Biochimica et Biophysica Acta, 512 (1978) 365—376

BBA 78136

INSERTION OF NEWLY SYNTHESIZED PROTEINS INTO THE OUTER MEMBRANE OF *ESCHERICHIA COLI*

LOE DE LEIJ, JAAP KINGMA and BERNARD WITHOLT

Department of Biochemistry, University of Groningen, Nijenborgh 16, Groningen (The Netherlands)

(Received February 16th, 1978)

Summary

The insertion of newly synthesized proteins into the outer membrane of *Escherichia coli* has been examined. The results show that there is no precurser pool of outer membrane proteins in the cytoplasmic membrane because first, the incorporation of a [^{35}S]methionine pulse into outer membrane proteins completely parallels its incorporation into cytoplasmic membrane proteins, and second, under optimal isolation conditions, no outer membrane proteins are found in the cytoplasmic membrane, even when the membranes are analysed after being labeled for only 15 s.

The [^{35}S]methionine present in the outer membrane after a pulse of 15 s was found in protein fragments of varying sizes rather than in specific outer membrane proteins. This label could however be chased into specific proteins within 30—120 s, depending on the size of the protein, indicating that although unfinished protein fragments were present in the outer membrane, they were completed by subsequent chain elongation.

Thus, outer membrane proteins are inserted into the outer membrane while still attached to ribosomes. Since ribosomes which are linked to the cell envelope by nascent polypeptide chains are stationary, the mRNA which is being translated by these ribosomes moves along the inner cell surface.

Introduction

The cell envelope of Gram-negative bacteria consists of a cytoplasmic membrane, a murein layer and an outer membrane [1,2]. Much research has been focused on the structural organization of these layers, resulting in the formulation of progressively more sophisticated envelope models [2—6]. The assembly of the cell envelope has also received considerable attention; it is of particular interest since the outer membrane and murein components must first be

exported through the cytoplasmic membrane, after which they are integrated into their respective layers.

Murein subunits are synthesized at the endoplasmic surface of the cytoplasmic membrane and exported while linked to the C_{55} carrier lipid to be transferred to growing murein chains [7]. Lipopolysaccharides [8] and phospholipids [9,10] are synthesized as complete molecules at the endoplasmic face of the cytoplasmic membrane and subsequently transferred to the outer membrane, although the driving force by which these molecules are translocated remains obscure. Outer membrane proteins are synthesized on ribosomes which appear to be membrane associated [11]. It is not clear however how outer membrane proteins are exported. They may first be completed and then exported, as is the case with murein subunits, lipopolysaccharides, and phospholipids. Alternatively, they may be extruded during chain elongation, as appears to be the case with the periplasmic enzyme alkaline phosphatase [12] and the secretory proteins synthesized by Gram-positive bacteria [13] and eucaryotic systems [14].

In order to differentiate between these two possibilities, we have examined the process of outer membrane protein synthesis and translocation. Our results indicate that outer membrane proteins are incorporated directly into the outer membrane as nascent peptide chains.

Materials and Methods

Organism and growth conditions. Strain J_5, a mutant of *Escherichia coli* 0111:K58 (B4), which lacks uridinediphosphogalactose-4-epimerase, was grown in minimal medium [15]. Cell densities are expressed as mg (dry mass)/ml [16].

Sample preparation and isolation of cytoplasmic and outer membrane fractions. Exponentially growing cells were labeled with [^{3}H]methionine and/or [^{35}S]methionine (Radiochemical Centre, Amersham, U.K.) as indicated in the text. The incorporation of label was stopped by rapidly freezing culture portions. Before freezing, glycerol was added to the samples to a concentration of 15% (w/w), in order to protect the cells against the cryo-damage which occurs during freezing in liquid nitrogen [17]. Accordingly, samples of 1 ml were mixed with an equal volume of 30% (w/w) glycerol at 37° C 3 s before they were frozen in liquid nitrogen at the times indicated in the text and figures. Each frozen sample was thawed and processed at 0° C as follows. The sample was diluted with 30 mg similarly treated unlabeled cells, unless otherwise stated. Cells were pelleted by centrifugation (15 min, 5000 × *g*), washed once by suspending in 10 ml 200 mM Tris · HCl/1 mM $MgCl_2$ (pH 8.0), and centrifuged as above. This washing step was necessary to remove glycerol, which interfered with subsequent membrane isolations.

Cells were spheroplasted [15,18] and disrupted by passage through a French press at an operating pressure of 600 atm, after which the cytoplasmic and outer membranes were isolated on a sucrose gradient as described [19], with the following modification. Before the administration of EDTA and subsequent disruption in the French press, the spheroplasts were either collected by cen-

trifugation (10 min, 5000 × *g*) and resuspended in 10 mM Tris · HCl (pH 8.0), containing 0.05 mg/ml RNAase (EC 3.1.4.22, Miles-Seravac Ltd., Berkshire, U.K.) or, when unlabeled cells had not been added, they were directly diluted 10-fold in the same buffer.

