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NATURE OF THE REGIONS INVOLVED IN THE INSERTION OF NEWLY SYNTHESIZED PROTEIN INTO THE OUTER MEMBRANE OF *ESCHERICHIA COLI*

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Summary

Outer membrane proteins are synthesized by cytoplasmic membrane-bound polysomes, and inserted at insertion sites which cover about 10% of the total outer membrane when cells grow with a generation time of 1 h. A membrane fraction enriched in outer membrane insertion regions was isolated and partly characterized. The rate at which newly inserted proteins are transferred from such insertion regions into the rest of the outer membrane was found to be very fast; the new protein content of insertion regions and that of the remaining outer membrane equilibrate completely within about 20 s at 25°C.

Given the rather rigid structure of the outer membrane and the multiple interactions between outer membane components and the murein layer, lateral diffusion of newly inserted proteins from insertion sites to the remaining outer membrane is not likely to explain this rapid equilibration. Instead, the data support a model in which mobile insertion regions move along the cell surface, leaving behind stationary, newly inserted outer membrane proteins.

Introduction

The insertion of proteins into the outer membrane of *Escherichia coli* raises several questions. How are proteins translocated from their sites of synthesis to the outer membrane? Do outer membrane proteins, once inserted, remain stationary, do they diffuse, or are they actively moved in the plane of the outer membrane? Does protein insertion take place at fixed sites in the cell envelope?

With respect to the first question, there is evidence that outer membrane proteins are synthesized on membrane bound polysomes [1,2]. Ribosomes

interact with the cytoplasmic membrane via nascent protein chains [3], which have signal sequences [2,4,5] analogous to those of secretory proteins in eucaryotic [6] as well as procaryotic [7] systems. In addition, we have recently shown that outer membrane proteins are inserted directly into the outer membrane as nascent chains [8]. An interesting consequence of this mode of insertion is that mRNA's involved in the synthesis of outer membrane proteins must move relative to stationary ribosomes, as outlined in Fig. 1, because nascent chains which penetrate through the murein-outer membrane complex must be severely restricted in their lateral mobility [8]. New ribosomes are added to the 5'-end of an mRNA as it moves along, and newly initiated nascent chains are subsequently inserted into the outer membrane. Given the dimensions of a ribosome, the distance between successive nascent chains must be at least 20—25 nm [8]. Since new ribosomes are continuously added and old ribosomes are continuously released from a moving mRNA, the polysome as a whole will move relatively to the envelope.

With respect to the second question, there is a considerable body of evidence which suggests that proteins, once inserted into the outer membrane, remain stationary. First, the outer membrane is a rigid structure compared to normal 'fluid mosaic' membranes [9]: Braun's lipoprotein is bound covalently to the murein layer at a density of about 40 000 molecules/μm^2 [10], for an average distance of only 5 nm between adjacent bound lipoprotein molecules, while the matrix protein [11] and various similar proteins [12] are bound very tightly to the murein layer at a density of about 17 000 molecules/μm^2 [11], or at average distances from one another of only 8 nm. Second, there are numerous interactions between various outer membrane components: free lipoprotein interacts with bound lipoprotein and matrix protein as shown by specific extraction procedures [13] and by spin labeling studies [14], lipopolysaccharides interact with several proteins in pores [15] and phage receptors [16], at least half of the phospholipids interact strongly with other outer membrane components [17], and various outer membrane proteins are found in close proximity to one another as shown by cross-linking experiments [18—20]. In view of these results, it is not surprising that the lateral mobility of phospholipids [17,21—23] and lipopolysaccharides [24,25] is limited, while several proteins appear to be essentially immobile. Thus, Ryter et al. [26] have shown directly that newly inserted λ receptors fail to be redistributed and remain stationary for at least 15 min when protein synthesis is inhibited. Similarly, Begg and Donachie [27] have shown that T6 receptors, which are inserted into the outer membrane at random, remain together in the same cell surface area for at least one generation, indicating there is no free diffusion of these molecules. In addition, Smit and Nikaido [28] have recently shown that newly inserted matrix proteins do not move laterally after insertion into the outer membrane. Given the rigid structure of the outer membrane, there is little reason to assume that other outer membrane proteins behave differently, and it is to be expected therefore that in general outer membrane proteins remain at their insertion points.

