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Uncoupler-induced relocation of elongation factor Tu to the outer membrane in an uncoupler-resistant mutant of *Escherichia coli*

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***Escherichia coli* UV6, a mutant which is resistant to the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), when grown in the presence of CCCP, but not in its absence, incorporated a new protein (M_r, 42000) into the cell envelope. This protein was found in both cytoplasmic and outer-membrane fractions. In the outer membrane it was one of three or four most abundant proteins. The protein was tightly bound to the membranes and was not solubilized by several detergents. Solubilization was achieved with sodium lauroylsarcosinate (sarkosyl). The protein was purified close to homogeneity by affinity chromatography on a column of GDP-Sepharose. It was identified as elongation factor Tu (EF-Tu) on the basis of electrophoretic mobility, profiles of peptide fragments produced by proteolysis, and by its ability to bind to GDP-Sepharose. Disruption of cells in the presence of CCCP or incubation of envelopes with EF-Tu did not result in incorporation of EF-Tu into the membranes. It is suggested that this protein is incorporated into the outer membrane as a consequence of an alteration in the normal protein biosynthetic mechanisms of the mutant induced by the presence of CCCP.**

Introduction

In a previous paper, we have described the bioenergetic properties of a mutant of *Escherichia coli* which is resistant to the uncoupler CCCP [1]. The mutant is able to grow in the presence of 250 μM CCCP, a lethal concentration for other strains. If the cells were harvested following growth in the presence of the uncoupler, it was evident from the intense yellow colour of the cell pellet that CCCP, or a metabolite of it, was accumulated. The principle sites of accumulation were the outer and inner (cytoplasmic) membranes of the organism. In a further study [2], we have examined the properties of the lipids in the membranes of the mutant by electron spin resonance using the spin probe 5-doxylstearic acid. Growth in the presence of CCCP resulted in a decrease in the fluidity of the membrane lipids. However, there was an increase in the proportion of unsaturated fatty acids in membranes from cells grown with the uncoupler. This increase was reflected in the fluidity of the lipids extracted from the membranes. Since there was a concomitant increase in the ratio of protein to lipid in the membranes, the decreased fluidity of the lipid in the membrane was attributed to the greater proportion of protein.

In the present paper, we describe a study of the nature of the protein which is incorporated into the outer membrane during growth of the mutant in the presence of uncoupler. This protein has been identified as elongation factor Tu, a protein involved in protein synthesis and other functions in the bacterial cell [3].

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EF-Tu, elongation factor Tu; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; sarkosyl, sodium *N*-lauroylsarcosinate; SDS, sodium dodecyl sulfate.

Materials and Methods

Bacterial strains. *E. coli* AN180 (*thi argE mtl xyl rpsL*) is the parent of the CCCP-resistant strain UV6 used in this study. Some of the characteristics of strain UV6 are described in Ref. 1.

Growth of cells. Strains AN180 and UV6 were grown at 37°C to the stationary phase from a 0.1% (v/v) inoculum on Penassay Broth (Difco) in static cultures. Strain UV6 was grown initially in the presence or absence of 50 μM CCCP as required. This culture was used to give a 0.1% inoculum for larger scale growth at the required concentration of CCCP (Sigma).

Isolation of membranes. Total cell envelopes and isolated inner and outer membrane fractions were prepared as previously described [2] except that DNAase and RNAase were omitted from the buffer when cells were disrupted by passage through a French pressure cell. Cell envelopes were prepared by centrifuging the supernatant at $160\,000 \times g$ for 1.5 h in a Beckman 42.1 rotor. Protein content was assayed by the method of Lowry et al. [4].

Osmotic shock treatment. Cells were harvested and washed twice in 10 mM Tris-HCl (pH 8.0). 0.5 g (wet weight) of cells were resuspended in 40 ml of 30 mM Tris-HCl (pH 8.0) containing 20% sucrose, at room temperature. After adding EDTA to a final concentration of 1 mM, the cell suspension was stirred for 10 min at room temperature and then centrifuged at $12\,000 \times g$ for 10 min. The cell pellet was resuspended in 40 ml of cold distilled water, stirred on ice for 10 min and then centrifuged again at $12\,000 \times g$ for 10 min. The shock fluid was decanted and concentrated to 1.0 ml by ultrafiltration. Cell envelopes were prepared from the osmotically shocked cells by French press treatment as previously described [2]. The supernatant fraction from the first $160\,000 \times g$ centrifugation of the membranes from osmotically shocked cells was retained and concentrated by ultrafiltration with an Amicon PM10 filter to 2.0 ml prior to analysis on SDS-polyacrylamide gels. This fraction is referred to as the cytosolic fraction.