In order to prevent possible binding of [^{35}S]methionine to various membrane fractions unlabeled methionine was added to all buffers to a concentration of 1 mM.

Counting procedures. After diluting a sample with unlabeled carrier cells as described above, the amount of acid-precipitable label in the sample was determined by filtration of 5% trichloroacetic acid-treated aliquots onto glass-fiber filters (Whatman GF/C). The filters were washed twice with 96% ethanol before counting. The distribution of label in sucrose gradients was determined by taking 0.1 ml aliquots in duplicate from the extruded fractions, which were counted directly. Glass-fiber filters and aqueous samples were added to polyethylene counting vials (Otan, Rijsbergen, The Netherlands) and counted in a Nuclear Chicago scintillation counter (Mark I) after the addition of 10 ml scintillation fluid (2.4 g diphenyloxazole, 48 mg *p*-bis-(*o*-methylstyryl) benzene, 300 ml Triton X-100, 600 ml toluene and 122.5 ml H_2O.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. After sodium dodecyl sulfate polyacrylamide gel electrophoresis [20], proteins were fixed and stained [21] to determine the position of the unlabeled reference proteins. After drying gels on Whatman 3 MM paper under vacuum, autoradiographs were made by overlaying the gels with Kodak RP Royal X-Omat film.

Results

Pulse labeling of cells and sample preparation. In order to follow the fate of newly synthesized proteins at very early stages, it was necessary to stop the incorporation of [^{35}S]methionine within a few seconds, which was achieved by freezing samples in liquid nitrogen. The effectiveness of this procedure is illustrated in Fig. 1. Samples frozen at the times indicated were thawed, diluted with unlabeled carrier cells and the incorporation of label was determined. Since the percentage incorporation can be extrapolated to zero at zero time, methionine incorporation was stopped immediately after sampling. Fig. 1 also shows that a chase with a 3000-fold excess of unlabeled methionine stopped the incorporation within 3 s.

Incorporation of pulse label in the cytoplasmic and outer membrane fraction. Cells (0.14 mg/ml) were (pre)labeled with [^{3}H]methionine (19 μM, 33 μCi/ml) and grown to 0.4 mg/ml. At that time all [^{3}H]methionine was consumed and incorporated into trichloroacetic acid-precipitable material. Subsequently, [^{35}S]methionine (pulse label) was added (1.8 μM, 108 μCi/ml) and samples were taken as described in Fig. 1. During pulse-chase labeling, a chase was performed after 30 s by the addition of unlabeled methionine to a concentration of 6 mM.

Fig. 2 shows that reproducible membrane separations were obtained, as indicated by the distribution of [^{3}H]methionine (prelabel). The distribution of [^{35}S]methionine (pulse label) was dependent on the pulse time however. After a 13 s pulse there was considerably more incorporation of [^{35}S]-

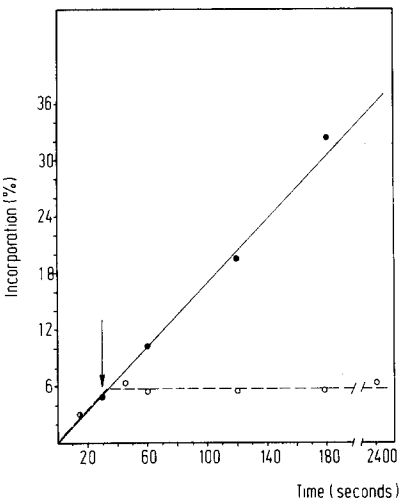

Fig. 1. Incorporation of pulse label into total cell protein. Cells (0.4 mg/ml) were pulse labeled with [^{35}S]methionine (1.9 μM, 108 μCi/ml). Incorporation is expressed as a percentage of the total added label. ●——●, no chase; ○- - -○, the label was chased at 30 s (arrow) with a 3000-fold excess of unlabeled methionine.

methionine in the cytoplasmic membrane fraction than in the outer membrane fraction, compared to the ^{3}H label distribution (Fig. 2A). This difference diminished at longer incubation times (Fig. 2, B and C), but did not disappear altogether. To investigate this effect, cells (0.07 mg/ml) were labeled continuously for two generations after the simultaneous addition of [^{3}H]-methionine and [^{35}S]methionine to radioactive concentrations of 6.2 μCi/ml and 28 μCi/ml, respectively, while the methionine concentration was 38 μM. Fig. 2D shows that, even though in long term label experiments the incorporation of [^{35}S]methionine into *E. coli* membranes should parallel the incorporation of [^{3}H]methionine, this was not the case. We have not investigated the reason for this discrepancy, which might be due to differences in the incorporation and/or degradation of either [^{35}S]methionine or [^{3}H]methionine in the cytoplasmic and outer membranes. Instead, we have circumvented this discrepancy by normalizing all ^{35}S/^{3}H ratios to the steady-state ratio of Fig. 2D.

