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Synthesis of a Precursor to the B Subunit of Heat-Labile Enterotoxin in *Escherichia coli*

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Escherichia coli K-12 minicells were employed to investigate the biosynthesis of plasmid-encoded, heat-labile enterotoxin of *E. coli*. Two polypeptide species related to the B subunit of the toxin were expressed in the minicells. One of these polypeptides (molecular weight, 11,500) was immunoprecipitated by antiserum to cholera toxin. Because the B subunits of heat-labile enterotoxin and cholera toxin have common antigenic sites, we concluded that this species was the mature B subunit. The larger polypeptide (molecular weight, 13,000) is likely to be a precursor of the B subunit because it could be chased into the mature form. This conversion was inhibited by compounds which dissipate proton motive force, suggesting that processing requires energy.

The heat-labile enterotoxin (LT) produced by *Escherichia coli* is closely related to the cholera toxin produced by *Vibrio cholerae* (for review, see reference 15). The toxins have similar subunit structures; both contain one A subunit (molecular weight, 28,000) and multiple copies of a smaller B subunit (molecular weight, 11,500) (3, 17) which serves to bind the toxin to its receptor on the eucaryotic target cell in the intestinal mucosa of humans and domestic animals. The toxic function resides in the A subunit, which activates adenylate cyclase by catalyzing a covalent ADP ribosylation of the regulatory subunit of that enzyme (2). Increased cellular levels of cyclic AMP result in active secretion of chloride ions into the intestinal lumen and a consequent loss of fluid characteristic of diarrheal disease.

The toxins have been well characterized biochemically, but little is known about their biosynthesis and export from bacteria. The heat-labile enterotoxin of *E. coli* is genetically encoded on a plasmid (10). Dallas and Falkow (4) and So et al. (23) isolated a gene which encodes the toxin from a large natural plasmid and, using recombinant DNA technology, linked it to the cloning vehicle, plasmid pBR313.

Dallas and Falkow (4) then introduced this plasmid into a minicell-producing strain of *E. coli* and demonstrated synthesis of the A and B subunits. Here we report synthesis in plasmid-containing minicells of a precursor of the B subunit as well as the mature form. The conversion of the precursor to the mature form is inhibited by treatment with compounds which dissipate proton motive force, suggesting that energy is necessary for maturation of the B subunit. A similar energy requirement for the processing of M13 coat protein precursor has been reported (6).

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MATERIALS AND METHODS

Strains. Plasmid DNA was isolated (plasmid EWD299, constructed by insertion of a DNA fragment with a mass of 1.2×10^6 daltons derived from plasmid P307 into plasmid pBR313 [23], the gift of S. Falkow) and used to transform the minicell-producing *E. coli* K-12 strain M2141 (1). A total of 23 independent isolates were tested for the production of enterotoxin with the G_{M1}-enzyme-linked immunosorbent assay, which assays the binding of the B subunit to its receptor ganglioside, G_{M1}, as described previously (24). The isolate which produced the highest level of enterotoxin was used in this study. As a control the minicell-producing strain was also transformed with plasmid pBR322.

Labeling of polypeptides synthesized by isolated minicells. Minicells were isolated as described previously (9) and suspended at an optical density (OD) of 1 (equivalent to 5×10^8 whole cells per ml) in 100 μl of labeling medium, which contained 1.25% (wt/vol) methionine assay medium (Difco Laboratories), M9 salts (21), 0.8% (wt/vol) glucose, and 0.005% (wt/vol) vitamin B_1. After incubation at 37°C for 5 min, compounds to be tested were added as specified in the

figure legends. After an additional 3 min of incubation at 37°C, 25 μCi of [^{35}S]methionine (ca. 800 Ci/mmol; New England Nuclear Corp.) was added, and incubation was continued for 60 min. Incorporation of isotope was stopped by the addition of trichloroacetic acid to a final concentration of 5%. Minicells were centrifuged, and the pellet was washed with 95% ethanol. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide (15%) gel electrophoresis and autoradiography.