Electrophoresis of proteins. SDS-polyacrylamide gel electrophoresis was performed using the discontinuous buffer system of Laemmli [5]. Vertical slab gels of 13% polyacrylamide, with stacking gels of 4% acrylamide, were used. All samples were treated at 100°C for 10 min in 1.15% dodecylsulfate/2.5% 2-mercaptoethanol before gel electrophoresis. Gels were stained with Coomassie blue.

Peptide mapping. Limited proteolysis of proteins and subsequent analysis by electrophoresis were carried out according to the method of Cleveland et al. [6]. Chymotrypsin (Calbiochem) or *Staphylococcus aureus* V8 proteinase (Miles Laboratories) were used.

Extraction of cell envelopes with sodium lauroylsarcosinate. Cell envelopes prepared from 5 g (wet weight) of cells were resuspended in 50 ml of 10 mM Hepes (pH 7.4) containing 1% sodium lauroylsarcosinate (sarkosyl). After 30 min incubation at room temperature, non-solubilized material was pelleted by centrifugation at $160\,000 \times g$ for 1.5 h. The supernatant fraction, containing the sarkosyl-solubilized proteins, was dialysed for 16 h at 0°C against 4×1 liter changes of 10 mM Hepes (pH 7.4) containing 1.0 mM dithiothreitol (DTT). The dialysed solution was concentrated to 10 ml by ultrafiltration.

Purification of the 42 kDa protein. The protein was purified from sarkosyl-solubilized membrane fractions by the affinity column chromatography method of Jacobson and Rosenbusch [7]. A guanosine-5′-diphosphate (GDP)-affinity column was constructed by coupling periodate-oxidized GDP to an aminohexyl-Sepharose 4B (AH-Sepharose (Pharmacia)) matrix which was subsequently reduced with KBH_4. 10 ml of solubilized membrane fraction (40 mg protein/ml) was mixed with an equal volume of GDP-Sepharose and dialysed for 16 h at 0°C against the following buffer (buffer A): 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.35 M NaCl, 1.0 mM dithiothreitol. A column was then poured and washed with buffer A until the absorbance at 280 nm was low and constant. The column was then developed with buffer A containing 1.0 mM GDP. The eluted material, containing the purified protein, was concentrated to 2.0 ml by ultrafiltration. Authentic elongation factor-Tu was prepared from the cytosolic fraction of *E. coli* AN180 by the affinity column chromatography method of Jacobson and Rosenbusch [7].

Results

Insertion of a specific protein into the outer membrane of E. coli UV6 as a consequence of the presence of CCCP

Growth of strain UV6, a CCCP-resistant mutant of *E. coli*, in the presence of the uncoupler results in an increase in the ratio of protein to phospholipid both in the outer and inner (cytoplasmic) membranes [2]. Membrane fractions were examined by SDS-polyacrylamide gel electrophoresis to determine if the increase was due to the formation of a specific protein. The gels of Fig. 1 show that growth in the presence of CCCP resulted in the presence of a new protein component in envelope, inner- and outer-membrane fractions. The new protein became one of the three or four most abundant proteins of the outer membrane. The molecular weight of the new protein was found to be about 42000, determined by comparison with the rates of migration during SDS-polyacrylamide gel electrophoresis of proteins of known molecular weights. For the purposes of this paper the protein will now be referred to as the '42 kDa protein'.