Fig. 3 shows how the resulting corrected ratio of pulse label (^{35}S) to steady-state label (^{3}H) increased in the cytoplasmic and outer membranes under different conditions. Following a pulse of [^{35}S]methionine, the outer membrane as well as the cytoplasmic membrane was labeled very rapidly; the incorporation of new label into the outer membrane fraction paralleled the incorporation of new label into the cytoplasmic membrane fraction with a small delay of about 3 s. After chasing, the incorporation of new label ceased almost immediately in both fractions.

Protein composition of the cytoplasmic and outer membrane fraction after pulse-chase labeling. Cells (0.4 mg/ml) were pulsed with [^{35}S]methionine (1 μM, 90 μCi/ml) and chased after 30 s. Samples were frozen 15, 45, 60, 120, 180 and 2400 s after the addition of [^{35}S]methionine, and processed without added unlabeled cells in order to keep the specific activity of the isolated

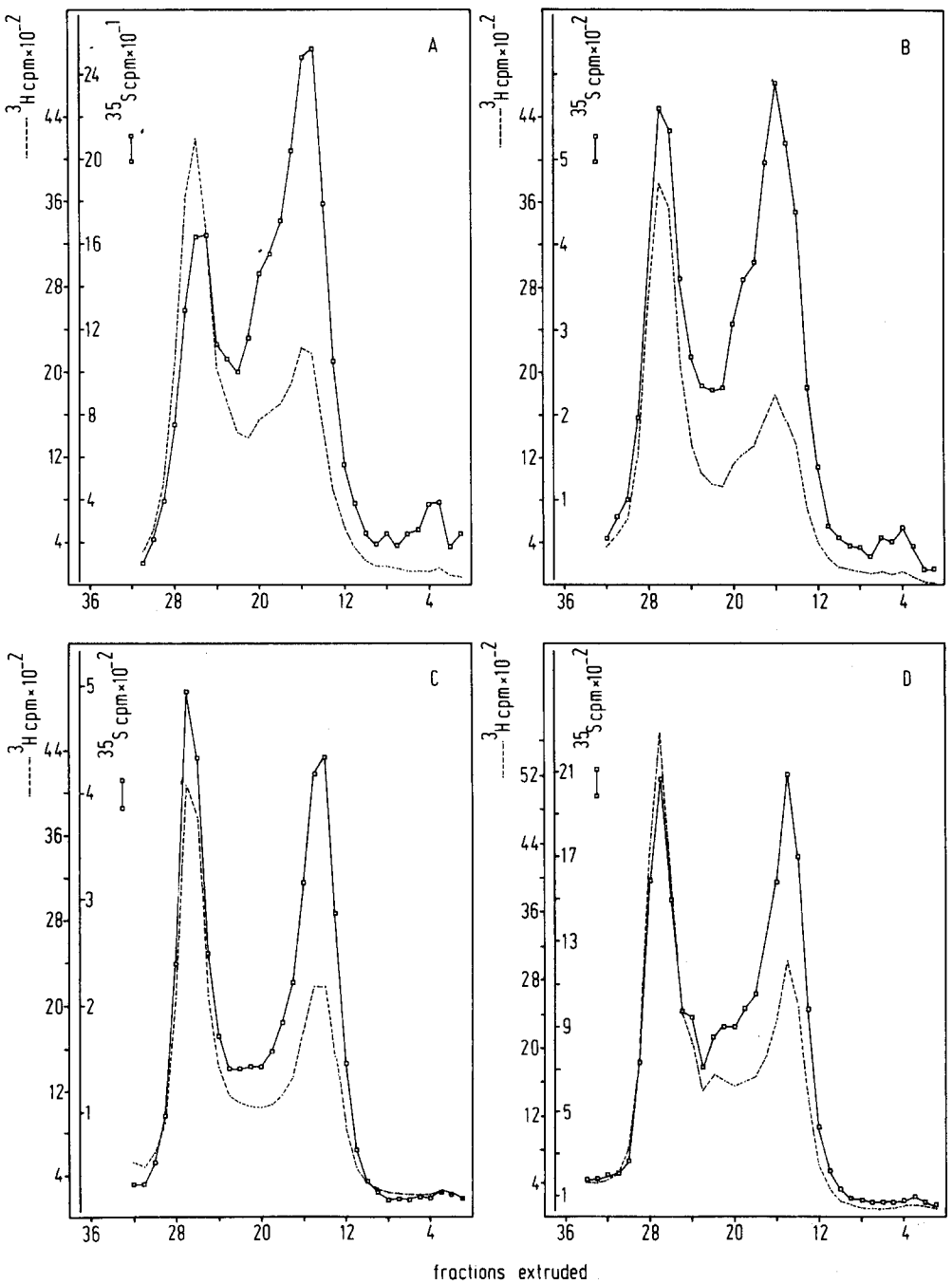

Fig. 2. Separation of membranes by sucrose gradient centrifugation. Cells were double labeled with [^{3}H]methionine (prelabel) and [^{35}S]methionine (pulse label), membranes were isolated and separated. Fractions 12—16 contain the cytoplasmic membrane, while fractions 24—28 contain the outer membrane. The distribution of ^{3}H and ^{35}S label over the membranes is shown after pulse labeling for 13 s (panel A) and 90 s (panel B), after pulse chase labeling for 190 s (panel C) and after continuous labeling for 175 min (panel D).

