Final steps of the maturation of Omp F, a major protein from the outer membrane of *Escherichia coli*

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Pulse-labelling experiments with *E. coli* cells allowed us to follow the incorporation of de novo proteins into the outer membrane of the cell envelope. Labelled membrane samples containing increasingly different levels of newly synthesized Omp F protein were subjected to chemical cross-linking with a bifunctional cleavable reagent in order to investigate the process of trimer formation of the protein. From the results obtained, we conclude that the formation of functional Omp F trimers is substantially delayed to, and can be distinguished from, the incorporation of Omp F monomers to the outer membrane.

E. coli *Outer membrane* *Porin protein* *Omp F* *Membrane biogenesis* *Chemical cross-linking*

1. INTRODUCTION

Great effort has been devoted to the topic of protein export and localization in procaryote organisms during the last few years. In *Escherichia coli*, in particular, studies on synthesis and translocation of both periplasmic and outer membrane proteins constitute a major field in bacterial biochemistry (reviews [1,2]). Some crucial aspects of protein translocation through and location in *E. coli* membranes involved an export machinery coupled to protein translation. Many features of this machinery still remain obscure [3], for example, a signal peptide needed for export of most, but not all, proteins. However, in spite of a great deal of data being available, little is known at the molecular level about the last steps of the locations process of a protein in bacterial cells. We have focused our attention on the last events which occur during the maturation of Omp F protein of *E. coli*. This protein is a major component of the *E. coli* outer membrane and is organized into a trimeric assembly that constitutes the functional units. They form channels for the permeation of small hydrophilic compounds (review [4]). Here, we offer data on the formation of the trimeric function unit from newly synthesized monomers of Omp F protein.

2. MATERIALS AND METHODS

2.1. *Bacterial strain*

E. coli W7 (dap A, lys A) was used throughout. Most work on outer membrane biogenesis has been done on the K-12 strain. The protein studied here corresponds to K-12 Omp F for the following reasons: (i) it is a very abundant protein whose M_r is a little higher than that of Omp A; (ii) the W7 protein forms hydrophilic pores when assayed for permeability to β-lactam antibiotics as described by Barbas et al. [5] using vesicles made from pure protein (unpublished); (iii) like K-12 protein, it is strongly linked to peptidoglycan and they only became dissociated from each other by incubation with 2% SDS at a high temperature; and (iv) the W7 protein should not correspond to Omp C as the latter is scarcely expressed, or not expressed at all under the conditions used here, i.e., low temperature and low ionic strength of the cultures [6,7].

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2.2. *Pulse labelling of membrane protein*

E. coli cells were grown in M9 medium [8] supplemented with 4 mg/ml D-glucose, 10 μg/ml, 2,6-*meso*-diaminopimelic acid and 40 μg/ml L-lysine, at 37°C under forced aeration. When the culture reached $A_{550}=0.9$, its temperature was shifted down to 20°C and incubation was continued for a further 15 min. L-[^{35}S]Methionine (1000 Ci/mmol) (Amersham International, Bucks) was rapidly added to the strongly shaken culture at a concentration of 2.5 μCi/ml. Radioactivity incorporation was stopped at various times after the addition of the label by rapid mixing of a culture aliquot with a similar amount of finely divided shaved ice at −20°C, made from water containing 0.01% (w/v) chloramphenicol.

2.3. *Isolation of inner (IM) and outer (OM) membranes*

After L-[^{35}S]methionine pulses, cells were harvested by rapid cooling and centrifugation. Envelopes were prepared from cell spheroplasts [9]. IM and OM were isolated by isopycnic centrifugation in sucrose gradients following the method of Osborn et al. [9]. Purity of membrane fractions was assessed by studying membrane markers as recently described [10]. Cross-contamination between IM and OM was lower than 3%.

2.4. *Cross-linking of membrane proteins and detection of cross-linked species*

Among those tried, the bifunctional cleavable reagent 2-iminothiolane (Pierce Eurochemie, Oud-Beijerland, The Netherlands) yields better results. We followed essentially the procedure of Traut et al. [11]. The concentration of membrane protein to be cross-linked was 5 mg/ml as estimated by the Folin phenol reagent method. The optimal concentration of the cross-linker we found to be 25 mM. Cross-linked membranes were subjected to two-dimensional SDS-polyacrylamide gel electrophoresis. The first dimension was run in 12.5% acrylamide gels described in [12] as 'high resolution gel'. The second dimension was carried out as described [13]. The separation gel of the second dimension was that described by Lugtenberg et al. [14]. Gels were stained, destained [15], dried under vacuum, and then exposed for autoradiography on prefogged X-Omat X-ray films [16].

3. RESULTS AND DISCUSSION

Kinetic studies on the assembly of both IM and OM proteins have been carried out by Ito et al. [17]) and Lin and Wu [18]. They found that the incorporation of proteins into the OM was slower than incorporation into the IM. Fig. 1 shows a se-

