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Precursors of three exported proteins in *Escherichia coli*

(protein synthesis/periplasmic binding proteins/membrane-bound polysomes/λ receptor/secretion)

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ABSTRACT **Arabinose-binding protein, maltose-binding protein, and λ receptor are synthesized *in vitro* on membrane-bound polysomes from *Escherichia coli*. All three proteins are exported from the cytoplasm of *E. coli* and all three are made *in vitro* in a form a few thousand daltons larger than the authentic protein. The larger form of arabinose-binding protein is also detected *in vivo* by pulse labeling. It is concluded that the larger forms of the exported proteins are precursors containing an extra sequence. In contrast to the above, when the intracellular protein elongation factor Tu is synthesized *in vitro* on free polysomes, it is not detectably larger than the authentic form.**

In eukaryotes, secreted proteins are synthesized on ribosomes bound to endoplasmic reticulum (reviewed in ref. 1). However, we do not fully understand how the ribosomes that synthesize proteins destined for transport across membranes come to be membrane-bound. A current hypothesis suggests that specialized NH_2-terminal sequences of nascent proteins function to bind polysomes to the membrane. While the nascent peptide chains are traversing the membrane, these signal sequences are removed by proteolysis (2–4). Several laboratories have provided evidence that supports this hypothesis. Proteins that are secreted *in vivo* have been synthesized *in vitro* in precursor forms containing additional hydrophobic stretches of 15 to 30 amino acids at the NH_2 terminus (5–7).

Gram-negative bacteria export two classes of protein, soluble periplasmic proteins analogous to eukaryotic secreted proteins and proteins that have their final location in outer membrane, a structure external to cytoplasmic membrane. The mechanism of transport of these proteins across cytoplasmic membrane may involve synthesis of larger precursor forms on membrane-bound ribosomes, as in eukaryotes. A previous investigation (8) demonstrated that 80% of nascent chains of alkaline phosphatase were associated with membrane-bound polysomes. Nevertheless, the physiological significance of membrane-bound polysomes has remained controversial. In our previous publication (9), we directly demonstrated that proteins to be exported from the cytoplasm are synthesized on polysomes bound to membrane. In addition, Smith *et al.* (10) have shown by extracellular labeling that some nascent chains of membrane-bound polysomes traverse the membrane *in vivo*. Thus, the existence of functionally significant membrane-bound polysomes in prokaryotes is firmly established.

Putative precursors of secreted proteins have been identified in prokaryotic systems. Alkaline phosphatase was synthesized in a larger than authentic form in a coupled transcription–translation system (11). Three outer membrane proteins synthesized in toluene-treated cells were also produced in forms larger than the corresponding proteins produced *in vivo* (12, 13). The precursor of the lipoprotein of outer membrane has been thoroughly analyzed. It contains an NH_2-terminal peptide extension of 20 amino acids (12).

