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Intermediate Location in the Assembly of the Matrix Protein or Porin into the Outer Membrane of *Escherichia coli*

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Evidence from pulse-chase experiments indicates that the outer membrane matrix protein or porin of *Escherichia coli* B/r passes through a Sarkosyl-soluble membrane pool on the way to its eventual Sarkosyl-insoluble state in the outer membrane.

The outermost layer of the cell envelope of gram-negative bacteria such as *Escherichia coli* is the outer membrane, a bilayer structure consisting of protein, phospholipid, and lipopolysaccharide (21). This membrane forms a barrier against toxic compounds such as bile salts, dyes, and certain antibiotics (20) but is freely permeable to hydrophilic substances of molecular weight less than ca. 600 because of the presence of pore-forming proteins or porins (18, 19).

The biogenesis of the outer membrane is of special interest as an example of a complex extracellular assembly process and also because outer membrane assembly appears to be coordinated with the growth of other layers of the cell surface (5). The proteins of the outer membrane are synthesized upon membrane-bound polysomes (22), but the mechanism whereby the proteins are translocated into the outer membrane is unknown. Amino-terminal leader or signal sequences (4) have been reported for several outer membrane proteins synthesized in vitro and in cells grown under conditions which block the removal of leader peptides (10, 12, 22, 24). These findings do not, however, constitute a solution to the problem of the assembly of specific proteins into the outer membrane because proteins of the periplasm (11, 22) and of the cytoplasmic membrane (6, 26) also possess leader sequences. In addition, it seems that some proteins encoded by the sex factor F are exported to the outer membrane without the involvement of a proteolytically removed leader (1). The specificity of assembly into the outer membrane must, therefore, be determined by other unique, structural features of outer membrane proteins. This specificity might be expressed during translation, resulting in the direct extrusion of proteins into the outer membrane, or it might be expressed post-translationally, in which case the proteins would reside transiently in the periplasm or the cytoplasmic membrane before entering the outer membrane.

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Our approach to this question was to study the kinetics of synthesis and assembly of the 36,500-dalton (36.5K) outer membrane matrix protein of *E. coli* B/r, which is the major pore-forming entity in this strain (2, 18, 23), in the hope of detecting intermediate stages in the assembly pathway of this typical outer membrane protein. The abundance of this protein in *E. coli* B/r permits the direct measurement of its rate of synthesis by the analysis of the corresponding band seen in cell lysates in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5). This technique enabled us to extend previous kinetic studies of outer membrane protein synthesis (7, 13, 16) which have been largely restricted to measurements of bulk protein fractions.

The pulse-chase technique we used to detect intermediates in the assembly pathway of the matrix protein involved a brief (30-s) pulse-labeling with [^{35}S]methionine (incorporation of the radioactive label was stopped by the addition of excess unlabeled methionine). The chasing of the label into various cell fractions and gel bands during the ensuing period was analyzed. Figures 1 and 2 show that after a 30-s labeling, approximately 70% of the label in RNA polymerase $\beta\beta'$ subunits was in incomplete molecules. As the synthesis of these polypeptides was completed during the chase period, they gradually contributed to the radioactivity of the $\beta\beta'$ subunits analyzed as the doublet of gel bands of approximately 160,000 molecular weight (14). The time at which the radioactivity in an individual gel band reached a constant and maximum level (the run-out time) was the time when the last labeled copy of the corresponding polypeptide was completed.

The run-out time for an outer membrane protein, if analyzed in outer membrane fractions, will include any time elapsing between the completion of translation and the assembly of the molecule into the outer membrane. We therefore analyzed the run-out kinetics of the 36.5K matrix protein at 30°C in whole cell ly-

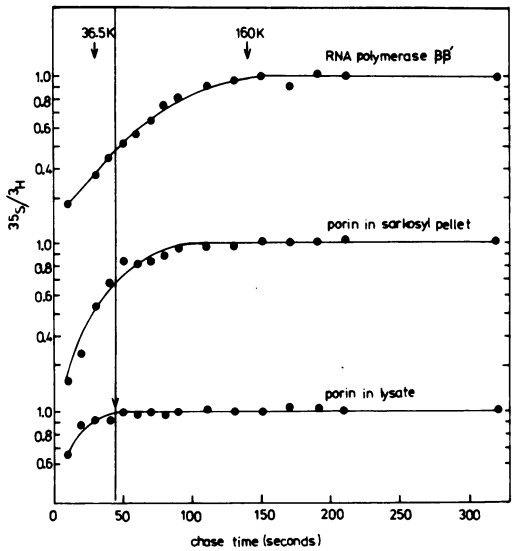

FIG. 1. *A culture of E. coli B/r LEB18 (5) was grown at 30°C in M9-glucose medium supplemented with 1 μg of [^{3}H]leucine (4 μCi/μg) per ml to an absorbancy at 450 nm of 0.15 and then pulse-labeled by the addition of 2.5 μCi of [^{35}S]methionine (680 Ci/mmol; 0.55 ng/ml) per ml. After 30 s, unlabeled methionine was added (final concentration, 150 μg/ml), and then samples (approximate volume, 1 ml) were withdrawn at frequent intervals into 10 ml of ice-cold M9 medium containing chloramphenicol (final concentration, 600 μg/ml). Portions of each sample were removed for the preparation of sodium dodecyl sulfate lysates, and then membrane fractions were prepared from the remainder of each sample by sonic lysis, differential centrifugation, and Sarkosyl fractionation (9). Sodium dodecyl sulfate lysates and Sarkosyl-insoluble membranes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The 36.5K band and the RNA polymerase ββ' 160K band doublet were cut out from the stained gel. Radioactivity in each band was measured in NCS tissue solubilizer (Amersham/Searle)-based scintillation fluid. Details of methods may be found in reference 5. All data are presented as the ratio of ^{35}S to ^{3}H radioctivities normalized to the final plateau value. The arrow marked 36.5K is the predicted run-out time estimated from the 160K band run-out time. The vertical line through all curves passes through the estimated run-out time for the 36.5K band in the sodium dodecyl sulfate lysate.*