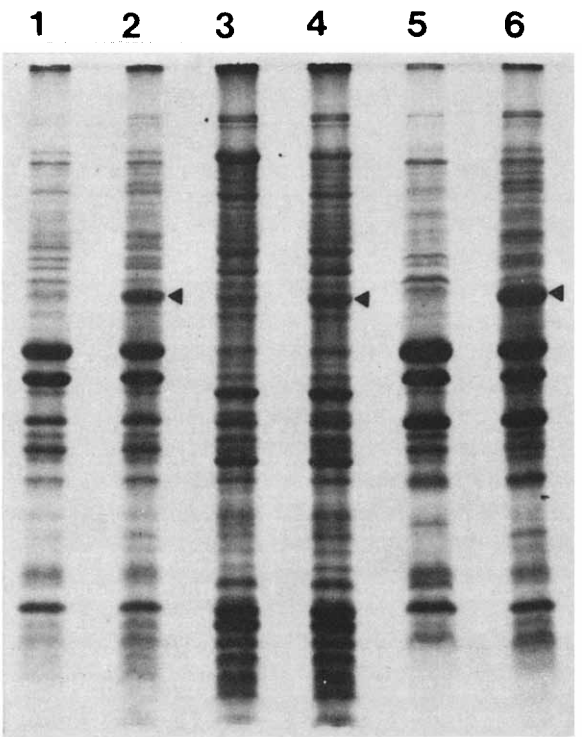


Fig. 1. SDS-polyacrylamide gel electrophoresis of cell envelopes (lanes 1, 2), cytoplasmic (lanes 3, 4) and outer membranes of strain UV6 grown with (lanes 2, 4, 6) or without (lanes 1, 3, 5) 75 μM CCCP. The 42 kDa protein is indicated by the arrow head.

The factors influencing the incorporation of this protein into cell envelopes are shown in Fig. 2. Only minor amounts, if any, were present when cells of the mutant were grown in the absence of CCCP. Optimal incorporation of the protein occurred at 50–75 μM CCCP (left panel). Incorporation was favoured by growth in non-aerated, standing cultures (right panel). Maximum incorporation occurred towards the end of exponential growth (centre panel, 10). Little incorporation had occurred by the beginning (centre panel, 7) or middle (centre panel, 8 and 9) of the exponential phase of growth of a non-aerated culture in the presence of 75 μM CCCP. In studies of the incorporation of the 42 kDa protein into the membranes of strain UV6 we have standardized the growth conditions to harvest the cells after 16 h of growth (stationary phase) in a non-aerated culture in the presence of 75 μM CCCP. The 42 kDa protein was not found in the envelope of the parent strain AN180, and was not incorporated significantly if the cells were grown in the presence of 4% ethanol [8]. It was not induced by heat shock [9]. The protein is not peptidoglycan-associated or heat-modifiable like some outer membrane proteins [10].

Since the 42 kDa protein appeared in the membranes late in the growth phase, we investigated the possibility that it had been synthesized earlier in growth but had not been incorporated. Cells were subjected to osmotic shocking to release the proteins from the periplasmic space. The envelope and cytoplasmic fractions were then prepared from the shocked cells. The periplasmic fraction from cells grown to the early exponential and stationary phases of growth, with and without CCCP, contained a prominent component with an apparent molecular weight of 42000 (Fig. 3, lanes A). This component comigrated during SDS-polyacrylamide gel electrophoresis with the 42 kDa protein (Fig. 3, lane 4B). That these two proteins are probably the same was shown by comparison of the products of partial proteolytic digestion. The protein bands were excised from SDS-polyacrylamide gels and digested with *Staphylococcus*