Other techniques. Immunoprecipitation, gel electrophoresis, and autoradiography were as described previously (22).

Thin-layer chromatography of tryptic peptides. ^{35}S-labeled proteins were separated by sodium dodecyl sulfate-polyacrylamide (15%) gel electrophoresis and then excised from dried gels with a razor blade. The gel pieces were rehydrated in approximately 1 ml of 0.05 M NH_4HCO_3 (pH 8), and the protein digested within the gel piece at a concentration of 50 μg of trypsin (TPCK treated; Worthington Biochemicals Corp.) per ml as described previously (8). The gel piece was removed from the solution after 16 h of incubation at 37°C. The peptides in the solution were lyophilized and suspended in 0.01 ml of water. Samples were analyzed on silica gel-coated thin-layer chromatography plates (Merck & Co., Inc.). Chromatography in the first dimension was done in solution 1 (methanol-chloroform-concentrated ammonia, 2:2:1). The plates were dried and then chromatographed in the second dimension in solution 2 (pyridine-glacial acetic acid-*n*-butanol-water, 40:14:68:25). The plates were dried and sprayed with En^{3}Hance spray (New England Nuclear Corp.), and the peptides were visualized by fluorography.

Reversible effect of ethanol. Minicells were incubated for 15 min at 37°C in labeling medium containing 8.3% ethanol and [^{35}S]methionine (as above). Chloramphenicol (100 μg/ml) was then added to ensure no further synthesis of protein. Appropriate control experiments were done to show that there was no residual, preferential synthesis of mature B subunit which could account for the observed appearance of the mature subunit during subsequent incubations. The mixture was then divided into two portions and centrifuged to pellet the minicells. Each portion of minicells was washed twice with labeling medium containing either 100 μg of chloramphenicol or 100 μg of chloramphenicol plus 8.3% ethanol per ml. Minicells were then suspended and incubated in their respective wash solutions at 37°C, and, at the times indicated in Fig. 5, samples were removed into 10% trichloroacetic acid and prepared for analysis by electrophoresis and autoradiography, as described above.

Fractionation of minicells. Minicells (1 ml at an OD of 1) labeled with [^{35}S]methionine were removed from the labeling medium by centrifugation, and a periplasmic fraction was obtained by the standard osmotic shock procedure (13) adapted for small quantities of cells (12). The shocked minicells were then treated with 10 mM EDTA–175 μg of lysozyme per ml for 15 min on ice and lysed by sonication (4 times at 30 s each in an ice-salt bath). The lysate was centrifuged at 100,000 × *g* for 16 h to separate cytoplasmic proteins (supernatant) from envelope proteins (pellet).

RESULTS

E. coli strains carrying mutations in *minA* and *minB* divide abnormally, resulting in production of minicells which do not contain chromosomal DNA. In contrast, plasmid DNA is segregated into minicells (9). Thus, isolated minicells provide a convenient system for analysis of plasmid-encoded polypeptides.

The polypeptides synthesized in minicells containing plasmid EWD299, which encodes heat-labile enterotoxin (LT), were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by autoradiography (Fig. 1, lane 2). Five predominant polypeptides were synthesized. The two uppermost polypeptides migrating with apparent molecular weights of 28,000 and 30,000 are probably related to β-lactamase, the enzyme responsible for the am-

