PRECURSORS OF MAJOR OUTER MEMBRANE PROTEINS OF *Escherichia coli*

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SUMMARY: The production of two major outer membrane proteins of *Escherichia coli*, the matrix protein, and the tolG protein was examined in cells mildly treated with toluene. Toluene treatment is known to result in the accumulation of the prolipoprotein, a putative precursor of the lipoprotein of the *E.coli* outer membrane (Halegoua *et al.*,(1977) J. Biol. Chem. *252*, 2324-2330). It was found that in the toluene-treated cells ^{35}S-methionine was not incorporated into the bands of the matrix protein and the tolG protein in sodium dodecyl sulfate-gel electrophoresis. Instead, two new bands appeared and each of them migrated slower than the corresponding band of the *in vivo* product by almost the same extent. The new products were shown to be closely related to the matrix protein and the tolG protein. In both cases the molecular weights of the new products were found to be larger by about 2,000 than those of the corresponding *in vivo* products. These results strongly suggest that both the matrix protein and the tolG protein are produced from their precursors, the promatrix protein and the protol-G protein, as is the case with the lipoprotein.

We have recently demonstrated the existence of a putative precursor of one of the major outer membrane proteins of *Escherichia coli*, the lipoprotein (1,2). This precursor, prolipoprotein, has a peptide extension of 20 amino acid residues at the amino terminal end of the lipoprotein molecule. Furthermore, the complete amino acid sequence of the extended peptide was determined (1). It was found that the extended peptide consists of a very unique amino acid sequence, which may have an important function(s) in translocation of the protein across the cytoplasmic membrane and into the outer membrane (1).

It is an intriguing question whether other outer membrane proteins of *E.coli* are also produced by a similar mechanism. In this paper, we present data indicating that two other major outer membrane proteins are also produced from their precursors.

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MATERIALS AND METHODS

Bacterial Strain and Medium: E.coli C90 (Hfr Cavalli phoS9, relA1, tonA22, $T2^R$) (3) obtained from the E.coli Genetic Stock Center, Yale University, School of Medicine, New Haven, Connecticut was used. Cells were grown in M9-minimal medium supplemented with 0.4% glucose.

Toluene Treatment: The toluene treatment was carried out in the same way as described previously (4) except that cells were treated with toluene for only 1.5 min. The toluene treated cells ($6x10^9$ cells) thus prepared were incubated with 35 μCi ^{35}S-methionine (New England Nuclear) at 37°C for 10 min in the complete reaction mixture (4). After the reaction, the membrane fraction was prepared as described previously (5). The membrane fraction of the intact cells of E.coli C90 ($6x10^9$ cells) labeled with ^{35}S-methionine was also prepared as described previously (5) except that 15 μCi ^{35}S-methionine was used instead of radioactive arginine. The membrane fractions were solubilized at 100°C for 8 min in 200 μl of the solubilizing solution (2% sodium dodecyl sulfate (SDS) and 10% glycerol in 80 mM Tris-HCl buffer, pH 6.8).

Immunoprecipitation: To 100 μl of the solubilized membrane fraction prepared above were added 100 μl of non-radioactive solubilized membrane fraction from $1x10^{10}$ cells. Then 900 μl of anti-serum against the matrix protein were added to the mixture. The final mixture was incubated at room temperature for 1 hour and at 4° C for overnight. The precipitate thus formed was collected by centrifugation, and washed once with 0.15 M NaCl solution containing 0.05% SDS. The final pellet was solubilized in 50 μl of the solubilizing solution described above.

SDS-slab gel electrophoresis: SDS-slab gel electrophoresis was carried out using 17.5% acrylamide according to the method of Anderson et al. (6) After gel electrophoresis, the gel was first stained with Coomassie blue and then dried for autoradiography.