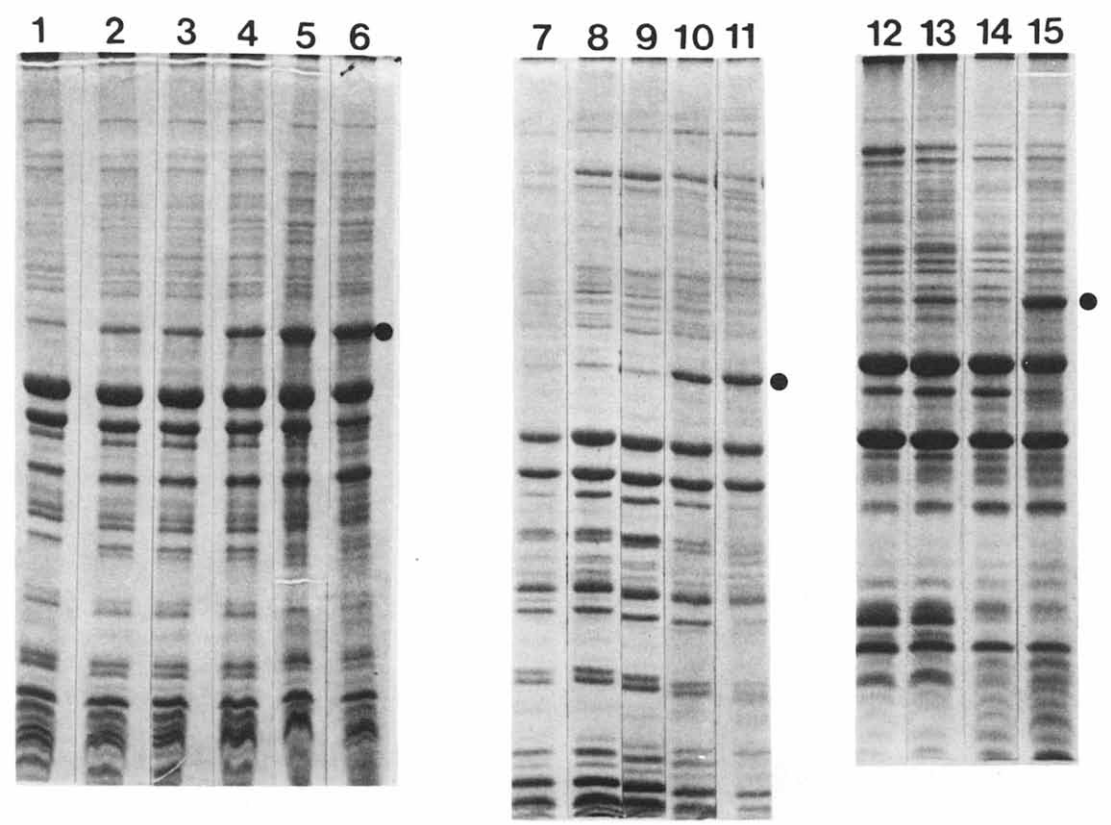


Fig. 2. SDS-polyacrylamide gels to show conditions affecting the incorporation of the 42 kDa protein into the cell envelope of strain UV6. Left panel: strain UV6 was grown in non-aerated cultures without CCCP (lane 1) or in the presence of 5, 10, 25, 50 and 75 μM CCCP (lanes 2, 3, 4, 5 and 6, respectively). Centre panel: strain UV6 was grown in non-aerated cultures with 75 μM CCCP and envelopes were harvested at 7.5, 8.7, 9.75, 11.75 and 32.5 h (lanes 7, 8, 9, 10 and 11, respectively). The absorbances at 660 nm of these cultures at the time of harvesting were 0.028, 0.06, 0.102, 0.15 and 0.115, respectively. Right panel: strain UV6 was grown under aerated (lanes 12, 13) or non-aerated conditions (lanes 14, 15) to the stationary phase without (lanes 12, 14) or with 75 μM CCCP (lanes 13, 15). The 42 kDa protein is indicated by the black dot.

V8 protease and with chymotrypsin by the procedure of Cleveland et al. [6]. The two proteins yielded identical patterns of peptide fragments (Fig. 4).

The experiments described above suggest that a protein of 42000 molecular weight, normally present in the periplasm, is incorporated into the outer membrane at the end of the exponential phase of growth if CCCP is present.

Identification of the 42 kDa protein

Application of the Creighton procedure [11] to the 42 kDa protein revealed that three cysteine residues per molecule were present. Examination of the gene-protein index of Neidhardt et al. [12] suggested that the 42 kDa protein might be elongation factor Tu (EF-Tu). This protein has a molecular weight of 43225 and contains three cysteine residues [13]. EF-Tu binds GDP and may be purified by chromatography on GDP-Sepharose affinity columns [7].

In order to compare the 42 kDa protein with EF-Tu a purification procedure had to be devised. The 42 kDa protein is tightly bound to envelope and outer-membrane fractions from strain UV6 grown in the presence of CCCP. It was not released by treatment with low ionic strength buffer containing EDTA (1 mM Tris-HCl (pH 7.5), containing 1 mM EDTA and 1 mM dithiothreitol), or with 1 M KCl, or with 1% (w/v) octyl glucoside or 2% (v/v) Triton X-100 in 10 mM Hepes buffer (pH 7.4). The effect of treating the envelope frac-