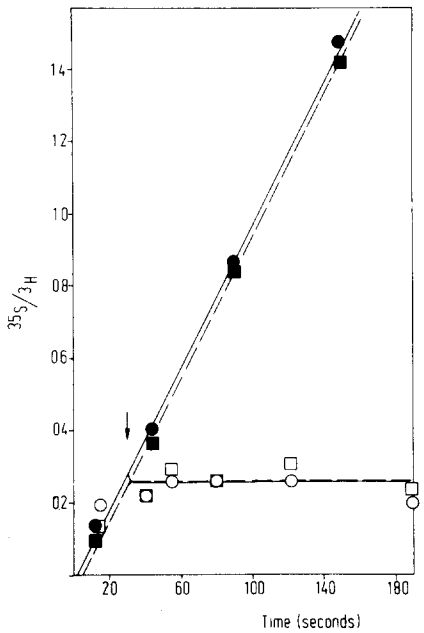

Fig. 3. Incorporation of pulse label in the cytoplasmic and outer membrane. The ratio of pulse label (^{35}S) to prelabel (^{3}H) in the membranes was determined by averaging the ratios in the top three fractions of each membrane fraction and by normalizing these ratios to the steady-state ratio of Fig. 2D. Incorporation was determined in the cytoplasmic membrane (circles, continuous lines) and in the outer membrane (squares, broken lines) during pulse (closed circles and squares) and pulse-chase (open circles and squares) labeling.

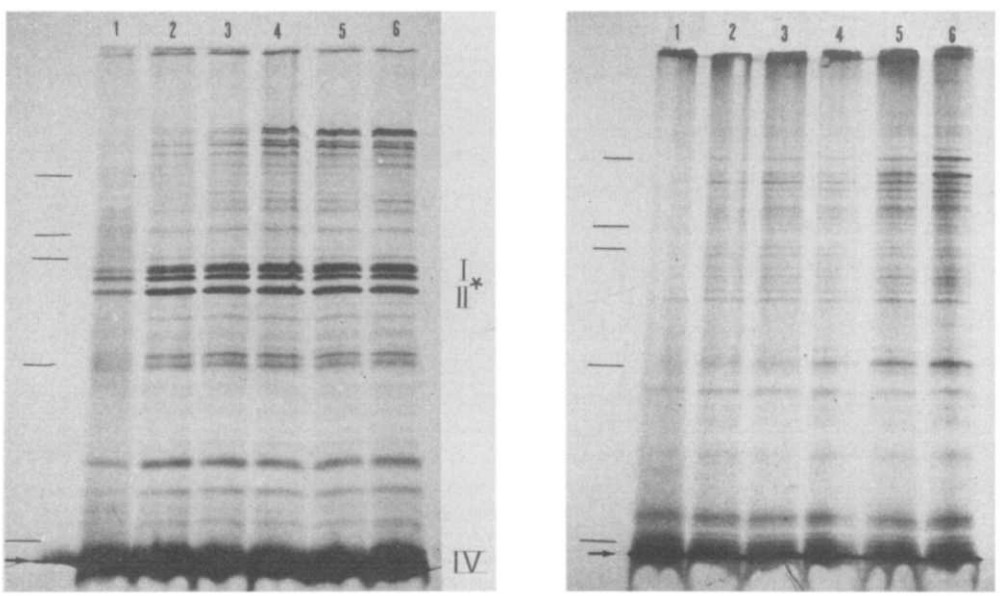

Fig. 4. Protein composition of membranes during and after pulse chase labeling. [^{35}S]Methionine was added at time zero, and chased with a 6000-fold excess of unlabeled methionine at 30 s. Samples were removed at 15, 45, 60, 120, 180 and 2400 s (slots 1—6, respectively), membranes were isolated from each

membrane fractions as high as possible. After isolating the cytoplasmic and outer membrane fraction, the distribution of ^{35}S-labeled proteins in these fractions was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by autoradiography.

Label appeared in all the major outer membrane proteins (Fig. 4a). However, the quantity of label detected at the running front in each slot amounted to about 20% of the total of applied label (see Fig. 6), which is considerably more than expected on the basis of protein distributions normally observed in stained gels [19]. Since free [^{35}S]methionine also runs with the front in this gel system (unpublished observation), the following was carried out to confirm the protein nature of the label detected at the front and to exclude the possibility that free [^{35}S]methionine was also present.