With respect to the third question, two basic models can be envisioned. In the first model, insertion takes place in small, fixed areas of the cell envelope; these areas are clearly differentiated from the remainder of the cell envelope by

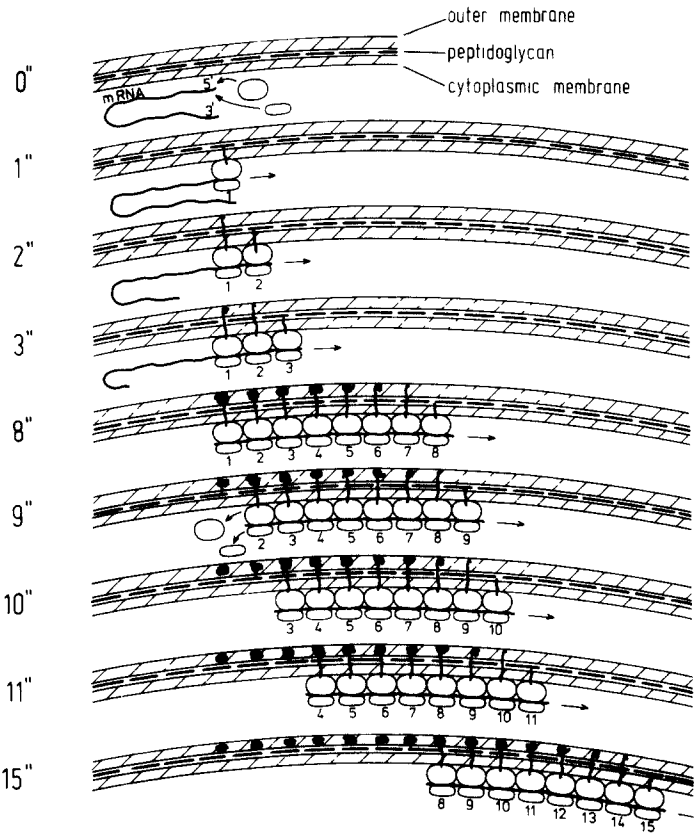

Fig. 1. Model for the movement of an outer membrane protein encoding mRNA. A time sequence of interactions between an outer membrane protein encoding mRNA, ribosomes, nascent chains and the envelope is given. To simplify the model it is assumed that the mRNA is initially present in the cell in a free form, even though ribosomes are attached to the mRNA as soon as this becomes transcribed from the DNA [54]. As we have discussed before [8], mRNA travels past ribosomes at a rate of about 20 nm/s. Given their dimensions (19 X 20 X 24 nm), new ribosomes can therefore bind to the mRNA maximally once every second. In their fully extended state, nascent protein chains grow at a rate of 5—6 nm/s Further details are given in the text

a set of (unknown) structural characteristics. In this model (fixed insertion region model) binding of nascent outer membrane protein chains and thus polysome movement (see Fig. 1) occur within these fixed envelope regions. Since newly inserted proteins remain in the proximity of their insertion points, this model implies localised membrane growth (at least with respect to the protein component); the number of growth zones depending on the number of insertion regions *,†.

* The outer membrane protein content is about 6% of the total cell protein or 4.2% of the total cell dry mass [39—41]. The cell surface to cell mass ratio of cylindrical bacteria is about $1.6 \cdot 10^{10}$ μm^2/mg cell dry mass [24,42,43]. Proteins are synthesized in *E. coli* at a rate of 18 amino acids/s at 37°C [44,45]. From this, and taking an average molecular weight of 110/amino acid, it follows that it will take 216 continuously active ribosomes 1 h to synthesize the outer membrane proteins found in 1 μm^2 of cell surface. Taking into account additional polymerization of the N-terminal signal sequences, it can be estimated that about 250 ribosomes/μm^2 of cell surface are involved in the synthesis of outer membrane proteins if cells grow with a generation time of about 1 h. Since ribosomes measure about 20 X 19 X 24 nm [46], a single ribosome occupies about 400 nm^2 of the inner cell surface. Thus, 250 ribosomes occupy about 10^5 nm^2, or about 10% of the 1 μm^2 of inner cell surface available to these ribosomes.