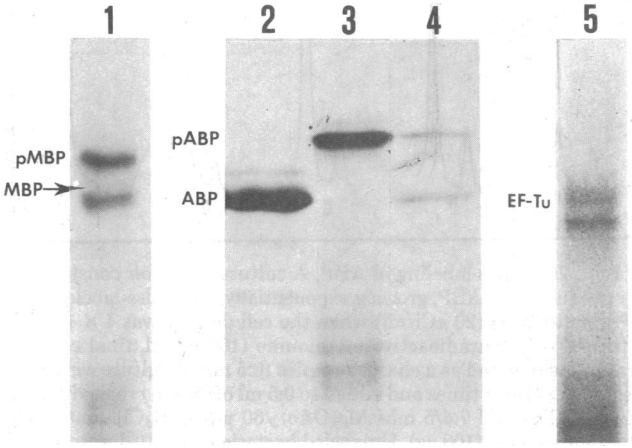

FIG. 1. Gel electrophoresis and autoradiography of [^{35}S]methionine-labeled immune precipitates. Lane 1. Membrane-bound polysomes from SH7 grown with maltose as carbon source were used *in vitro* in a protein-synthesizing system. After incubation for 20 min at 37°, incorporation of [^{35}S]methionine had ceased and the reaction mixture was incubated with puromycin (10 μg/ml, 5 min, 37°). The released soluble products were separated from envelope by centrifugation (15 min, 8000 × *g*) and immune precipitated with antiserum to MBP. The immune precipitate was analyzed by sodium dodecyl sulfate/10% polyacrylamide gel electrophoresis followed by autoradiography. Lane 2. Authentic ABP immune precipitated from a lysate of G6 constitutive for ABP labeled *in vivo* with [^{35}S]methionine and analyzed as described above. Lane 3. The same as Lane 1 except G6 constitutive for ABP grown on glycerol was used as source of membrane-bound polysomes, and immunoprecipitation was carried out with antiserum to ABP. Lane 4. G6 constitutive for ABP was pulse-labeled with [^{35}S]methionine for 15 sec immediately prior to harvesting. Membrane fraction (fraction M of ref. 9) was prepared and incubated with 2% Triton X-100/5 mM EDTA/10 mM Tris·HCl, pH 7.6, for 5 min at 37° and the soluble released material was immunoprecipitated and analyzed as described above. Lane 5. Products synthesized *in vitro* by free polysomes were released with puromycin, immunoprecipitated with antiserum to EF-Tu, and analyzed as described above. Molecular weights: MBP, 38,500; ABP, 34,000; EF-Tu, 44,000.

Here we report the *in vitro* synthesis, on membrane-bound polysomes, of two secreted periplasmic proteins, maltose-binding protein (MBP) and arabinose-binding protein (ABP), and one outer membrane protein, the λ receptor. All three exported proteins were synthesized in forms larger than the authentic proteins. In contrast, elongation factor Tu (EF-Tu), the major product of soluble polysomes *in vitro*, was not detected in a form larger than authentic EF-Tu.

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Abbreviations: ABP, arabinose-binding protein; pABP, "precursor" ABP synthesized *in vitro*; MBP, maltose-binding protein; pMBP, "precursor" MBP synthesized *in vitro*; EF-Tu, elongation factor Tu; pλ, "precursor" λ receptor.

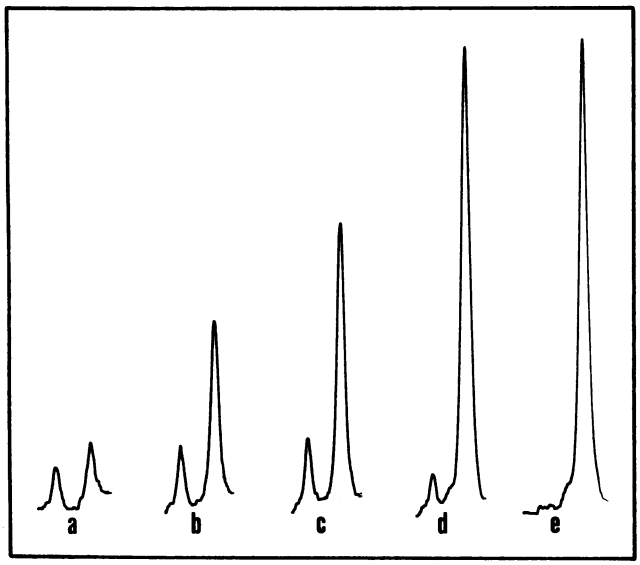

FIG. 2. Pulse labeling of ABP. A culture of *E. coli* constitutive for production of ABP, growing exponentially, was pulse-labeled with [^{35}S]methionine (20 μCi/ml) when the cell density was 4×10^8/ml. After 16 sec, nonradioactive methionine (100 μg/ml, final concentration) was added as a chase. Samples (0.5 ml) of culture were harvested at various times and added to 0.5 ml of frozen crushed buffer [10 mM Tris, pH 7.4/5 mM $Mg(OAc)_2$/60 mM NH_4Cl] containing chloramphenicol (100 μg). Unlabeled bacteria (2.5×10^9) were added as carrier, and the cells were collected by centrifugation, washed once, and resuspended in the same buffer. They were lysed by sonication and the labeled ABP was immune precipitated from the envelope fraction. The immune precipitate was subjected to electrophoresis and autoradiography as in Fig. 1. The autoradiogram was scanned by using a Joyce–Loebl microdensitometer. Precursor to ABP (pABP) is the left peak; authentic ABP is the right peak. The times of sampling were: a, 12 sec of pulse; b, 4 sec of chase; c, 9 sec of chase; d, 14 sec of chase; e, 34 sec of chase.

