al., 1983); this results in saturation of export sites and in accumulation of pre-PhoS both in the inner membrane and in the cytoplasm as evidenced by electron microscopy and cell fractionation (Anba et al., 1984; Pages et al., 1984).

If this saturation resulted in a delay in completion of synthesis of precursor polypeptide chains, one would expect an increase in the apparent half-life of pre-PhoS under conditions of overproduction. This half-life was thus determined in strains C600 and C600(pAJ202) by using pulse and chase experiments (Fig. 3). There was a marked retardation in the PhoS overproducer as compared to the wild-type: the half-life of pre-PhoS was extended from 11 s to 22 s. Under conditions of PhoS overproduction, a biphasic exponential decay was observed. This is consistent with our previous report of a slow two-step cleavage of cytoplasmic pre-PhoS, the membrane-associated pre-PhoS being rapidly matured (Pages et al., 1984).
Protein export in *E. coli*

Fig. 3. Hyperproduction of PhoS induces a delay in maturation of pre-PhoS. At 2 h after transfer in low-phosphate medium, cells of strains C600 (○), C600(pAJ202) (●) or C600(pAJ202, pTD101) (●) were pulse-labelled for 15 s with ^14^C-labelled amino acids and an excess of unlabelled amino acids was then added with 1 mM-phosphate. Samples were subsequently removed during the chase. Immunoprecipitation by antiserum against PhoS was carried out and the immunoprecipitates were analysed by SDS-PAGE. The half-life of pre-PhoS was determined by densitometer scanning of fluorograms from three independent experiments.

Fig. 4. Hyperproduction of PhoS induces a delay in maturation of precursor forms of periplasmic proteins. (a, b) The conditions of pulse-labelling and chase with cells of strains C600(pBR322) (a) and C600(pBR322, pAJ202) (b) were identical to those described for Fig. 3. The first sample was removed after a 15 s pulse (lane 1), and subsequent samples were removed during the chase at 15 s (lane 2), 30 s (lane 3), 45 s (lane 4), 60 s (lane 5), 2 min (lane 6), 3 min (lane 7) and 10 min (lane 8). The arrows indicate the positions of mature and precursor forms of β-lactamase. (c) The half-life of pre-β-lactamase was determined by densitometer scannings of fluorograms from three independent experiments. ○, C600(pBR322); ●, C600(pBR322, pAJ202).

The effect of PhoS hyperproduction on the export of two other periplasmic proteins, β-lactamase and alkaline phosphatase (PhoA), was investigated. The accumulation of both pre-PhoS and pre-β-lactamase was clearly detected in whole-cell extracts (data not shown). As mentioned above for β-lactamase under normal PhoS synthesis conditions, the half-life of pre-β-lactamase was about 42 s (Fig. 1). Hyperproduction of PhoS markedly slowed the conversion of pre-β-lactamase to β-lactamase, the half-life of pre-β-lactamase being increased to 100 s (Fig. 4). Similar results (not shown) were observed for PhoA.

These results suggest that some common step in the export pathway of Bla, PhoS, and PhoA is altered by PhoS hyperproduction. Since there is a delay in precursor maturation, the possibility exists that leader peptidase activity becomes rate-limiting. This possibility was tested.

**Effect of leader peptidase overproduction on pre-PhoS processing under export site saturation conditions**

The kinetics of pre-PhoS maturation were studied in strain C600 carrying both plasmid pTD101, encoding leader peptidase, and pAJ202, encoding PhoS. The overproduction of leader peptidase did not result in acceleration of pre-PhoS processing (Fig. 3).
DISCUSSION

In this study we have attempted to answer two questions. The first concerns the effect of hyperproduction of PhoS protein on the kinetics of maturation of pre-β-lactamase and pre-alkaline phosphatase. The second concerns the effects of overproduction of leader peptidase on the kinetics of maturation of exported proteins. Our results suggest that hyperproduction of PhoS interferes with normal export of other proteins. The interference observed might reflect a competition between nascent pre-PhoS and other exported proteins for one or more components of the secretory apparatus. A role has been proposed (Ferro-Novick et al., 1984) for this machinery in coupling the synthesis and export of proteins by a mechanism similar to that of the signal recognition particle and its receptor in eukaryotic cells (Walter et al., 1984). Competition would result in the occupation of the said component for an inordinate period. Consequently, the reduced availability of this component to participate in normal protein export becomes a limiting factor, whereas this is not usually the case. Similar conclusions have recently been reached using a different experimental approach. Bankaitis & Bassford (1984) have demonstrated that the synthesis of proteins defective in export properties can interfere with normal protein export in E. coli. Both we and they conclude that slowed maturation kinetics for precursor forms in strains either hyperproducing normal exported proteins or concomitantly synthesizing an export-defective protein actually reflect a problem at an early step in the secretory pathway rather than in the maturation step itself.