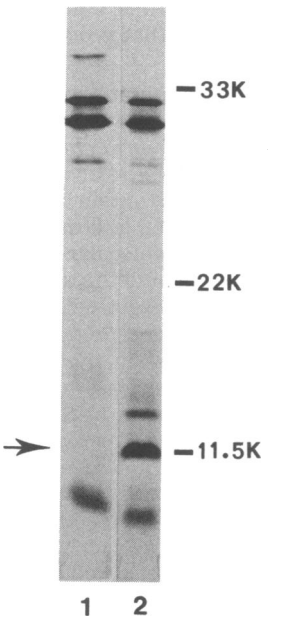

FIG. 1. *Polypeptides synthesized in plasmid-containing minicells. Minicells were isolated and labeled as described in the text, the synthesized products were analyzed by sodium dodecyl sulfate-polyacrylamide (15%) gel electrophoresis and autoradiography. Sample slots contained polypeptides synthesized in minicells containing plasmid pBR322 (lane 1) and plasmid EWD299, which carries the enterotoxin genes (lane 2). The arrow indicates the position of the mature B subunit of cholera toxin. The molecular weight markers used were as follows: protein II*, 33,000 (33K) (14); cholera toxin peptide A_1, 22,000 (22K) (19); and cholera toxin B subunit, 11,500 (11.5K) (18). Each sample slot contained minicells equivalent to an OD of 0.03.*

picillin resistance carried by the plasmid, because they were not specific for plasmid EWD299 but were also present in minicells harboring a related plasmid, pBR322, which carries the β-lactamase (7) but not the enterotoxin genes (Fig. 1, compare lanes 1 and 2). Two bands in the lower-molecular-weight region were specific for plasmid EWD299 (Fig. 1, compare lanes 1 and 2). The smaller of the two migrated with an apparent molecular weight of 11,500, a molecular weight identical to that of the B subunit of cholera toxin (position indicated by arrow, Fig. 1). In addition, the polypeptide was specifically precipitated by antiserum raised to purified cholera toxin, which has antigenic sites in common with the enterotoxin (Fig. 2, lane 2). Therefore, we concluded that this polypeptide was the B subunit.

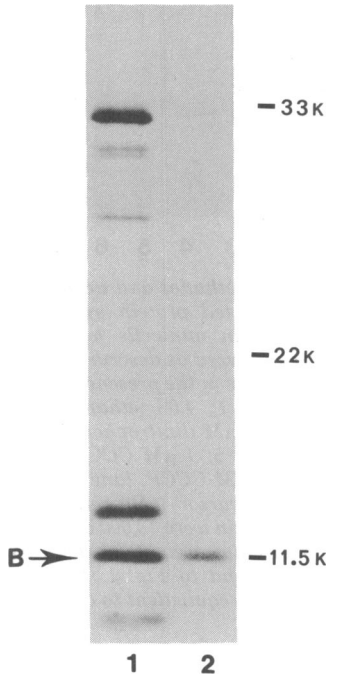

FIG. 2. *Immunoprecipitation of the mature B subunit of enterotoxin. An envelope fraction was isolated from labeled minicells as described in the text and solubilized with 2% (wt/vol) Triton X-100–10 mM EDTA–5 mM Tris-hydrochloride (pH 7.6). Rabbit antiserum raised to purified cholera toxin and nonradioactive cholera toxin (to achieve equivalence) were added to the solubilized sample, which was then incubated for 3 h on ice. The immunoprecipitate was analyzed by sodium dodecyl sulfate-polyacrylamide (15%) gel electrophoresis and autoradiography. Sample slots contained solubilized envelope fraction (lane 1) and immunoprecipitate (lane 2). The arrow indicates the position of the mature B subunit of enterotoxin. The molecular weight markers were as described in the legend to Fig. 1.*