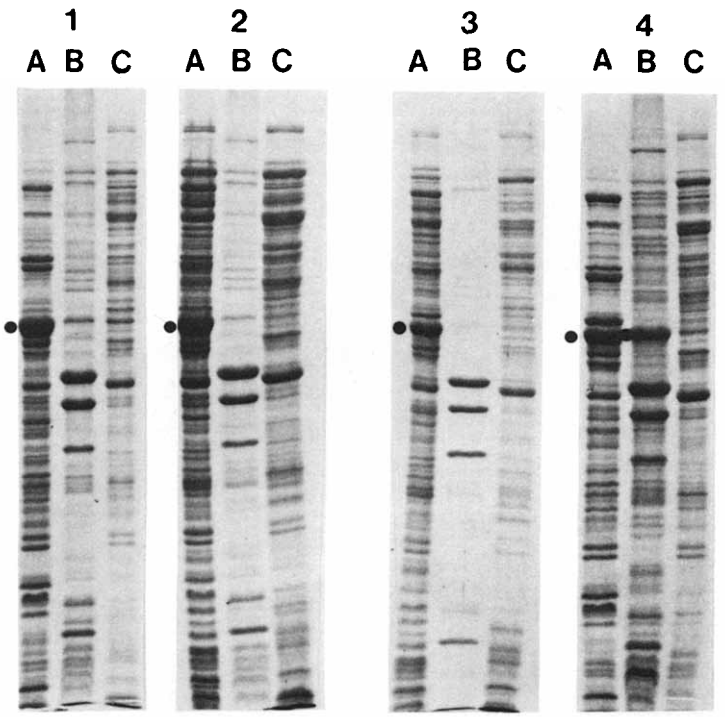


Fig. 3. Effect of growth phase on the cellular location of the 42 kDa protien. SDS-polyacrylamide gels of proteins released by osmotic shocking (lanes A), cell envelope fraction (lanes B) and cytosolic fraction (lanes C) prepared from strain UV6 grown to the early exponential phase (lanes 1, 2), or to the stationary phase (lanes 3, 4), without (lanes 1, 3) or with (lanes 2, 4) 75 μM CCCP. The 42 kDa protein is indicated by the black dot.

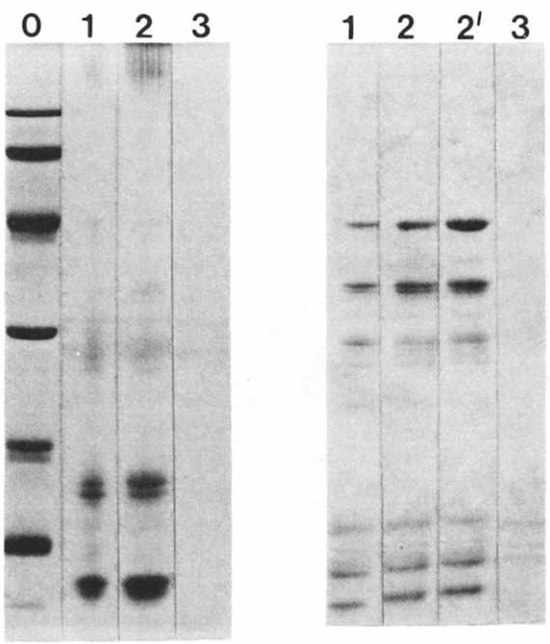
tion with other detergents is shown in Fig. 5. Under the conditions of the experiment, cholate, deoxycholate, and Zwittergent 3–12 were ineffective in solubilizing the 42 kDa protein. In some experiments the protein was solubilzied by 2% (w/v) deoxycholate, but not consistently. 1% (w/v) sarkosyl effectively solubilized the 42 kDa protein (Fig. 5, lanes 3). The 42 kDa protein was purified from the sarkosyl extract, following dialysis to remove detergent, by application to a GDP-Sepharose affinity column. The column was washed well with buffer to remove unbound proteins. The 42 kDa protein was then eluted from the column in an almost homogeneous state with GDP. It comigrated on SDS-polyacrylamide gel electrophoresis with authentic EF-Tu prepared from *E. coli* by the method of Jacobsen and Rosenbusch [7] (Fig. 6, left panel).

The polypeptides resulting from partial digestion by chymotrypsin of authentic EF-Tu and of the 42 kDa protein were compared. The two proteins were excised from SDS-polyacrylamide gels and digested by the procedure of Cleveland et al. [6]. In some experiments the 42 kDa protein was obtained from the sarkosyl extract of the envelope. In others, it had been purified by affinity chromatography. As shown in Fig. 6 (centre panel), the 42 kDa protein and EF-Tu gave identical digestion products. The same intermediate polypeptide fragment (M_r, 30000) in the digestion was detected also when a shorter time of incubation with chymotrypsin was used (Fig. 6, right panel). To further confirm the identification of the 42 kDa protein with EF-Tu, we examined the ability of the purified protein to bind [^{14}C]GDP using the centrifuged column technique of Penefsky [14]. In spite of repeated efforts, weak and variable binding only was observed, presumably due to partial denaturation of the protein during detergent extraction and subsequent purification.

Attempts to incorporate EF-Tu into membranes

The above results indicate that EF-Tu is incorporated into the envelope of strain UV6 when it is grown to the stationary phase with CCCP. The EF-Tu is firmly embedded in the membrane and is not released until the membrane has been disaggregated by a strong detergent such as sarkosyl. We have attempted to find the factors involved in the incorporation by attempting to simulate it in vitro.