In the second model (mobile insertion region model), binding of nascent chains is not restricted to specialized envelope areas, but occurs randomly. As a result, polysomes and the associated 'insertion regions' (defined in this model as those membrane areas where protein insertion happens to be taking place at any one time) can move all over the surface of the envelope. This could account for a rapid distribution of newly inserted protein over the outer membrane in the absence of lateral diffusion of inserted proteins.

To test the above two models, we have followed the rate at which newly inserted proteins and 'insertion regions' are separated. The results obtained favor a model in which 'insertion regions' are mobile relative to the cell envelope.

Materials and Methods

Organism and growth conditions. Strain J5, a mutant of *E. coli* 0111 : K58 (B4) lacking uridinediphosphogalactose-4-epimerase, was grown in minimal medium [29] at an incubation temperature of 25°C. The generation time was 150 min under these conditions. Cell densities are expressed as mg (dry mass)/ml [30].

Sample preparation and isolation of membrane fractions. Culture portions (1 ml) of exponentially growing cells were labeled by the addition of [^{3}H]-leucine and [^{35}S]methionine as described in the text and figures. Incorporation was stopped by freezing the cultures in liquid nitrogen in the presence of glycerol [8]; to improve the timing accuracy, duplicate cultures were frozen for each time point. After subsequent thawing, the duplicate cultures were mixed and membranes were prepared in the absence of unlabeled carrier cells as described [8], except that RNAase was omitted. The operating pressure in the French press was 480 atm, except when stated otherwise.

Counting procedures. The distribution of label in the sucrose gradients was determined by taking 0.1 ml aliquots in duplicate from the extruded fractions. After the addition of 10 ml scintillation fluid (900 ml Emulsifier Scintillator-299, Packard Becker BV Chemical Operations, Groningen, The Netherlands, supplemented with 122.5 ml H_2O), the samples were counted in an automatic liquid scintillation analyser (Philips PW 4510/01, Philips, Eindhoven, The Netherlands).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis [31], followed by fixation and staining [32], the gels were prepared for fluorography [33]. After drying the gels on Whatman 3 MM paper, fluorographs were made by overlaying the gels with Kodak RP Royal X-Omat film; exposure took place at −80°C.

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† For the major outer membrane proteins about 340 amino acids have to be polymerized (see footnote †, p. 233). Thus polysomes involved in the synthesis of such proteins may be expected to consist of 10—15 ribosomes [48,49], occupying a surface of about 4000—6000 nm^2 or about 0.1% of the total cell surface. While polysomes for smaller proteins such as the lipoprotein [50] or larger proteins such as the enterochelin binding protein [51,52] should be correspondingly smaller or larger, respectively, it can be estimated that the 10% of the cell surface involved in the synthesis of outer membrane proteins (see footnote *, p. 226) should contain some 100 polysomes, which could be associated with at most 100 separate insertion regions.

Results

Isolation of a membrane fraction enriched in 'insertion regions'

Outer membrane and periplasmic proteins are synthesized by cytoplasmic membrane-bound ribosomes [1,34], and it seems likely that this applies to cytoplasmic membrane proteins as well. Thus, the cytoplasmic membrane should, at least partly, be covered by ribosomes, as has in fact been observed [35]. It is not known whether, and to what extent, such ribosomes are organized spatially. It is clear, however, that the smallest organizational unit must be equivalent to, or greater than, a single polysome. On that basis a cell contains about 100 outer membrane protein insertion regions *, each consisting of nothing more than a single polysome and the associated envelope layers; there will be fewer such insertion regions however, if single insertion regions contain several polysomes. The term 'insertion region' as used here implies nothing about the molecular structure of these regions: 'insertion regions' might be specific structures in the cell envelope, or they might simply be envelope areas where the cytoplasmic and outer membranes are joined temporarily as a result of the insertion and translocation of nascent outer membrane proteins.

To isolate a membrane fraction containing these outer membrane protein insertion regions, we have taken advantage of the fact that in both cases outer membrane protein nascent chains should link the cytoplasmic membrane to the outer membane when insertion is taking place [8]; the mixed membrane fraction should therefore be enriched with such regions. The mixed membrane fraction is not likely to contain insertion regions only, however. It will also contain aspecific unseparated outer and cytoplasmic membrane vesicles [36], high density cytoplasmic membrane vesicles and low density outer membrane vesicles [37]. Thus, isolation conditions have been sought which enrich the mixed membrane fraction with newly inserted proteins.