The other polypeptide specific to plasmid EWD299 had an apparent molecular weight of 13,000. Comparison of the peptide patterns generated by partial proteolytic digestion with trypsin (Fig. 3) showed that this polypeptide was related chemically to the B subunit. There were clearly 20 peptides common to the tryptic digests of both proteins, and 3 additional peptides were found in the digest of the larger species. When minicells synthesized protein in the presence of 8.3% ethanol, the species with a molecular weight of 13,000 was made, but no mature subunit was present (Fig. 4, lane 3). A precursor-product relationship between this species and the B subunit was suggested by its apparent conversion to the mature size after removal of the ethanol (Fig. 5). The conversion occurred in the presence of chloramphenicol, indicating that the labeled mature subunit which appeared came from previously synthesized protein. Proteolytic cleavage of the signal sequence to generate the mature protein involves the removal of one of the six methionines (5) found in the fully elongated protein. Thus, the total amount of radioactivity distributed between the positions of precursor protein and mature subunit should decrease as processing proceeds. Figure 5 shows a reasonable correlation between the calculated and observed recoveries of radioactivity. Thus, we concluded that the higher-molecular-weight species was a precursor to the mature B subunit.

In the presence of 3 mM dinitrophenol or 50 μM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), minicells containing plasmid EWD299 synthesized the putative precursor but not the mature B subunit (Fig. 4, lanes 4 through 8). When the compounds were added after protein synthesis was terminated by the addition of chloramphenicol, there was no effect on the pattern of proteins present (data not shown) as compared with a control (Fig. 4, lane 1). Dinitrophenol and CCCP are uncouplers (11), and thus it is likely that the inhibition of processing was directly or indirectly the result of dissipation of proton motive force. Evidence that these compounds have an uncoupling action at the concentrations at which processing was inhibited was obtained by examining the motility of treated cells. Because the energy source for bacterial motility is proton motive force, uncouplers inhibit motility (20). A suspension of highly motile cells maintained normal swimming upon addition of 5 μM CCCP but became nonmotile upon addition of 50 μM CCCP. Dinitrophenol had no effect at 0.5 mM; inhibition was partial

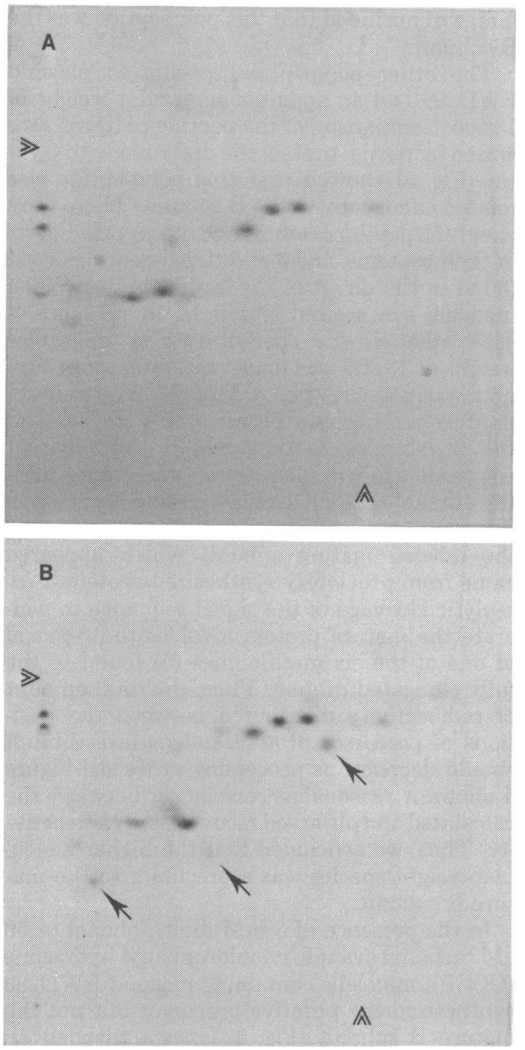

FIG. 3. *Comparison of tryptic digests of the mature B subunit and the putative precursor. ^{35}S-labeled polypeptides synthesized in minicells were isolated and digested with trypsin, and the peptides were analyzed by thin-layer chromatography as described in the text. (A) Tryptic digest of the mature B subunit, corresponding to the band indicated by arrow B in Fig. 4. (B) Tryptic digest of the putative precursor, corresponding to the band indicated by arrow pB in Fig. 4. The arrows indicate additional peptides found in the precursor form. Chromatography in the first dimension was from bottom to top, and that in the second dimension was from left to right. The position of the double arrowheads indicates an R_f value of 0.5. The peptides which are visible in (B) but not in (A) and which are not indicated by arrows are also present in (A) but do not show in the reproduction of the autoradiogram.*