CCCP is known to react with sulfhydryl groups



on proteins [15]. Thus, binding of EF-Tu to the envelope might involve linkage to CCCP dissolved in the membrane lipids. We attempted to simulate this situation by disrupting the parental strain AN180 in the presence and absence of 75 μM CCCP. Although CCCP was incorporated into the envelope, as shown by its yellow colour, binding of EF-Tu was not observed.

Fig. 4. Comparison of the 42 kDa protein released by osmotic shocking with that found in the envelope or outer-membrane fraction. The fractions were run on SDS-polyacrylamide gels, and the region of the gel containing the 42 kDa protein was excised. The proteins were digested with V8 proteinase (left panel) or chymotrypsin (right panel) by the method of Cleveland et al. [6]. 42 kDa protein from shock fluid (lane 1), cell envelope (lane 2), outer membrane (lane 2′). Lanes 3 (left and right panels) contained the amount of V8 proteinase and chymotrypsin, respectively, used in the digestions. Lane 0, standard proteins of M_r, 92500, 66200, 45000, 31000, 21500 and 14400.

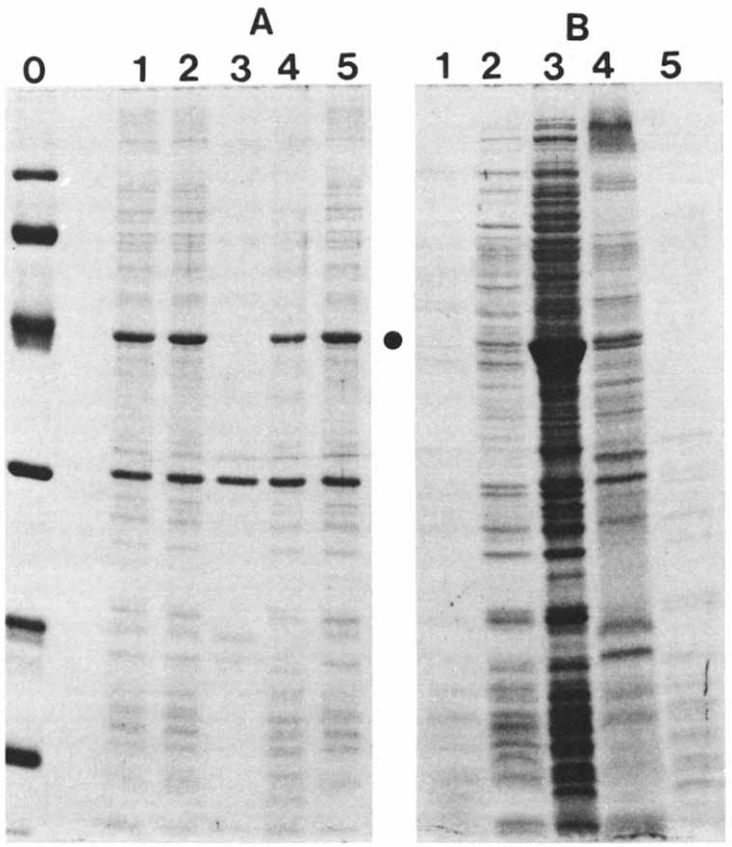


Fig. 5. SDS-polyacrylamide gels of cell envelopes of strain UV6, grown with 75 μM CCCP, treated with detergent (A), and of the proteins released by this treatment (B). Envelopes (5 mg protein/ml) in 10 mM Hepes-KOH buffer (pH 7.4) were stirred with the detergent at 22°C for 30 min. The envelopes were sedimented by centrifugation at 13000 × *g* for 2 min in a Fisher 235B microfuge. The supernatant and the pellet, after washing in Hepes buffer, were examined by SDS-polyacrylamide gel electrophoresis. Lanes 1, 1% (w/v) sodium cholate; lanes 2, 1% (w/v) sodium deoxycholate, lanes 3, 1% (w/v) sarkosyl; lanes 4, 1% (w/v) Zwittergent 3–12; lanes 5, no detergent. Lane 0, standard proteins of M_r 92500, 66200, 45000, 31000, 21500 and 14400. The 42 kDa protein is indicated by the black dot.