First, a large excess of unlabeled methionine was included in all steps of the membrane isolations to prevent possible binding of free [^{35}S]methionine to membrane preparations. To test the effectiveness of this procedure, the samples of Fig. 4a were chromatographed over a Biogel P6 column in the presence of 0.1% sodium dodecyl sulfate and 8 M urea. All ^{35}S radioactivity was recovered quantitatively in the void volume, while similarly treated free [^{35}S]-methionine was eluted with the total volume. Thus, all radioactivity observed at the front in Fig. 4a is due to proteins with molecular weights of at least 5000.

Second, although the resolution of low molecular weight proteins on our slab gels was variable, it was sometimes possible to resolve proteins with molecular weights below 10 000. In such cases, the material migrating at the front in Fig. 4a was seen to have an apparent molecular weight of 9000 (Fig. 5, slot 1), although it still did not stain with Fast Green. Furthermore, in the presence of chloramphenicol (80 μg/ml) labeling of this material was inhibited to the same extent as that of other protein bands (Fig. 5, slot 3 compared to slot 1) and we conclude therefore that it probably represents lipoprotein [3] or a closely related protein, which is somehow not detected with Fast Green.

Fig. 4a shows that label incorporated into the outer membrane immediately after the addition of [^{35}S]methionine appeared mostly as a smear of label between specific outer membrane protein bands. This material is chased into specific outer membrane proteins following the [^{35}S]methionine pulse, as shown by an increase in the label present in specific outer membrane proteins and a decrease in the radioactivity between outer membrane protein bands. To demonstrate this effect more clearly, the autoradiogram of Fig. 4a was scanned with a rotating drum densitometer and ^{35}S profiles were generated as described

---

sample, and membrane proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Autoradiographs were made after determination of the migration position of the molecular weight standard proteins by staining. Standards (from top to bottom: bovine serum albumin (67 000), ovalbumin (43 000), aldolase (40 000), chymotrypsinogen (26 000) and cytochrome *c* (12 000) are indicated as bars. The arrow indicates the position of the front. a. Outer membrane proteins. In each slot 81 700 cpm was applied and the film was exposed for 7 days. The migrating position of the major outer membrane proteins is indicated according to the nomenclature of Henning et al. [27] *. b. Cytoplasmic membrane proteins. In each slot 50 300 cpm was applied and the film was exposed for 12 days.

* Although it is clear that protein I consists of three bands, we refer to this group of proteins as 'I' to simplify the discussion.

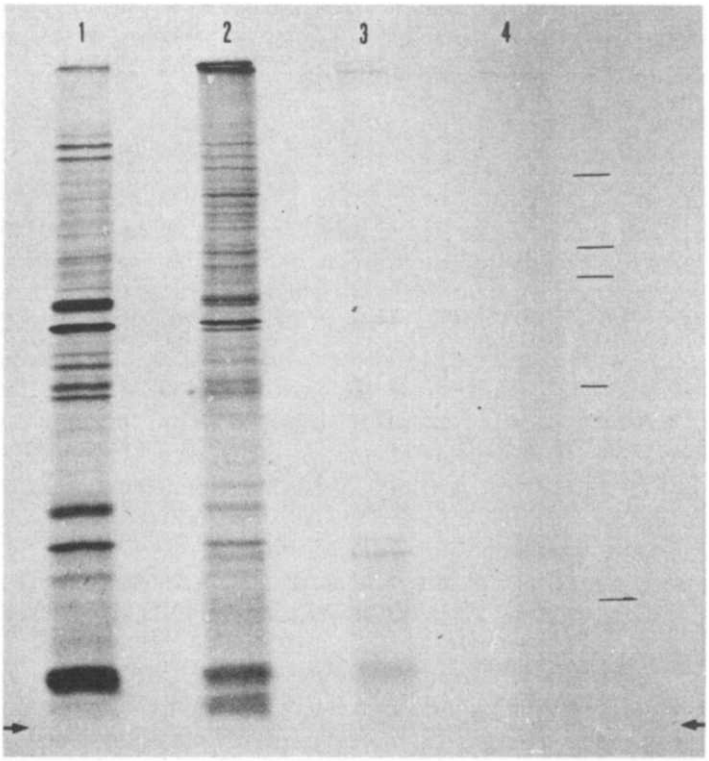

Fig. 5. Effect of chloramphenicol on the incorporation of pulse label into the cytoplasmic and outer membrane. Cells (0.3 mg/ml) were pulsed with [^{35}S]methionine (0.98 μM, 76 μCi/ml) during 70 s and subsequently frozen in liquid nitrogen. Chloramphenicol (final concentration 80 μg/ml) was either added 60 s after (slots 1 and 2) or 5 min before (slots 3 and 4) the addition of label. Membranes were isolated, the respective cytoplasmic and outer membrane fractions were resuspended in equal amounts of denaturation buffer, after which equal volumes were applied to the gel. After electrophoresis, the position of the standards was determined and an autoradiogram was made as described in Fig. 4. Slots 1 and 3, outer membrane fractions labeled in the presence or absence of chloramphenicol, respectively; slots 2 and 4, corresponding cytoplasmic membrane fractions. Arrows indicate the position of the front.

previously [21]; Fig. 6 shows the ^{35}S profiles of the slots 1 and 6 of Fig. 4a as examples.