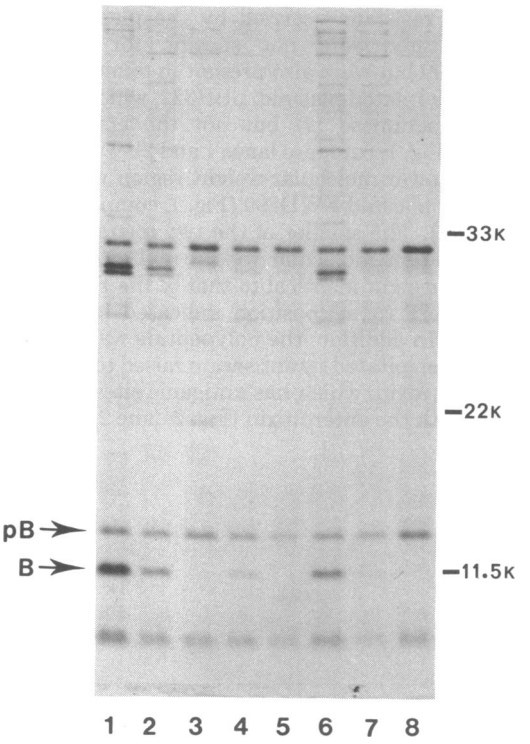

FIG. 4. *Effect of ethanol and uncouplers on plasmid EWD299-directed protein synthesis. Polypeptides synthesized in minicells harboring plasmid EWD299 were analyzed as described in the legend to Fig. 1. Synthesis was in the presence of the following: no addition, lane 1; 4.0% ethanol, lane 2; 8.3% ethanol, lane 3; 1 mM dinitrophenol, lane 4; 3 mM dinitrophenol, lane 5; 1 μM CCCP, lane 6; 10 μM CCCP, lane 7; 50 μM CCCP, lane 8. The molecular weights of the precursor (pB) and the mature (B) subunit of enterotoxin were 13,000 and 11,500, respectively. The molecular weight markers were as described in the legend to Fig. 1. Each sample slot contained minicells equivalent to an OD of 0.01.*

at 1.0 mM and was complete at 10 mM.

The possibility that ethanol could dissipate the electrochemical membrane potential at the concentration at which it inhibited processing was investigated by determining the effect of ethanol on motility. At an ethanol concentration of 4.0% there was no effect, whereas motility was abolished at an ethanol concentration of 8.3%. Thus, we suggest that ethanol inhibited processing by dissipating proton motive force, as did CCCP and dinitrophenol.

For determining the cellular location of the B subunit and its precursor, minicells were labeled in the presence and absence of ethanol and subsequently separated from the incubation medium. The cells were fractionated into periplasm, cytoplasm, and total envelope. When the medium and cell fractions were examined for the presence of the two forms of the B subunit, both the precursor and the mature form were found