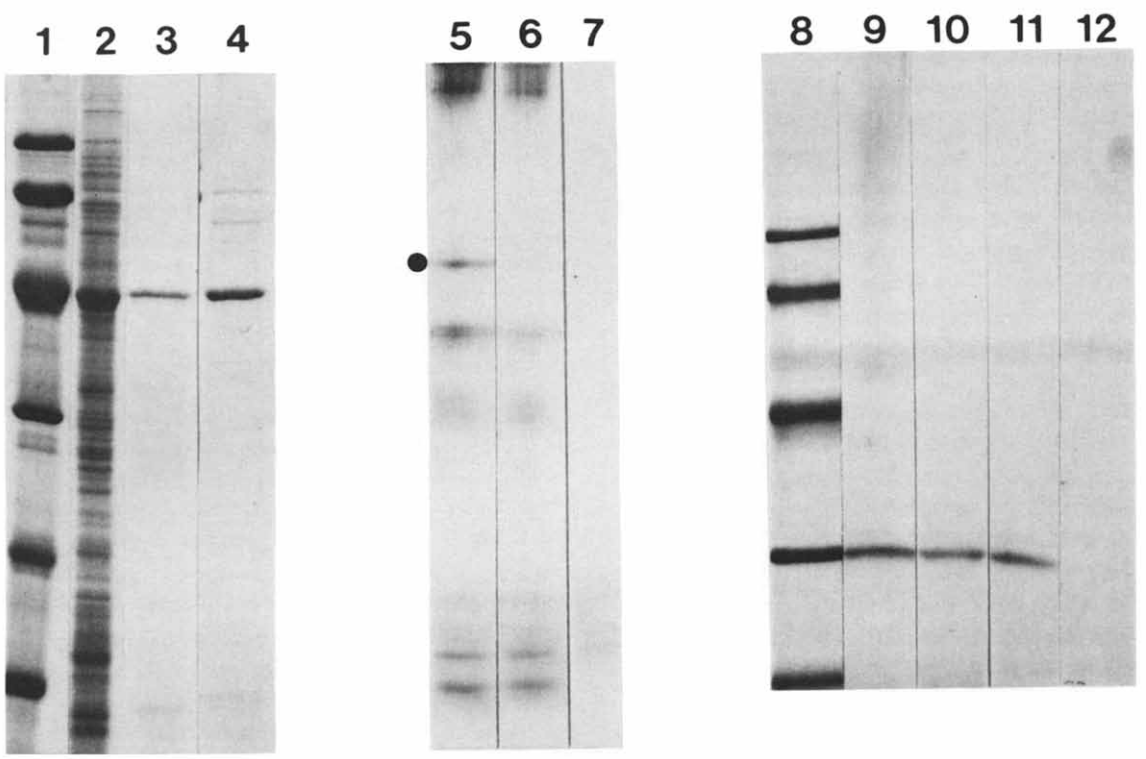


Fig. 6. Purification of 42 kDa protein and comparison with authentic EF-Tu. Left panel: SDS-polyacrylamide gels of proteins solubilized by 1% (w/v) sarkosyl from envelopes of strain UV6 grown with 75 μM CCCP (lane 2), proteins of sarkosyl extract adsorbed on GDP-Sepharose and eluted by GDP (lane 3), authentic EF-Tu (lane 4), molecular weight marker proteins (lane 1). Centre and right panels: the sarkosyl-solubilized envelope proteins (lanes 5, 9), authentic EF-Tu (lanes 6, 10), and GDP-Sepharose purified 42 kDa protein (lane 11) were submitted to SDS-polyacrylamide gels electrophoresis. The gel containing 42 kDa protein was excised and treated with chymotrypsin for 20 (right panel) or 30 min (centre panel) by the method of Cleveland et al. [6]. Lanes 7 and 12 contain the amount of chymotrypsin used in the digestions. Lane 8, molecular weight marker proteins. The black dot in the centre panel indicates the position of migration of undigested 42 kDa protein/EF-Tu.

Envelopes, prepared from strain UV6 grown without CCCP at 20°C and 37°C, were sonicated, in the presence or absence of 75 μM CCCP, with a cytosolic fraction containing EF-Tu. EF-Tu was not incorporated into the envelope. Thus, sonication, or the more fluid membrane lipid of envelopes from cells grown at 30°C, did not induce incorporation.

Finally, incubation or sonication in the presence or absence of 75 μM CCCP of an exhaustively-dialyzed preparation of sarkosyl-solubilized EF-Tu (from the envelope fraction of strain UV6 cells grown with CCCP) with the envelopes of the parental strain AN180 did not lead to incorporation of EF-Tu into the membranes.

These results show that EF-Tu is not artifactually trapped in the vesicle fractions.