Bands of lower molecular weight proteins were labeled faster than bands of higher molecular weight (Fig. 4a). In fact, there was a good inverse correlation between the rate of labeling and apparent molecular weight. Similar results were obtained after pulse labeling the cells without chase.

Fig. 4b shows the corresponding protein profiles in the cytoplasmic membrane after pulse chase labeling. As was the case in Fig. 4a, the amount of label detected at the front of each slot in Fig. 4b was greater than expected on the basis of stained gels [19]. This material, which represents protein, since chloramphenicol inhibited its labeling to the same extent as that of the other cytoplasmic membrane proteins (Fig. 5, slot 4 compared to 2), is heterogeneous with molecular weights of 8000 and 9000; the 8000 dalton component clearly differs from the low molecular weight protein in the outer membrane (Fig. 5,

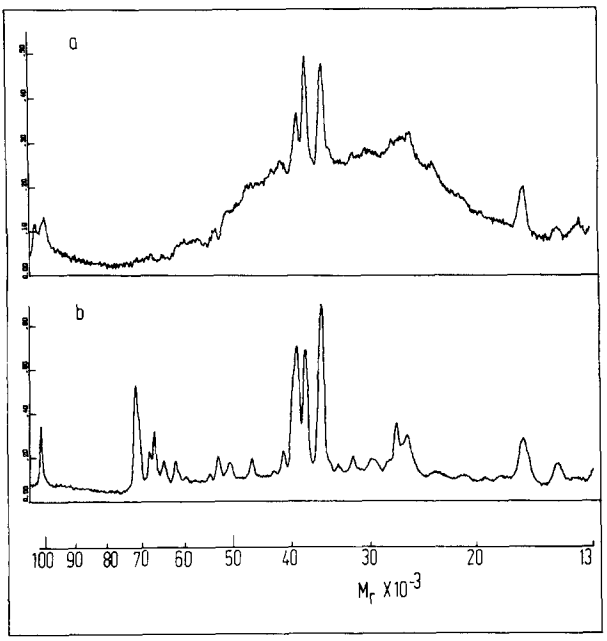

Fig. 6. Radioactivity profiles generated from the autoradiogram of Fig. 4a. The original autoradiogram was scanned with a rotating drum densitometer [21]; 100 density values per mm^2 of the autoradiogram were transferred to magnetic tape. Several computer programs were used to determine the density as a function of migrating distance as well as the integrated density for each slot. The computer plotted scans a and b (slots 1 and 6, respectively, of Fig. 4a) show density data only for proteins greater than 13 000 daltons; the front (see Fig. 4a), which contained in all cases 20 ± 1% of the radioactivity applied to the slots (as determined with a second autoradiogram obtained after exposure for only one day) was not included in the plots to allow the other major outer membrane proteins to be plotted at full scale. As expected, since the same amount of label was applied to each slot, there was little variation in the integrated total densities (18, 19, 20, 22, 19 and $20 \cdot 10^3$ arbitrary density units for slots 1—6 of Fig. 4a, respectively). The molecular weight scale, constructed from the standards of Fig. 4a, is reliable to about 50 000; the higher molecular weight values are given only for reference purposes. Note that the density scale in profile a is expanded twice compared to profile b.

compare slots 2 and 1) and therefore represents a cytoplasmic membrane protein.

Although in some experiments cytoplasmic membrane fractions are contaminated with outer membrane material (Fig. 5), a temporary accumulation of outer membrane proteins in the cytoplasmic membrane was generally not detected (Fig. 4b), in agreement with the findings of Lee et al. [22]. This was true even when the membranes were labeled only 15 s, at which time the cytoplasmic membrane contains about 20% more pulse label than the outer membrane (Fig. 3) and only proteins of relatively low molecular weight have been incorporated into the outer membrane (Fig. 4a).

Although equal amounts of label were applied in each slot, the amount of label detected in discrete bands increased with time, as was the case in the outer membrane fraction. The inverse relationship between apparent molecular weight and rate of incorporation was, however, not as strict as was the case in the outer membrane fraction. Thus, a protein with an apparent molecular weight of 26 000 became labeled only after longer incubation times; this

phenomenon might also be due to protein degradation in the cytoplasmic membrane.

Discussion

In this paper we have focused on the incorporation of proteins into the outer membrane, a process which consists of several stages. First, outer membrane proteins must be synthesized. This might occur either in the cell cytoplasm or on ribosomes bound to the cytoplasmic membrane. Second, the finished outer membrane proteins must be transferred through the cytoplasmic membrane and the murein layer. They might either be transferred very rapidly (possibly through specialized sites in the cytoplasmic membrane) or they might accumulate in the cytoplasmic membrane to form a precursor pool of outer membrane proteins, which are transferred after a suitable delay. Third, at least one outer membrane protein [23], and possibly others as well [24], is modified. This might occur at various times during their synthesis and transfer to the outer membrane. Alternatively, although the above process has been divided into several stages, it is also possible that synthesis, transfer and modification occur simultaneously, in which case the N-terminal portion of an outer membrane protein might be in the outer membrane before the C-terminal region has been completed.