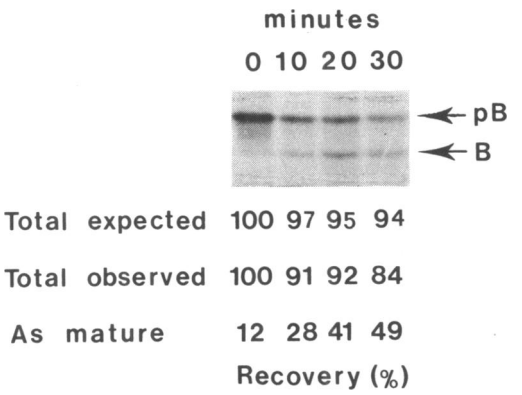

FIG. 5. *Conversion of the precursor to the mature B subunit. Polypeptides synthesized in minicells were labeled in the presence of 8.3% ethanol for 15 min, and then chloramphenicol was added to a final concentration of 100 μg/ml. The ethanol was removed by two washings, followed by a further incubation at 37°C. During this incubation, samples were removed at the times indicated, precipitated with 10% trichloroacetic acid, and analyzed as described in the text. The positions of the precursor (pB) and the mature (B) subunit of enterotoxin are indicated by the arrows. The amount of protein in each band of enterotoxin was quantitated by densitometric scanning of the autoradiogram and quantitation of the area under the peaks. Each sample slot contained minicells equivalent to an OD of 0.01.*

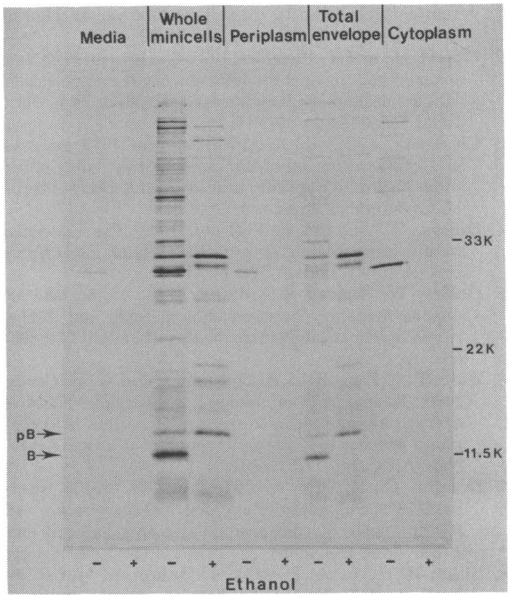

FIG. 6. *Localization of polypeptides related to the B subunit. Minicells were labeled in the presence and absence of 8.3% ethanol and separated into the indicated cell fractions as described in the text. Samples were applied to a polyacrylamide gel in relative amounts corresponding to an OD of minicells of 0.02, with the amount of whole cells corresponding to a half quantity (an OD of 0.01). The arrows indicate the positions of the precursor (pB) and the mature B subunit (B). The molecular weight markers were as described in the legend to Fig. 1.*

predominantly in the cell envelope fractions (Fig. 6).

DISCUSSION

Minicells harboring plasmid EWD299, which encodes enterotoxin LT, synthesize both the A and B subunits, as demonstrated by Dallas and Falkow (4). Here we discuss data concerning only the B subunit, as the presence of large amounts of β-lactamase, which has an apparent molecular weight very similar to that of the A subunit, makes interpretation of the data concerning the A subunit difficult.

The plasmid-containing minicells synthesize two polypeptides related to the B subunit; one migrates in sodium dodecyl sulfate-polyacrylamide gels at the same position as the authentic B subunit from cholera toxin. Cholera toxin B subunit has antigenic determinants in common with the B subunit from the heat-labile enterotoxin, thus allowing definitive identification of this polypeptide as the B subunit by specific immunoprecipitation. The other polypeptide is larger than the mature subunit by mass of 1,500 daltons. Comparison of tryptic peptide patterns shows that although the two species are closely related, the larger polypeptide contains peptides which are not present in the mature form. A precursor-product relationship is suggested by the conversion of the larger species to the smaller form in the absence of protein synthesis. Thus, the additional peptides are likely to arise from a sequence which is removed by proteolysis to generate the mature form, as in the case of other proteins exported by *E. coli* (16). Direct evidence for the existence of such an extra amino acid sequence in the precursor comes from DNA sequencing studies (5).

The processing which occurs in minicells is inhibited by the presence of ethanol, dinitrophenol, and CCCP at concentrations which also inhibit cell motility. This indicates that energy may be essential for processing of the enterotoxin.

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