MATERIALS AND METHODS

Materials were obtained from the following sources: [^{35}S]methionine (approx. 800 Ci/mmol), from the Radiochemical Centre, Amersham; total tRNA from *E. coli*, from Boehringer/Mannheim Ltd.; *Staphylococcus aureus* V8 protease, from Miles Laboratories, Elkhart, IN; puromycin, from Sigma Chemical Co., Saint Louis, MO.

Strains. *E. coli* K12 SH7, Hfr Met$^-$ was used in studies of MBP. *E. coli* K12 HfrG6 His$^-$ made constitutive for production of ABP by transduction to araC^c (14) was used in studies of ABP. We thank B. Berneholm for construction of this strain.

Ribosome Preparation. Preparation of free (fraction P) and membrane-bound (fraction M) polysomes was as described (9).

Protein Synthesis *In Vitro*. Completion of nascent proteins of polysomes was carried out as described (9). No initiation of polypeptides occurs in this system.

Limited Proteolysis. Peptide patterns that are highly reproducible and characteristic of the protein substrate can be generated by limited proteolysis as described (15). After drying and autoradiography of gels (9), bands to be digested were excised, soaked in gel sample buffer for 30 min, inserted in sample slot of a second gel, and overlaid with *S. aureus* V8 protease in 20 μl of sample buffer. Electrophoresis was stopped for 40 min (before the tracking dye entered the separation gel) to allow proteolysis to proceed in the stacking gel. Electrophoresis was then continued and the gel was processed as usual. The amount of protease per gel slot is as indicated in the legend to Fig. 3.

Other Techniques. Gel electrophoresis, autoradiography, and immune precipitation were as described (9).

RESULTS

Proteins synthesized *in vitro* by membrane-bound polysomes can be separated by centrifugation into those that are soluble