The data presented in this paper provide strong evidence for this last alternative.

There is no precursor pool of outer membrane proteins in the cytoplasmic membrane. We have found that newly synthesized protein appears in the outer membane as fast as it appears in the cytoplasmic membrane; if there is a delay it cannot exceed 3 s, as shown in Fig. 3. Thus, there cannot be a precursor pool of outer membrane proteins in the cytoplasmic membrane, or if there is such a precursor pool, it contains less than 0.1% of the total outer membrane proteins of the cell *. This conclusion is further supported by the fact that neither we (Fig. 4b) nor others [22,25] could ever detect an accumulation of outer membrane proteins in the cytoplasmic membrane after pulse labeling. This was true even after labeling for only 15 s, when very few completed proteins are seen in the outer membrane (Fig. 4a).

The outer membrane proteins which are sometimes seen in isolated cytoplasmic membrane vesicles (refs. 26 and 27, see also Fig. 5, slot 2) which may contain 5—10% of the total outer membrane proteins must therefore be contaminants or if they have been incorporated in the cytoplasmic membrane, they cannot be in equilibrium with newly synthesized outer membrane proteins. Thus, although perhaps as much as 5—10% of the total outer membrane proteins accumulate irreversibly in the cytoplasmic membrane, the bulk of outer membrane proteins is inserted essentially directly into the outer membrane.

Outer membrane proteins are inserted into the outer membrane before chain elongation has been completed. If outer membrane proteins were com-

* Since the cell doubling time, and hence the time necessary to double the amount of outer membrane proteins in a cell, is 83 min in these experiments, the outer membrane protein pool in the cytoplasmic membrane amounts to only 0.06% (3/(83 × 60)) of the total outer membrane protein.

pleted before being transferred to the outer membrane, all outer membrane label would be expected to be associated with specific outer membrane proteins in sodium dodecyl sulfate polyacrylamide gels. In fact however, when cells are labeled for only 15 s, the outer membrane contains relatively little label in specific outer membrane proteins. Instead, much of the label is present in smears between specific outer membrane proteins. This result indicates that incomplete proteins are inserted into the outer membrane, since such incomplete proteins may have any length up to that of the corresponding complete proteins. The disappearance of these incomplete proteins and the concomitant appearance of specific outer membrane proteins during a chase with unlabeled methionine (Fig. 4a) indicate that these unfinished proteins are still attached to ribosomes and are completed after insertion into the outer membrane. Thus, outer membrane proteins appear in the outer membrane while chain elongation is still in progress. Additional evidence for this conclusion stems from the fact that puromycin, when administrated at the beginning of the chase, inhibited this conversion of smeared label to a large extent (data not shown), indicating that protein synthesis is necessary for the completion of outer membrane proteins already partly inserted into the outer membrane.

Nature of insertion regions. A polysome involved in the synthesis of an outer membrane protein might be envisaged as follows. A ribosome binds to a messenger RNA which codes for an outer membrane protein. The nascent polypeptide penetrates through the cytoplasmic membrane, the periplasmic space, the murein layer, and into the outer membrane, where it begins to fold into its tertiary structure. The growing polypeptide may be processed at one or more of these stages.

The mobility of proteins in the outer membrane is very low [28,29]; moreover, the nascent chain will have little or no freedom of lateral movement during its passage through the rigid peptidoglycan layer. As a result, the ribosome must remain stationary relative to the envelope, while the mRNA moves relative to the ribosome, and hence relative to the cell envelope. Since peptide chain elongation occurs at 16—20 residues per s [30,31], mRNA travels past the ribosome at a rate of 48—60 bases/s or, depending on its secondary structure when associated with the ribosome, at a rate of about 20—40 nm/s. Given their dimensions ($20 \times 19 \times 24$ nm [32]), new ribosomes can therefore bind to the mRNA approximately once every second, implying that a new nascent chain is initiated about once per second. Since in their fully extended state these nascent chains grow at a rate of only about 5—6 nm/s, it seems unlikely that neighbouring nascent chains, which must originate at least 20 nm apart, are inserted at a single cytoplasmic membrane site.

Instead, it is likely that the entire polysome becomes fixed to the cell envelope at a number of different spots, which must lie about 20 nm apart from one another. As nascent protein chains are completed and released into the outer membrane, ribosomes are released from the mRNA and possibly also from the cytoplasmic membrane. Since old ribosomes are continuously released from, and new ribosomes are continuously added to the mRNA chain, the entire polysome must move relative to the cell envelope, again at a rate of about 20—40 nm/s. The total distance travelled by a polysome depends on the lifetime of the mRNA; while this is usually 1 or 2 min [33,34],

the lipoprotein mRNA has a lifetime of about 10 min [34]. Thus, polysomes engaged in the synthesis of outer membrane proteins may travel as much as 12—24 μm during their lifetimes.