Fig. 2 shows that spheroplast lysis in the French press at a moderate operating pressure (480 atm) results in a substantial enrichment of newly inserted protein in the mixed membrane fraction; the ratio of new to old protein in the mixed membrane fraction is considerably higher than that expected if the mixed membrane fraction were only composed of aspecific complexes of outer and cytoplasmic membranes of various densities. When the pre- and pulse labels were reversed, the $^{3}H/^{35}S$ ratio profile was identical to the $^{35}S/^{3}H$ ratio profile shown in panel b. When [^{3}H]leucine and [^{35}S]methionine were administered simultaneously as prelabels, no fluctuation in the $^{35}S/^{3}H$ ratio profile was found throughout the gradient. Thus, at an operating pressure of 480 atm lysis was mild enough to preserve an appreciable part of the linking nascent chains, while membrane separation was extensive enough to minimize contamination of the mixed membrane fraction by aspecific unseparated outer and cytoplasmic membranes. Lysis at higher pressure (880 atm) apparently severed the linking chains, since in that case no specific enrichment in the mixed membrane fraction could be detected (results not shown).

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* See footnote †, p. 227.

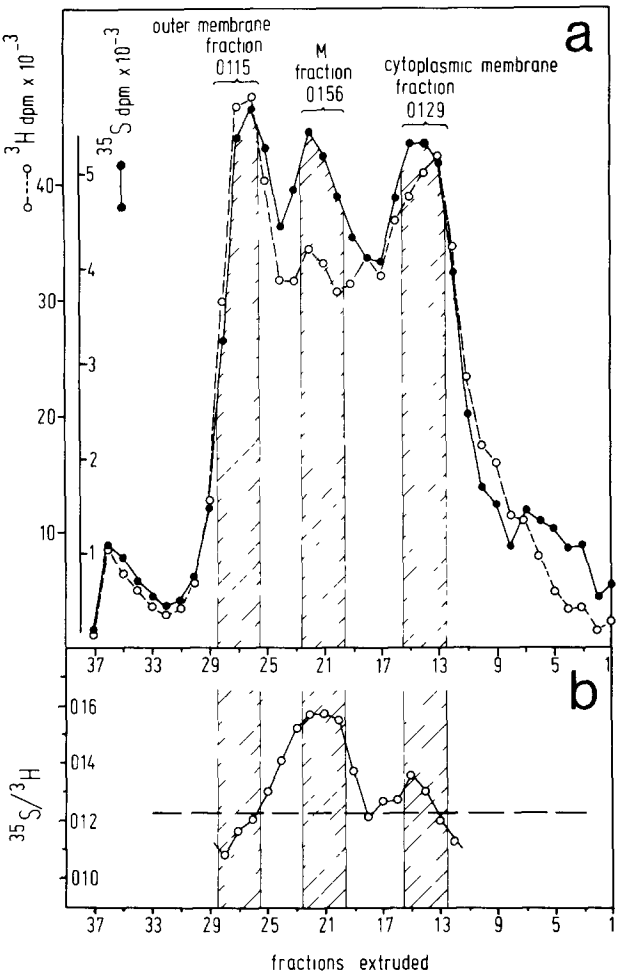

Fig. 2. Separation of membranes by sucrose gradient centrifugation. Cells were doubly labeled as described previously [8]. Pre- and pulse labeling occurred by the addition of [^{3}H]leucine (8.5 μM, 8.5 μCi/ml) and [^{35}S]methionine (0.75 μM, 30 μCi/ml), respectively. Duplicate cultures were frozen 20 s after addition of the pulse and membranes were isolated and separated. Panel a shows the ^{3}H and ^{35}S content, while panel b shows the ^{35}S/^{3}H ratio of the separated membranes. To calculate the overall ratio of new to old label in the different membrane fractions, total ^{35}S dpm were divided by total ^{3}H dpm in the hatched areas of panel a. The obtained ratios are indicated in panel a. M fraction, mixed membrane fraction.