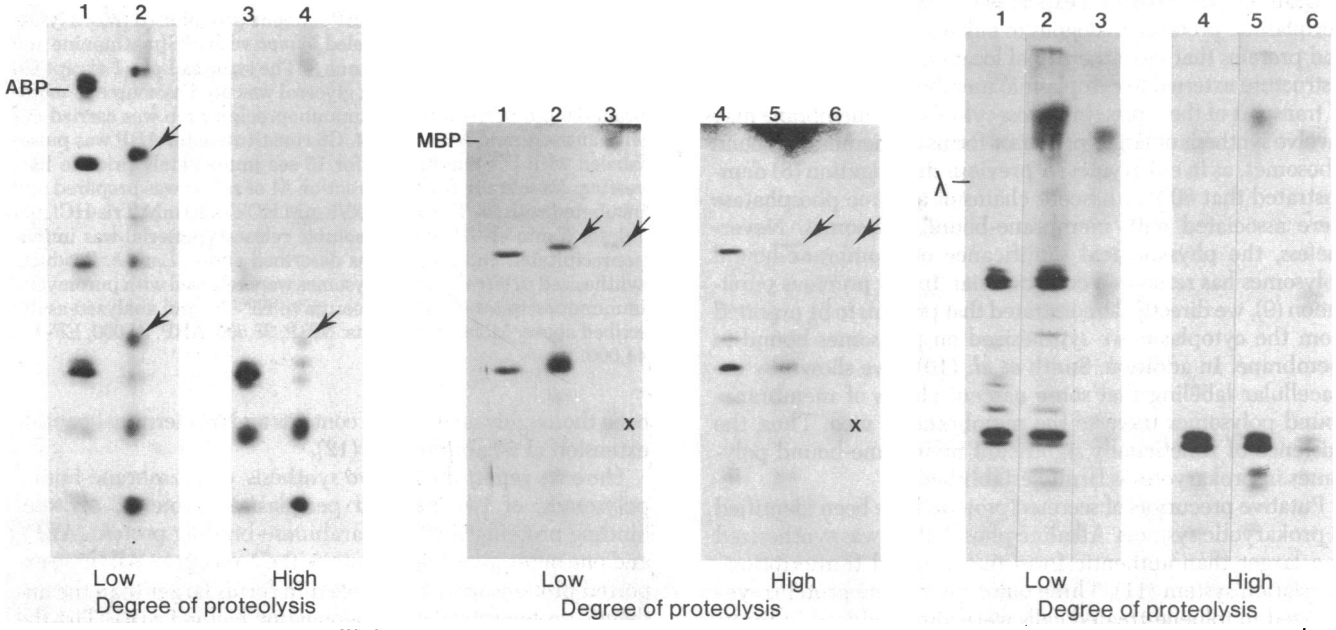

FIG. 3. Limited proteolysis of [^{35}S]methionine-labeled proteins. After autoradiography of gels similar to those shown in Figs. 1 and 2, radioactive bands were excised and subjected to limited proteolysis and electrophoresis on sodium dodecyl sulfate/15% polyacrylamide gels. Autoradiography was then carried out. (*Left* and *Center*) Excised bands were derived from gels of immune precipitates similar to those in Fig. 1. (*Right*) Excised bands were derived from a gel similar to that of Fig. 2. (*Left*) Lanes 1 and 3, authentic ABP; lanes 2 and 4, pABP. (*Center*) Lanes 1 and 4, authentic MBP; lanes 2 and 5, pMBP; lanes 3 and 6, MBP "processed" *in vitro*. The arrows indicate peptides that are characteristic of the larger precursors. X indicates a new peptide that appears after "processing" *in vitro*. (*Right*) Lanes 1 and 4, authentic λ receptor; lanes 2 and 5, pλ receptor; lanes 3 and 6, λ receptor "processed" *in vitro*. The amount of *S. aureus* V8 protease per gel sample slot was 0.05 μg for low degree of proteolysis and 0.5 μg for high degree of proteolysis. The molecular weight of the smallest peptides in the figure is approximately 10,000.

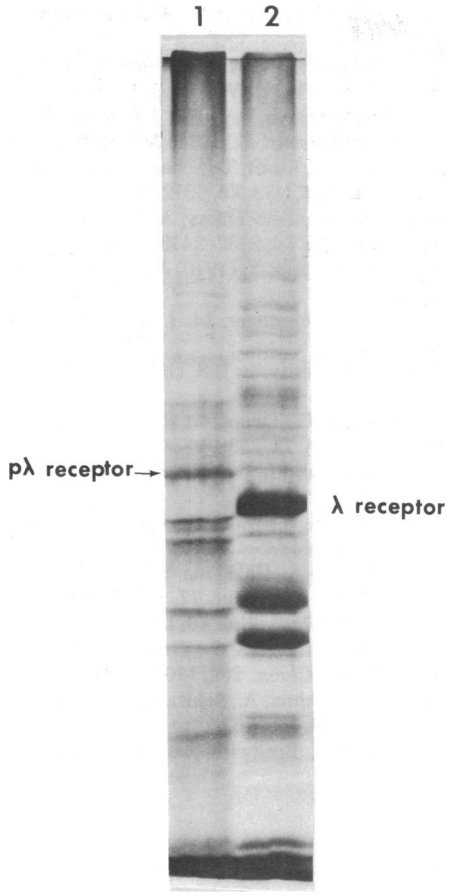

FIG. 4. Products of protein synthesis *in vitro* that remain membrane-bound. Lane 1. [^{35}S]Methionine-labeled products, synthesized *in vitro* by membrane-bound polysomes, that remain with envelope after puromycin release were analyzed by sodium dodecyl sulfate/10% polyacrylamide gel electrophoresis and autoradiography. Lane 2. Authentic outer membrane labeled with [^{35}S]methionine (a generous gift of E. T. Palva).

and those that remain associated with envelope fragments. We have previously shown (9) that, when cells are grown on maltose, one of the major soluble products of membrane-bound polysomes (fraction M) is MBP. The MBP synthesized in this system was found in two forms as shown by specific immunoprecipitation followed by gel electrophoresis and autoradiography (Fig. 1). One form was larger than authentic MBP by approximately 2000 daltons. The second form was slightly smaller than authentic MBP. When membrane-bound polysomes were prepared from a strain constitutive for production of ABP and the products synthesized *in vitro* were analyzed as described for MBP, all protein precipitated by antiserum to ABP was larger than authentic ABP (Fig. 1). A larger form of ABP also could be detected *in vivo*. Immune precipitates of membrane fraction isolated from pulse-labeled cells contained, in addition to authentic ABP, a protein that had the same molecular weight as the larger form synthesized *in vitro* (Fig. 1). Evidence that the larger form observed *in vivo* is a precursor of ABP was provided by pulse-chase experiments (Fig. 2). Therefore, we believe the larger forms of MBP and ABP made *in vitro* to be precursors and refer to them as pMBP and pABP.