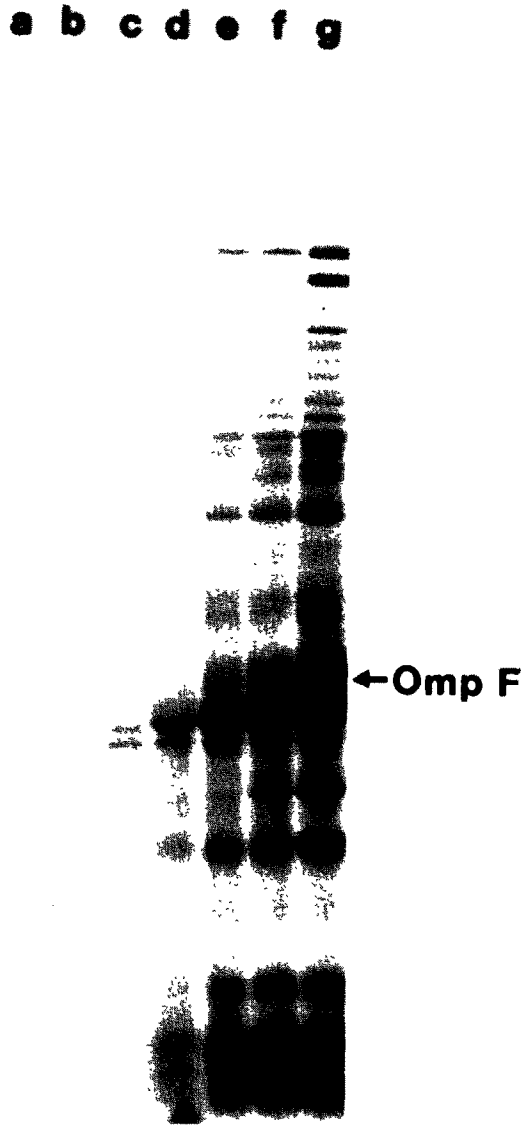

Fig.1. Autoradiograph of a gel electrophoresis of outer membrane proteins from pulse-labelled *E. coli* cells with L-[^{35}S]methionine. Cultured cells were labelled for (a) 10 s, (b) 20 s, (c) 30 s, (d) 45 s, (e), 60 s, (f) 90 s, and (g) 180 s. Cells were processed for the isolation of outer membranes that were electrophoresed as described in [14].

quential picture of the assembly of OM proteins under the labelling conditions described in Section 2. Data shown are not suitable to determine kinetic parameters because it is likely there was L-[^{35}S]methionine starvation in the longest pulses. However, these data may be useful for the purpose of comparison with cross-linked membranes (see below). Fig. 1 shows that Omp F became noticeable 20 s after the addition of the label to the cells. An apparent saturation of labelled Omp F was achieved after a 90 s pulse. The same batches of OM fractions shown in fig. 1 were used for our cross-linking experiments. The concentration of OM protein to be cross-linked was always constant. Fig. 2 shows the first dimension electrophoresis of cross-linked outer membranes. Each track was exposed for enough time to obtain a similar signal for the radioactivity associated to Omp F protein. After cross-linking treatment some minor protein bands were not detected in the electrophoretic system used [12], but those conditions were very suitable for the detection of Omp F trimers. In addition Omp F and Omp A displayed the same migration behaviour, probably due to intramolecular cross-linking of Omp A, which slowed down the electrophoretic mobility of this protein. As can be seen in fig. 2, trimeric species of Omp F protein were only elicited 90 s after labelling of cultured cells. We must point out that the 4 cross-linking experiments shown in fig. 2 were done simultaneously under carefully controlled identical conditions. Under such conditions, in all experiments, the amount of mature (cold) Omp F that was cross-linked accounted for about 60% of the total Omp F as estimated by densitometric tracing of Coomassie blue-stained gels (not shown). However, the appearance of radioactive cross-linked species of Omp F was greatly delayed compared to that of the monomeric species. The identity of cross-linked Omp F was established by a second dimension electrophoresis as shown in fig. 3. In this figure only the parallel f and g tracks of fig. 2 are shown because no trimeric species of Omp F was detected in the shorter labelling pulses. We believe that, although a proper quantification has not been made, even in the 180 s pulse, trimerization of nascent Omp F had not yet been fully achieved.

The data shown here indicate that, at least under our experimental conditions, Omp F was assembled in the outer membrane as a preterminal monomeric form. Trimerization of Omp F is a relatively slow process that can be distinguished from the incorporation of the monomer. Trimers of this protein can be isolated from the envelope under non-denaturing conditions [19,20]. In addition, cross-linking studies on mature species of Omp F have demonstrated its trimeric nature [21].

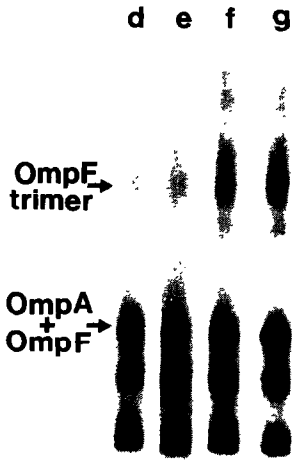

Fig.2. Autoradiograph of a gel electrophoresis of cross-linked outer membrane proteins from pulse-labelled *E. coli* cells. Samples and symbols correspond to those of fig. 1. The exposure period for each track was adjusted to produce a fairly even level of radioactivity associated with Omp F protein. The electrophoresis system used was that described in [12].

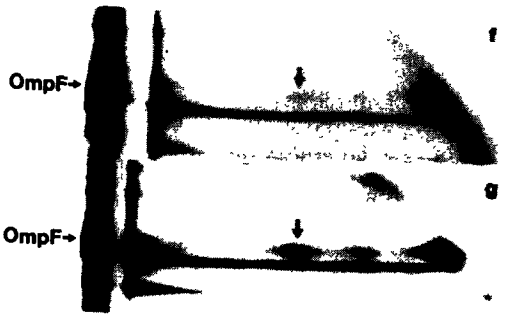

Fig.3. Autoradiographs of second dimension electrophoresis gels of cross-linked outer membrane proteins. Samples and symbols correspond to those of fig. 2. Arrows point to the trimeric aggregates of Omp F protein.

Here, we have shown a kinematic picture of how Omp F acquires a quaternary structure. However, doubts remain as to whether the trimers formed from nascent protein monomers are functional or further steps such a proper association with peptidoglycan or lipopolisaccharide are still needed for functionality of the porin.

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