The proteins which were newly inserted into the mixed membrane fraction after different pulse times were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by fluorography. Fig. 3 shows that, compared to the cytoplasmic and outer membrane fractions, the mixed membrane fraction is not specifically enriched in either outer or cytoplasmic membrane proteins. There was an enrichment in the extent of background label however, which was higher in the mixed membrane fraction, especially after a very short pulse time. This background label is likely to represent nascent outer membrane proteins [8], which may have any length up to that of the corresponding completed proteins, and which link the cytoplasmic and outer membranes together at the insertion regions. Cytoplasmic membrane and periplasmic pro-

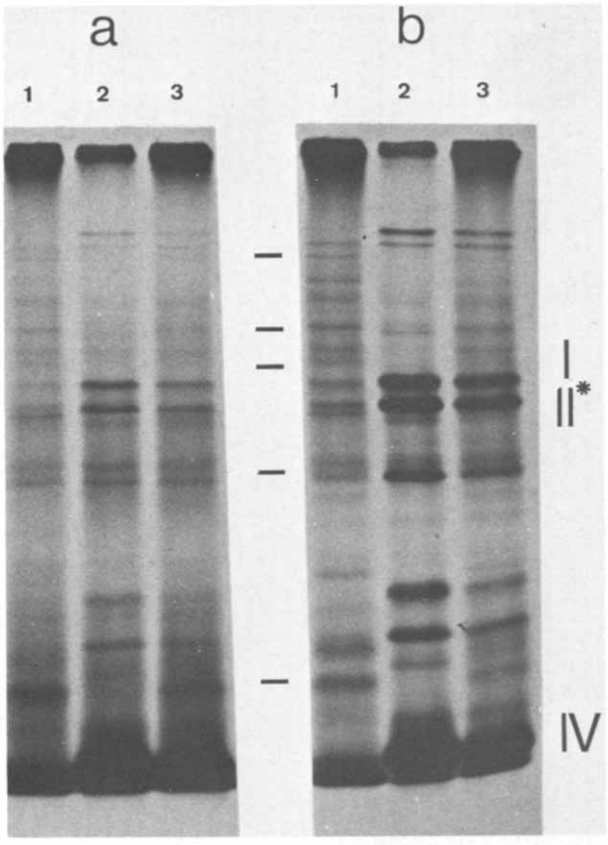

Fig. 3. Protein composition of membrane fractions during pulse labeling. Culture portions of exponentially growing cells (0.3 mg/ml) were labeled by addition of [^{35}S]methionine (0.19 μM for the 20 s sample and 1.2 μM in the case of the 120 s sample, while the radioactive concentration was 60 μCi/ml) and frozen after 20 and 120 s (panels a and b, respectively). Membrane fractions were isolated as described in Fig. 2 and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by fluorography. Slot 1 contains the cytoplasmic membrane fraction, slot 2 the outer membrane fraction and slot 3 the mixed membrane fraction. In each slot 20 000 dpm was applied and the film was exposed for 5 days. The migrating position of the major outer membrane proteins is indicated according to the nomenclature of Henning et al. [39]. The molecular weight standard proteins [8] are indicated as bars (from top to bottom: 67 000, 43 000, 40 000, 26 000 and 12 000).

tein insertion regions cannot be present preferentially in this membrane fraction to any significant extent, since nascent cytoplasmic membrane and periplasmic protein chains should not cross-link the cytoplasmic membrane to the outer membrane. Instead, such insertion regions are expected to be recovered predominantly in the cytoplasmic membrane fraction, which does in fact contain a large amount of background label as is shown in Fig. 3.

The outer membrane fraction also contains unfinished chains ([8], see also Fig. 3), indicating that although some of the insertion regions stay intact and can be isolated in the mixed membrane fraction, many of the insertion regions are disrupted and consequently the outer membrane component of these insertion regions becomes part of the outer membrane fraction upon isolation.