As regards the effect of overproduction of leader peptidase on maturation kinetics, one might argue that the overproduced leader peptidase might use the same export sites as periplasmic and outer membrane proteins because correct insertion of this enzyme also requires a functional secA gene product (Wolfe & Wickner, 1984). The insertion of overproduced leader peptidase might then be disrupted by the excess production of β-lactamase or PhoS. However, one can rule out this hypothesis for two reasons: (i) overproduction of leader peptidase, encoded by pTD101, the plasmid that we used, and which also encodes β-lactamase, results in vivo in a marked acceleration in the processing of the M13 pro-coat protein (Zimmermann et al., 1982); (ii) in experiments dealing with PhoS, the cells were transferred to phosphate-limiting medium only to induce synthesis of this protein and most of their growth took place in the absence of PhoS synthesis (Morita et al., 1983); thus, there is no reason why the overproduced leader peptidase should not be correctly inserted in the inner membrane, even if export sites were used for this insertion.

The difference observed in vivo between the effect obtained on processing of M13 phage prococat to coat protein and that obtained on processing precursor forms of periplasmic and outer membrane proteins raises an important question: why is the maturation step accelerated in the first case and not in the second? With β-lactamase, the situation is quite similar to that with M13 coat protein. The synthesis of both proteins is completed before maturation and both proteins are transferred post-translationally (Josefsson & Randall, 1981; Zimmermann et al., 1982) to their final location. This process depends upon the proton-motive force across the inner membrane (Date et al., 1980; Daniels et al., 1981; Pages & Lazdunski, 1982; Bakker & Randall, 1984). The only significant difference concerns the location of synthesis: the coat protein is produced in free polysomes in the cytoplasm (Ito et al., 1979) whereas β-lactamase is produced in membrane-bound polysomes (Baty et al., 1981). However, both Zimmermann et al. (1982) for the coat protein and Koshland & Botstein (1982) for β-lactamase have proposed that the precursor species first becomes associated with the inner side of the cytoplasmic membrane, the polypeptide being then transferred, regardless of whether any synthetic process occurs. Maturation by the leader peptidase, due to its topology in the membrane, could only occur after transfer. Since no change in kinetics of pre-β-lactamase maturation was observed after leader peptidase overproduction, the difference between the results reported here and those for M13 coat protein might be explained in two ways. Possibly the export mechanisms are different, the coat protein being inserted according to the membrane-triggered folding hypothesis (Wickner, 1980) and periplasmic and outer membrane proteins being exported by a secretion machinery involving many components (SecA, SecB, SecC, PrlA, PrlB, PrlD) (reviewed by Silhavy et al.,
1983). Alternatively, both proteins use the same export pathway but some other step in export may become rate-limiting in the case of M13 coat protein.

The transfer step is difficult to separate from the elongation step. If one assumes that for $\beta$-lactamase the elongation and transfer step was rate-limiting in the export pathway, even the 30-fold increase in peptidase level obtained with plasmid pTD101 (Date & Wickner, 1981) should not change the rate of processing. Such a change, if it existed, should have been detected by our technique since we could show an increase in the rate of membrane-associated pre-PhoS processing after cell disruption.

The biphasic kinetics of processing of pre-PhoS protein accumulated under overproduction conditions is also of interest. As proposed above, the fast processing is probably related to the membrane-associated pre-PhoS. The half-life of this precursor was approximately 11 s in wild-type cells and 22 s in PhoS overproducer cells. The slow processing with a half-life of 400 s probably corresponds, as previously reported (Pages et al., 1984), to the two-step cleavage of the signal peptide that occurs in the cytoplasm.

With regard to the maturation of pre-\(\beta\)-lactamase, we did not detect biphasic kinetics in cells that overproduced PhoS. This is somewhat surprising since one might expect in a simple model of competition between the two proteins that some fraction of the \(\beta\)-lactamase precursor, like a fraction of PhoS precursor, would not engage the export apparatus. As with \(\beta\)-lactamase, and for the same reasons, overproduction of leader peptidase will not accelerate the cleavage of membrane-bound pre-PhoS if synthesis and translocation of a polypeptide domain across the inner membrane is rate-limiting. Obviously, this overproduction should not alter the kinetics of cytoplasmic pre-PhoS processing for which unidentified cytoplasmic proteases are responsible. Concerning the lack of effect of overproduction of leader peptidase on processing of pre-PhoE, the same conclusion as that presented above for \(\beta\)-lactamase and PhoS probably holds true. This result again suggests that periplasmic and outer membrane proteins also share the same early steps in the secretory pathway: this corroborates the conclusion previously based on an exchange between respective signal peptides (Tommassen et al., 1983; Tommassen & Lugtenberg, 1984).

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