RESULTS

Toluene Treatment: In order to examine whether other major outer membrane proteins are also produced from their precursors, we used E.coli cells treated with toluene, in which we have shown that the prolipoprotein was accumulated (2,4). The toluene treatment was thought to cause inactivation of the processing enzyme which is required to cleave off the extended peptide of the precursor (2,4). In this study, we used the same toluene treatment as previously reported (2,4) except that the time of toluene treatment was reduced as described in MATERIALS AND METHODS. This mild toluene treatment allowed the cells to produce membrane proteins of higher molecular weights to a larger extent. The incorporation of radioactive amino acids into the higher molecular weight products was not due to intact cells remaining among the mildly toluene treated cells

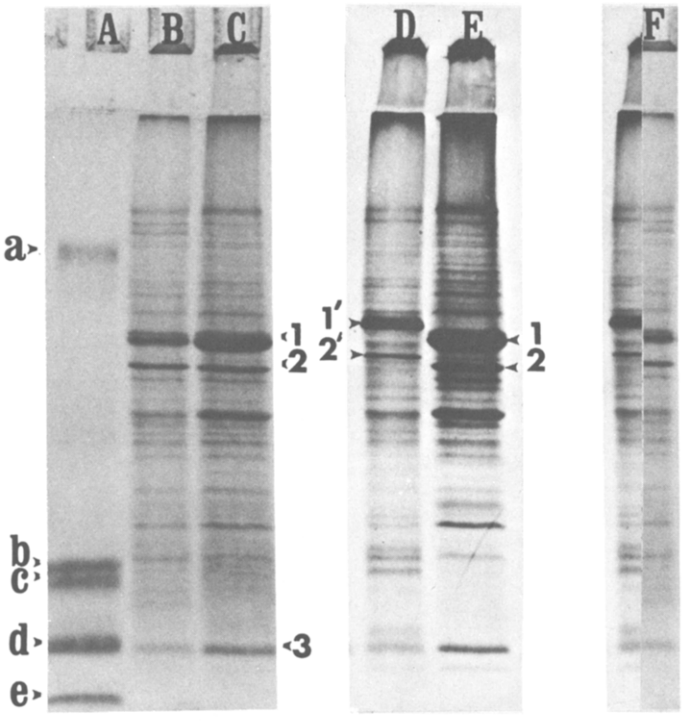

Figure 1. SDS-slab gel electrophoresis of the membrane fractions of the toluene treated cells and the intact cells of E.coli C90. The toluene treatment was carried out as described in MATERIALS AND METHODS . Fifteen µl of the solubilized membrane fractions were applied to SDS-slab gel electrophoresis. A, molecular weight standards; a, bovine serum albumin b, egg white lysozyme; c, cytochrome c; d, free form lipoprotein; e, insulin. B, stained gel pattern of the toluene treated cells. C, stained gel pattern of the intact cell membrane. D, autoradiogram of B. E, autoradiogram of C. F, composite gel pattern of the membrane fraction of the toluene treated cells. The left half and the right half come from the gel patterns of D and B, respectively.

because the incorporation was completely dependent upon the addition of ATP (data not shown), as previously shown (4). After 10 min incubation of the toluene treated cells with ^{35}S-methionine in the complete reaction mixture (4), the membrane fraction was prepared and subjected to SDS-slab gel electrophoresis as described in Fig. 1. After electrophoresis, the gel was first stained and then autoradiographed.

Fig. 1. shows the stained gel patterns and corresponding autoradiograms of the membrane fractions of toluene treated cells as well as of control

intact cells. It can be seen that the stained gel pattern of the toluene treated cells (Fig. 1-B) is identical to that of the intact cells (Fig. 1-C), indicating that the toluene treatment did not cause any changes which result in abnormally slower or faster migration of particular membrane proteins during gel electrophoresis. In contrast, one can find significant differences between the autoradiograms of toluene treated cells (Fig. 1-D) and the intact cells (Fig. 1-E). Two major bands 1 and 2 in the intact cells are missing in the toluene treated cells. Instead there are two new major bands 1' and 2' in the toluene treated cells, both of which migrated slower than bands 1 and 2 respectively by almost the same distance. This can be seen more easily in Fig. 1-F, a composite picture of the left half and the right half of the gels from Figs. 1-D and 1-B, respectively. One can see that many minor protein bands produced in the toluene treated cells (radioactive) migrated to the exact same positions as the pre-existing ones (stained). In the present gel system, we can not separate the prolipoprotein from the lipoprotein, both of which will migrate at the band 3 position.