We previously demonstrated that EF-Tu is the major product synthesized *in vitro* by free polysomes (fraction P) (9). Immunoprecipitation of EF-Tu synthesized *in vitro* revealed no polypeptides larger than authentic EF-Tu (Fig. 1). Several bands of lower molecular weight were apparent, and are likely to be degradation products.

Limited proteolysis showed that pMBP and pABP are related to the authentic proteins. The partial peptide pattern of authentic ABP labeled *in vivo* with [^{35}S]methionine was similar to that obtained from [^{35}S]methionine-labeled precursor form synthesized *in vitro* (Fig. 3 *left*). Two peptides derived from authentic protein are absent in precursor digests, and these appear to be replaced by larger peptides as would be expected if pABP contains an extra sequence of amino acids. The same was found for pMBP and MBP (Fig. 3 *center*).

When cells are grown on maltose as the carbon source, λ receptor is one of the major outer membrane proteins. Growth on maltose is required for the appearance of one of the products that remains associated with envelope after synthesis *in vitro* on membrane-bound polysomes (data not shown). This protein (designated pλ) is approximately 3000 daltons larger than authentic λ receptor as determined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and autoradiography (Fig. 4). Limited proteolysis clearly demonstrated that this protein is closely related to authentic λ receptor (Fig. 3 *right*). Because this protein was not precipitated with antiserum prior to proteolysis, the band cut from the gel may contain more than one protein species. Consequently, although differences are apparent in the peptide patterns, they are not interpretable. A protein band that migrated just below authentic λ receptor on sodium dodecyl sulfate/polyacrylamide gels (Fig. 4) also gave a limited proteolysis peptide pattern similar to that of authentic λ receptor (Fig. 3 *right*).

ABP synthesized *in vitro* was found only in its precursor form. In contrast, MBP and λ receptor were found in two forms, a larger precursor form and a form with a molecular weight slightly smaller than the authentic molecular weight. In the case of MBP, production of the smaller form *in vitro* is unrelated to processing which occurs *in vivo*. The smaller form is not derived from pMBP by removal of the extra sequence in the precursor as would be expected if it were processed to authentic MBP. Thus, the larger peptides characteristic of pMBP are present in the digest of the smaller form of MBP and new peptides, absent from both authentic MBP and pMBP, appear (Fig. 3 *center*).

DISCUSSION

We have identified three proteins synthesized by membrane-bound polysomes *in vitro*. Two, ABP and MBP, are soluble periplasmic proteins. The third is an outer membrane protein, λ receptor. All three proteins made *in vitro* are larger than the corresponding authentic forms. Synthesis of putative precursors in systems that initiate, such as toluene-treated cells (13, 16) and transcription–translation coupled systems (11, 17), is open to criticism on the basis of errors of initiation *in vitro*. Here we demonstrate production of precursor forms *in vitro* in a system that only completes polypeptide chains, thus eliminating initiation artifacts. In addition we have detected pABP *in vivo* by pulse-labeling. Thus, the larger polypeptides made *in vitro* probably represent precursor species that are present *in vivo*. The position of the extra sequences in the larger forms of the proteins has not been determined. However, it seems likely that they are NH_2 terminal because an exported outer membrane protein, the lipoprotein, contains an additional NH_2-terminal sequence when synthesized *in vitro* (12). In contrast to the situation for exported proteins, we could detect no larger form of EF-Tu synthesized *in vitro* by free polysomes.

The extra sequence of exported proteins is removed *in vivo* during maturation of the proteins. Recently it has been shown that the periplasmic protein alkaline phosphatase is made as

a larger than authentic form *in vitro* in a coupled transcription–translation system. The larger form is cleaved to the size of authentic alkaline phosphatase by an activity present in outer membrane preparations (11). We have detected forms of MBP and λ receptor that appear to be slightly smaller than the authentic proteins. However, in the case of MBP the smaller form we observe *in vitro* appears to be different from processed MBP. Not only is the final product smaller than authentic MBP, but also it is not the extra sequence that is removed. It seems likely that the smaller form results either from spurious cleavage of the precursor at its COOH terminus or from premature termination *in vitro*.

Processing of ABP *in vitro* clearly can occur after elongation of the polypeptide chain is complete or virtually complete, because we detected pABP *in vivo*.

The detailed mechanism of transport of proteins across membranes in bacteria is not yet known. However, the general mechanism is similar to that in eukaryotes in that proteins to be exported are synthesized in precursor-form containing an extra sequence.

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