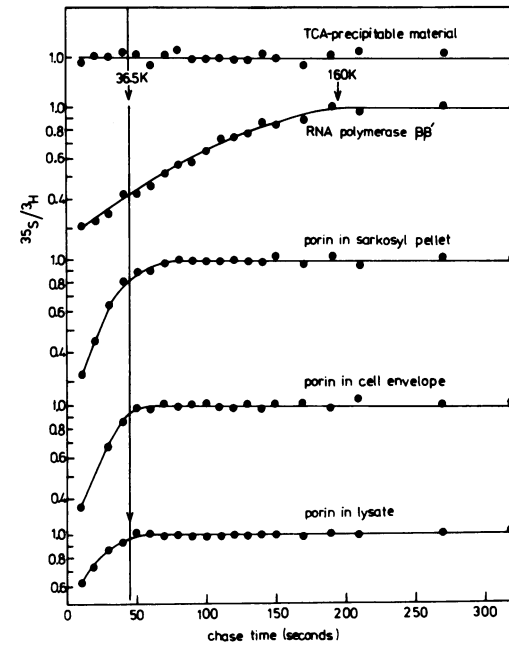

FIG. 2. *A culture of E. coli B/r LEB18 was grown at 25°C in M9-glucose medium to an absorbancy at 450 nm of 0.1 when 8 μCi of [^{3}H]leuine (53 Ci/mmol; 0.2 μg/ml) per ml was added. After 60 min, unlabeled leucine was added (final concentration, 100 μg/ml); 15 min later, [^{35}S]methionine was added for pulse-labeling. This and all subsequent procedures were as described in the legend to Fig. 1, except that an additional portion of each chase sample was removed for the determination of trichloroacetic acid (TCA)-precipitable radioactivity. Samples of washed cell envelopes were also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in this experiment.*

sates and in Sarkosyl-insoluble membrane fractions (Fig. 1). The data show a lag of at least 30 s between the time when the last labeled copy of the matrix protein was completed (lysate analysis) and the time when the last labeled copy stably entered the outer membrane (as defined by the criterion of insolubility in Sarkosyl). Also marked in Fig. 1 is the calculated run-out time for a 36.5K polypeptide as estimated from the 140-s run-out time for the 160K doublet. The reason for the discrepancy between this and the observed 36.5K run-out time is not known; however, it may simply reflect scatter in the early time points. This does not weaken the conclusion that there is a lag before assembly because the observed lysate run-out time was longer than the calculated time.

For determining whether this assembly intermediate was present in the soluble fraction or was envelope associated, a further experiment was performed in which the matrix protein run-out time was analyzed in unfractionated cell envelopes and in the Sarkosyl-insoluble fraction. The result of this experiment, carried out at 25°C, is shown in Fig. 2. Once again, there was a lag between completion of the last labeled copy and its appearance in the Sarkosyl-insoluble fraction. There was, however, no corresponding lag detectable before its appearance in the unfractionated cell envelope. Similar results were

obtained in an experiment conducted at 30°C (not shown).

Thus, the mature 36.5K matrix protein becomes associated with the sedimentable cell envelope fraction immediately upon synthesis but passes through a Sarkosyl-soluble pool in the envelope before attaining its final association with the Sarkosyl-insoluble outer membrane fraction. The former finding rules out the possibility of a transient soluble periplasmic form of the protein. We are left, therefore, with two of the possible routes into the outer membrane mentioned above, namely, initial insertion into the cytoplasmic membrane, followed by translocation into the outer membrane, or direct insertion into the outer membrane. Does the finding of a Sarkosyl-soluble kinetic intermediate help in choosing between these possibilities?

It is tempting to conclude that the Sarkosyl-soluble intermediate is in the cytoplasmic membrane. An alternative, and perhaps more likely, explanation for the detergent solubility of the newly synthesized matrix protein is that it exists in a Sarkosyl-soluble state in the outer membrane, remaining extractable until stabilizing noncovalent interactions with neighboring membrane components are established. Previous investigators (7, 16) have failed to detect kinetic intermediates when using density gradient procedures to separate outer and cytoplasmic membranes. This could be reconciled with our findings if the Sarkosyl-soluble nascent outer membrane protein cofractionates with the dense outer membrane. Clearly, both separation techniques are imperfect. Sarkosyl-extracted membranes and least-dense membranes always contain significant amounts of matrix protein; conversely, only two-thirds of the dense outer membrane peak from a gradient is resistant to Sarkosyl solubilization (unpublished data).

There are other indications that a Sarkosyl-soluble outer membrane state exists. Halegoua and Inouye (10) have reported that in the presence of phenethyl alcohol the matrix protein accumulates in the outer membrane, as defined by density, and yet is solubilized by Sarkosyl. Moreover, DiRienzo and Inouye (8) have shown that at low temperatures the unprocessed form of another outer membrane protein accumulates in a form which is accessible to extracellular trypsin yet is Sarkosyl soluble. In both of these cases the failure to remove the amino-terminal leader peptide may have blocked the maturation of the proteins into the Sarkosyl-insoluble state.

Several lines of evidence indicate that outer membrane components appear at the surface in localized areas (15, 17, 25), perhaps associated with the regions of intimate contact between the outer and cytoplasmic membranes (3). Our findings suggest that these growth zones might be separated from the cell envelope as Sarkosyl-soluble fractions of dense or intermediate gradient peaks; in this way, any polypeptides which are unique to these regions might be identified.

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