Transfer of newly synthesized proteins from an insertion region to the outer membrane

After being inserted into the outer membrane at insertion regions (which cover perhaps 10% of the cell surface * [38]), newly synthesized outer membrane proteins may become distributed over the remainder of the outer membrane. The rate at which this happens may provide information about the nature of an insertion region (fixed or mobile insertion regions, see Introduction); this rate has therefore been estimated in the experiments described below. Following a pulse of [^{35}S]methionine, insertion regions are initially labeled faster than the rest of the envelope (Fig. 4a, continuous lines). After 15—20 s these labeling rates equalize however, indicating that within this short period of time, a steady state is achieved between the insertion of pulse-labeled nascent chains into, and the subsequent removal of completed proteins from, the insertion regions. Thus, if there is a pool of newly inserted outer membrane proteins in insertion regions, the rate at which newly inserted outer membrane proteins disappear from this pool is quite high. This is corroborated by pulse-chase experiments (Fig. 4a, broken lines) in which the effect of a chase on the disappearance of newly labeled proteins from the insertion regions was determined; within 10—20 s after the chase, the ratio of newly labeled protein to old protein in the insertion regions is equal to that of the remainder of the cell envelope.

An additional method to distinguish between fixed and mobile insertion regions is to block protein chain elongation with chloramphenicol. Chloramphenicol should fix existing insertion sites, since nascent chains cannot be terminated. If protein 'redistribution' in the outer membrane is the result of moving mRNAs, the ratio of new to old protein should remain high in these locked insertion regions, compared to the total outer membrane. If instead proteins diffuse or are removed by some unknown mechanism from fixed insertion regions, all newly inserted proteins except those still in the process of being inserted (and hence not yet terminated) should be removed from the insertion regions and equilibrate with the remaining outer membrane.

Fig. 4b shows that upon the addition of chloramphenicol (arrow 1), the incorporation of pulse label in the mixed membrane fraction as well as in the cytoplasmic and outer membranes is inhibited (Fig. 4b, broken lines), indicating that the insertion of newly synthesized protein was slowed down considerably. The enrichment in pulse label in the mixed membrane fraction persisted almost completely, however, even after the subsequent performance of a chase (Fig. 4b, arrow 2, dotted lines). Thus, besides unfinished nascent chains, which should remain in insertion regions in either model (since nascent chains threaded through the cytoplasmic membrane, murein and outer membrane cannot move laterally), there is no transfer of newly finished outer membrane proteins away from insertion regions under these conditions to a significant extent. As a result it is necessary to invoke mobile insertion regions (Fig. 1) to explain the rapid loss of new protein following a pulse chase (Fig. 4a).

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* See footnote *, p. 226.