Discussion

E. coli UV6 was able to grow in the presence of 250 μM CCCP, a concentration lethal to normal strains. Growth in the presence of uncoupler resulted in distinct changes in the cell envelope of the bacterium [2]. CCCP, or a metabolite of it, was accumulated in the envelope to levels sufficient to impart a bright yellow colour. There was an increase in the proportion of unsaturated and cyclopropane fatty acids both in the outer and inner (cytoplasmic) membranes. Although the extracted lipids were more fluid as a consequence of this change, electron spin resonance measurements with 5-doxylstearic acid indicated that the lipids when in the membrane were actually less fluid as a consequence of the cells being grown with CCCP.

However, since there was a concomitant increase in the protein : lipid ratio, we attributed the decrease in lipid fluidity to this cause [2].

In this paper we have examined the nature of a protein the amount of which was increased by growth of strain UV6 in the presence of CCCP. About equal amounts of this protein were present in the cell envelope and cytoplasmic fractions. It was found in both inner and outer membrane fractions, and was one of the three or four most abundant proteins of the outer membrane. Incorporation of this protein occurred at the end of the exponential phase and was favoured by non-aerated conditions. Incorporation was dependent on the presence of CCCP.

This protein has been identified as elongation factor-Tu on the basis of comigration on SDS-polyacrylamide gels, the presence of three cysteine residues in both proteins, and identical proteolytic cleavage fragments. Both proteins bind to GDP-Sepharose and are eluted by this nucleotide.

EF-Tu is an abundant protein in *E. coli* (5% of the total cellular protein) [16]. It is required in protein biosynthesis to ensure the correct binding of aminoacyl-tRNAs to the acceptor (A-site) of the ribosome [3]. However, it has other roles in the cell. It is a subunit component of the $Q\beta$ replicase and thus participates in RNA synthesis [17].

Jacobsen et al. [16,18] found that EF-Tu was released from *E. coli* by osmotic shocking, but not when the cells were converted to spheroplasts. They suggested it was not a typical periplasmic protein but was located on the cytoplasmic surface of the inner membrane and was released into the shock fluid by rearrangement of the membrane proteins during shocking. The amount of this protein associated with the envelope fraction depended on the severity of the method used to break the cells. French pressing released greater than 90% of EF-Tu [18]. The results of our studies are difficult to reconcile with the hypothesis that EF-Tu is associated only with the cytoplasmic surface of the inner membrane, although like Jacobsen et al. [16,18] we have found it to be released by osmotic shocking. The amount of EF-Tu found in the cytoplasm was not noticeably decreased when strain UV6 was grown with CCCP. It was found in the outer membrane only when this strain was grown with the uncoupler. It was a major protein which remained in this location even after French pressing and required disruption of the membrane by sarkosyl before it was released. We have not been able to obtain incorporation or binding of EF-Tu to outer membrane and envelope fractions from the mutant or its parent under in vitro conditions designed to simulate those occurring during breakage of the cell. This leads us to conclude that the EF-Tu was inserted into the outer membrane of *E. coli* UV6 during biosynthesis. Biosynthesis of outer-membrane proteins usually involves the biosynthesis of a precursor form of the protein. This form is processed with the removal of its leader sequence concomitant with translocation [19]. EF-Tu is not synthesized with a leader sequence [13,20]. Thus, its occurrence in the outer membrane is surprising.

Since these studies were completed, we have become aware of the work of Dombou et al. [21] which shows some similarities to that described here. These investigators found that EF-Tu became incorporated into the outer membrane of a sucrose-dependent spectinomycin-resistant mutant of *E. coli* when it was grown in the absence of sucrose. This mutation caused an alteration in proteins S3, S4 or S5 of the 30-S ribosomal subunit and gave rise to hypersensitivity to antibiotics, dyes and detergents. They have speculated that movement of EF-Tu to the outer membrane might take place through adhesion sites between the outer and cytoplasmic membranes as a consequence of an abnormal interaction between the ribosomes and the cytoplasmic membrane. Since our mutant is not spectinomycin-resistant or hypersensitive to antibiotics, dyes and detergents, the mechanisms for incorporation of EF-Tu in the outer membrane may not be the same in the two mutants.

It seems unlikely that the incorporation of EF-Tu into the outer membrane of strain UV6, when grown in the presence of CCCP, had any significance in the mechanism of resistance of this organism to uncoupler. The protein was incorporated late in the growth phase. Presumably resistance needs to be manifested from the earliest stages for growth to occur.

Acknowledgement

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