Whether such movement occurs in specific areas (mRNAs moving in circles) or over the entire cell surface (more or less linear movement of mRNAs) remains to be determined; if the latter possibility applies, protein insertion regions cannot be stationary but must move along the cell surface at a rate of 20—40 nm/s.

Acknowledgements

We thank Mieke Boekhout for scanning the autoradiogram, Nico Panman for drawing the Figures, Klaas Gilissen for photography and Fredy Blaauw for typing the manuscript.

References

1 Murray, R.G., Steed, P. and Elson, H.E. (1965) Can. J. Microbiol. 11, 547—560
2 Costeron, J.W., Ingram, J.M. and Cheng, K.J. (1974) Bacteriol. Rev. 38, 87—110
3 Braun, V. and Rehn, K. (1969) Eur. J. Biochem. 10, 426—438
4 Nikaido, H. (1973) in Bacterial Membranes and Walls (Leive, L., ed.), pp. 131—208, Marcel Dekker, Inc., New York
5 Inouye, M. (1975) in Membrane Biogenesis (Tzagoloff, A., ed.), pp. 351—392, Plenum Publishing Corp., New York
6 Smit, J., Kamio, Y. and Nikaido, H. (1975) J. Bacteriol. 124, 942—958
7 Ghuysen, J.M. and Shockman, G.D. (1973) in Bacterial Membranes and Walls (Leive, L., ed.), pp. 37—130, Marcel Dekker, Inc., New York
8 Osborn, M.J., Gander, J.E. and Parisi, E. (1972) J. Biol. Chem. 247, 3973—3986
9 Bell, R.M., Davis, R.D., Osborn, M.J. and Vagelos, P.R. (1971) Biochim. Biophys. Acta 249, 628—635
10 White, D.A., Albright, F.R., Lennarz, W.J. and Schnaitman, C.A. (1971) Biochim. Biophys. Acta 249, 636—642
11 Randall, L.L. and Hardy, J.S. (1977) Eur. J. Biochem. 75, 43—53
12 Smith, W.P., Tai, P.C., Thompson, R.C. and Davis, B.D. (1977) Proc. Natl. Acad. Sci. U.S. 74, 2830—2834
13 Glenn, R.A. (1976) Annu. Rev. Microbiol. 30, 41—62
14 Blobel, G. and Dobberstein, B. (1975) J. Cell Biol. 67, 835—851
15 Witholt, B., Boekhout, M., Brock, M., Kingma, J., van Heerikhuizen, H. and de Leij, L. (1976) Anal. Biochem. 74, 160—170
16 Witholt, B. (1972) J. Bacteriol. 109, 350—364
17 Nanninga, N. (1973) in Freeze-Etching, Techniques and Applications (Benedetti, E.L. and Favard, P., eds.), pp. 151—180, Société Francaise de Microscopie Electronique, Paris
18 Witholt, B., van Heerikhuizen, H. and de Leij, L. (1976) Biochim. Biophys. Acta 443, 534—544
19 De Leij, L. and Witholt, B. (1977) Biochim. Biophys. Acta 471, 92—104
20 Laemmli, U.K. (1970) Nature 227, 680—685
21 Hoekstra, D., van der Laan, J.W., de Leij, L. and Witholt, B. (1976) Biochim. Biophys. Acta 455, 889—899
22 Lee, N. and Inouye, M. (1974) FEBS Lett. 39, 167—170
23 Inouye, S., Wang, S., Sekizawa, J., Halegoua, S. and Inouye, M. (1977) Proc. Natl. Acad. Sci. U.S. 74, 1004—1008
24 Sekizawa, J., Inouye, S., Halegoua, S. and Inouye, M. (1977) Biochem. Biophys. Res. Commun. 77, 1126—1133
25 Ito, K., Sato, T. and Yura, T. (1977) Cell 11, 551—559
26 Henning, U., Höhn, B. and Sonntag, I. (1973) Eur. J. Biochem. 39, 27—36
27 Schnaitman, C.A. (1971) J. Bacteriol. 108, 545—552
28 Ryter, A., Schuman, H. and Schwartz, M. (1975) J. Bacteriol. 122, 295—301
29 Mug-Opstelten, D. and Witholt, B. (1978) Biochim. Biophys. Acta 508, 287—295
30 Engbaek, F., Kjeldgaard, N.O. and Maaløe, O. (1975) J. Mol. Biol. 75, 109—118
31 Johnsen, K., Molin, S., Karlström, O. and Maaløe, O. (1977) J. Bacteriol. 131, 18—29
32 Kurland, C.G. (1977) Annu. Rev. of Biochem. 46, 173—200
33 Blundell, M., Craig, E. and Kenell, D. (1972) Nat. New Biol. 238, 46—49
34 Hirashima, A., Childs, G. and Inouye, M. (1973) J. Mol. Biol. 79, 373—389