Identification of Band 1' Protein: Judging from the mobility of band 1' protein, it may well be related to band 1 protein. In the present gel system, it has been established that band 1 protein corresponds to peak 4 protein (7) or matrix protein (8) and band 2 protein corresponds to peak 7 protein (7) or tolG protein (9) (unpublished data). In order to examine the relationship between band 1' and band 1 proteins, the SDS solubilized membrane fractions from both the toluene treated cells and the intact cells were treated with anti-serum against the purified matrix protein. Immunoprecipitates were subjected to SDS-slab gel electrophoresis followed by autoradiography. From the autoradiogram shown in Fig. 2, it can be clearly seen that band 1' protein was immunoprecipitated by anti-serum against the matrix protein (band 1 protein), indicating that band 1' protein shares common structure(s) with the matrix protein. In Fig. 2, the lipo-

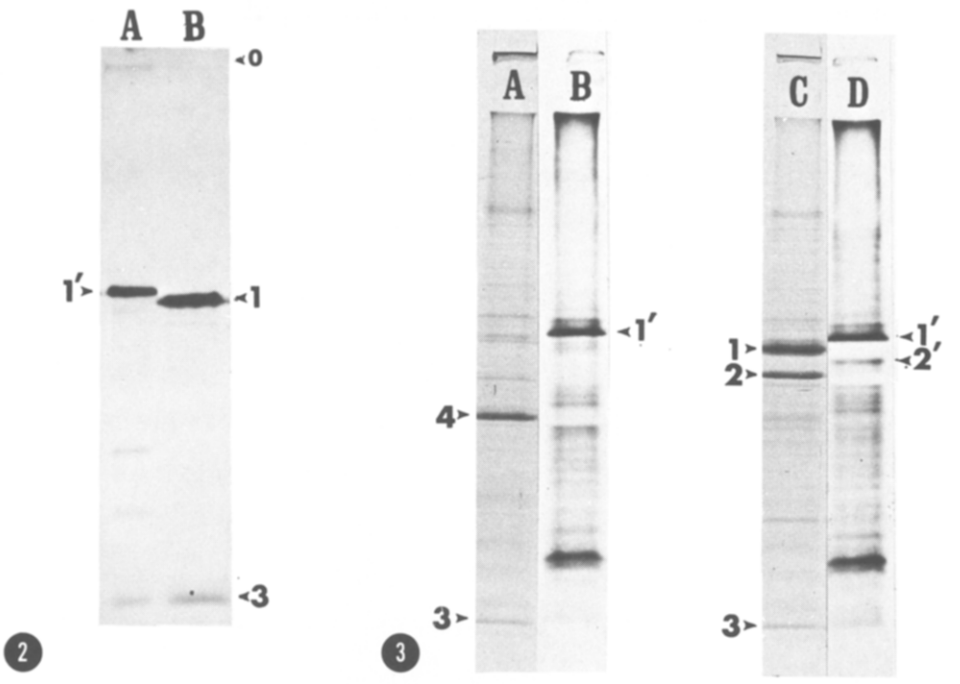

Figure 2. SDS-slab gel electrophoresis of the cross-reactive materials with anti-serum against the purified matrix protein from the membrane fractions of ^{35}S-methionine labeled toluene treated cells and intact cells. The preparation of membrane fractions and the immunoprecipitation were carried out as described in MATERIALS AND METHODS. Fifteen µl of the final solubilized solution were applied to SDS-slab gel electrophoresis. After drying the gel, the autoradiogram was taken. A, the toluene treated cells; B, the intact cells. An arrow with 0 indicates the origin for gel electrophoresis.

Figure 3. Abnormal migration of bands 2' and 2 in SDS-gel electrophoresis. The membrane fractions were prepared and solubilized as described in Fig. 1 except that they were solubilized in two different conditions; at 50°C for 20 min or 100°C for 5 min. SDS-gel electrophoresis and autoradiography were performed as described in MATERIALS AND METHODS. A, stained gel pattern of the membrane fraction of the toluene treated cells solubilized at 50° for 20 min; B, autoradiogram of A; C, stained gel pattern of the membrane fraction of the toluene treated cells solubilized at 100°C for 5 min; D, autoradiogram of C.

protein bands were also seen because the matrix protein injected as antigen for production of anti-serum contained a small amount of contaminating lipoprotein.