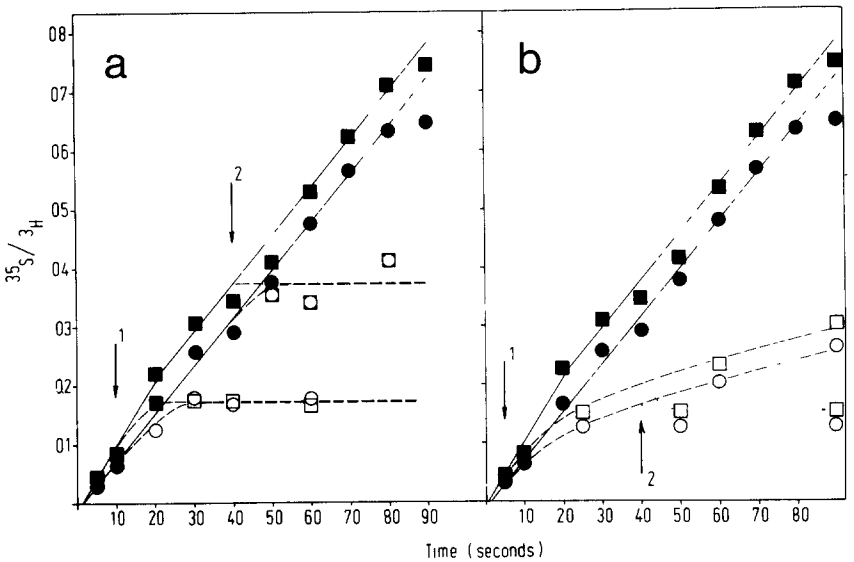

Fig. 4. Incorporation of pulse label in the mixed membrane fraction (squares) compared to the average incorporation in the cytoplasmic and outer membranes (circles) under different conditions. Each time point is determined in a separate experiment. To this end, a culture (0.1 mg/ml) was (pre)labeled with [^{3}H]leucine (8.5 μM, 8.5 μCi/ml) and subsequently grown to a density of 0.3 mg/ml [8]. At that time, the culture was divided in a number of 1 ml culture portions, which were pulse labeled by the addition of [^{35}S]methionine (0.75 μM, 30 μCi/ml) and frozen at the times indicated For each time point duplicate culture portions were labeled, and processed to membranes as described in Materials and Methods. The ratio of pulse ^{35}S to pre ^{3}H label was computed as indicated in Fig. 2. Incorporation was followed after pulse labeling (panels a and b: continuous lines, closed circles and squares), after pulse-chase labeling (panel a: broken lines, open circles and squares), after pulse labeling in the presence of chloramphenicol (panel b: broken lines, open circles and squares) and after pulse labeling in the presence of chloramphenicol followed by a chase (panel b: dotted lines, open circles and squares). A chase was performed by the addition of unlabeled methionine to a concentration of 6 mM (different administration times are indicated by arrows 1 and 2 in panel a and arrow 2 in panel b). Chloramphenicol was added to a concentration of 200 μg/ml (indicated by arrow 1 in panel b). A completely separate, duplicate experiment showed essentially the same results.

Discussion

In order to investigate the nature of outer membrane protein insertion regions, we have isolated a membrane fraction enriched with newly inserted outer membrane proteins, and have determined the rate at which these newly inserted proteins are transferred to the rest of the outer membrane. The results show that following a pulse of [^{35}S]methionine, these newly inserted proteins equilibrate rapidly over the total outer membrane; within 20 s after insertion newly synthesized proteins are as likely to be found in the remaining outer membrane as in insertion regions. Similarly, when a pulse of new label ([^{35}S]-methionine) is chased with unlabeled methionine, only 10—20 s are required to lower the content of ^{35}S-labeled proteins in insertion regions to that of the total membrane. Thus, following insertion, new outer membrane proteins and their original insertion regions are rapidly separated from one another. This can either be accomplished by the diffusion of newly inserted outer membrane proteins from stationary insertion regions, or by the movement of mobile insertion

regions away from stationary, newly inserted outer membrane proteins. Since there is little or no lateral movement of proteins in the outer membrane ([26—28], and see Introduction), the first alternative can be eliminated, leaving the second, perhaps less obvious model.

Thus, newly inserted proteins appear to 'equilibrate' rapidly over the entire outer membrane because they are part of an insertion region for only a short time (Fig. 1); when insertion of a group of nascent chains is complete, they no longer link the envelope layers and they become components of the total outer membrane rather than an insertion region.

Additional evidence for this model stems from the following observations. The protein profile of the mixed membrane fraction after pulse labeling for a short period (20 s) shows that, although there is in fact a considerable enrichment in pulse label in that fraction (28%, compared to the pulse label incorporation in the cytoplasmic and outer membrane (see Fig. 2)) this enrichment consists not of completed (outer membrane) proteins but solely of unfinished chains (Fig. 3), in agreement with the prediction of a mobile insertion region. Furthermore, if protein synthesis is blocked by the addition of chloramphenicol, insertion regions are fixed and newly synthesized proteins are not 'transferred' to the rest of the outer membrane, which is again in accordance with a mobile insertion region model. The rate at which insertion regions advance along the cell envelope depends on the exact direction and rate of polysome movement. Thus, an insertion region may be displaced to a new, non-overlapping envelope area in the time required to move the mRNA completely past a single ribosome. This applies whether an insertion region consists of an envelope area overlying a single or several polysomes moving in parallel. Translation of mRNAs coding for proteins I or II * should take about 30 s †; an insertion region located above one or more such mRNA chains (moving more or less in unison) would therefore also be expected to advance to a neighboring new area of the envelope every 30 s, which is in reasonable agreement with the results presented in this paper.

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† Proteins I and II* contain 336 and 292 amino acids, respectively [11,47], for an average of about 320 amino acids. Assuming N-terminal signal sequences of 20 amino acids [5], about 340 amino acids have to be polymerized before the mRNAs are completely translated. If the elongation rate is about 10—12 amino acids/s at 25°C [53], chain elongation should take about 30 s.

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