The molecular weight difference between band 1' and band 1 proteins was calculated to be about 2,000 (average of two experiments) on the basis

of the molecular weights of the matrix protein (36,500) (8) and the tolG protein (33,000) (10). This difference corresponds to about 20 amino acid residues. Thus these results indicate that band 1' protein is a putative precursor of the matrix protein (promatrix protein), which has about 20 extra amino acid residues over the matrix protein as is the case with the prolipoprotein (1).

Identification of Band 2' Protein: As discussed above for band 1' protein, it is also likely that band 2' protein is a putative precursor (pro-tolG protein) of band 2 protein (tolG protein). This was examined as follows: The tolG protein is known to have a unique conformation so that it migrates faster in SDS-gel electrophoresis when solubilized in SDS at temperatures lower than 70°C (see peak 7 protein in ref. 11). Thus the ^{35}S-methionine-labeled membrane fractions of the toluene treated cells were solubilized at 50°C and 100°C and analyzed by SDS-gel electrophoresis. Figs. 3-A and -C show stained SDS-gel patterns of the membrane fractions solubilized in 2% SDS at 50°C and 100°C, respectively. It has been shown that the tolG protein migrates at band 2 position (Fig. 3-C) when solubilized at 100°C, whereas it migrates at band 4 position (Fig. 3-A) when solubilized at 50°C (data not shown). Their corresponding autoradiograms are shown in Fig. 3-B for Fig. 3-A and Fig. 3-D for Fig. 3-C. When the membrane fraction was solubilized at 100°C, band 2' is seen in Fig. 3-D as previously shown in Fig. 1-D. However, no band is observed at band 2' position in Fig. 3-B when the membrane fraction was solubilized at 50°C. This indicates that band 2' protein also has an abnormal conformation as band 2 protein. At present we have not identified the position of band 2' protein in Fig. 3-B. Thus, band 2' and 2 proteins may well be related. The molecular weight difference between the two proteins was calculated to be about 1,700 (average of two experiments) in the same manner as for band 1' and 1 proteins.

It should be noted in Fig. 3-B that the promatrix protein (band 1')was easily solubilized in 2% SDS at 50°C. In contrast, the matrix protein

(band 1) which is known to have very high affinity for the peptidoglycan (8) was hardly solubilized in 2% SDS at 50°C (Fig. 3-A). This suggests that the promatrix protein may be bound to the cytoplasmic membrane and not yet associated with the peptidoglycan.

DISCUSSION

From the results presented above we concluded that besides the lipoprotein two other major outer membrane proteins, the matrix and tolG proteins, are also produced from precursors. Both precursor proteins seem to have about 20 extra amino acid residues as is the case with the prolipoprotein (1, 2). The determination of the amino acid sequences of the amino terminal regions of these precursors is now in progress. We have found that the amino terminus of the promatrix protein is methionine as is the case with the prolipoprotein. In addition, we have found that the distribution of leucine residues at the amino terminal region of the promatrix protein is completely different from that of the matrix protein, suggesting that the peptide extension is in fact located at the amino terminal end as with the prolipoprotein. The existence of precursors appears to be a general feature for biosynthesis of the major outer membrane proteins. In this regard, alkaline phosphatase, a periplasmic enzyme, has also recently been reported to be produced from a precursor (12). It has been demonstrated that secreted polypeptides or proteins are also produced from precursors in eukaryotic systems (13-18) and the signal hypothesis has been proposed to explain the function of the peptide extensions (18). As discussed previously (1,2), the amino acid sequence of the extended region may contain important information for directing the translocation of the proteins through the cytoplasmic membrane and to the outer membrane. The determination of the amino acid sequences of the extended peptides of the promatrix and the pro-tolG proteins should shed some light on the precise roles of the extended peptides.

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