Accumulation of a precursor of subunit S1 of pertussis toxin in cell envelopes of *Bordetella pertussis* in response to the membrane perturbant phenethyl alcohol

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Summary. Exponential cultures of *Bordetella pertussis* strain 18334 were treated with the membrane-perturbing agent phenethyl alcohol which, at a concentration of 0.075% v/v, blocked the synthesis of mature subunit S1 of pertussis toxin as revealed by Western blotting. It also caused the accumulation of a precursor, pS1, with an estimated mol. wt of 32×10^3 , that was located in the cytoplasmic membrane. These findings suggested that subunit S1 of pertussis toxin was exported in a signal peptide-dependent manner.

Introduction

Bordetella pertussis, the causative agent of whooping cough in man, produces at least four distinct toxins—heat-labile toxin, lipopolysaccharide endotoxin, tracheal cytotoxin and pertussis toxin (PT) (Wardlaw and Parton, 1982). PT, the major virulence determinant responsible for some of the clinical manifestations of whooping cough (Wardlaw and Parton, 1982), consists of five dissimilar subunits S1–5 with apparent mol. wts (10^3) of 28, 23, 22, 11 and 9, respectively (Tamura *et al.*, 1982). The largest subunit, S1, possesses ADP ribosyl-transferase activity (Tamura *et al.*, 1982).

Pertussis toxin is found as both cell-free and cell-associated protein in growing cultures (Perera *et al.*, 1986a) and culture supernates of *B. pertussis* have been used widely for purification of PT (Yajima *et al.*, 1978; Sekura *et al.*, 1983). Thus, PT is a protein synthesised intracellularly and released into the culture medium by some unknown mechanism. Extracellular PT does not result from cellular lysis (Perera *et al.*, 1986a).

The mechanisms of export of proteins in eukaryotic cells have been studied extensively. Precursor forms of these proteins are assembled on polysomes associated with the cytoplasmic membrane and usually contain a signal peptide (also referred to as a leader sequence) at the amino-terminus of the molecule (Blobel and Dobberstein, 1975). Such

proteins are exported by either co-translational or post-translational translocation (Silhavy *et al.*, 1983). These translocational events also occur in prokaryotes (Inouye and Halegoua, 1980; Silhavy *et al.*, 1983; Pugsley and Schwartz, 1985). Most exported proteins of bacteria are translocated across the plasma membrane in a signal peptide-dependent and energy-dependent manner. As a result of this energy dependence, the export process is sensitive to membrane perturbants and uncouplers that depolarise the membrane and dissipate the proton-motive force (Pugsley and Schwartz, 1985). This effect leads to blockage of export and accumulation of the precursor at the site of synthesis. The view that the cytoplasmic membrane is the site of synthesis of bacterial proteins destined for export has been substantiated by studies on proteins—including penicillinase, enterotoxins A, B and C, α -lysin—of *Staphylococcus aureus* (Yoshikawa *et al.*, 1974; Altenbern, 1977; Berkeley *et al.*, 1978) and outer-membrane and periplasmic proteins of *Escherichia coli* (Halegoua and Inouye, 1979; Pagès and Lazdunski, 1981).

The second method of protein export does not require the presence of a signal sequence. For example, colicins and some of the cytoplasmic-membrane proteins of *E. coli* appear to lack an amino-terminal signal sequence and are believed to be targeted in a signal peptide-independent manner (Pugsley and Schwartz, 1985).

Our objectives were to investigate the effect of the membrane-perturbing agent phenethyl alcohol

(PEA) on the synthesis of subunit S1 of PT and to detect, and locate the position in the cell envelope of, any precursor of subunit S1.

Materials and methods

Organism and growth conditions

B. pertussis strain 18334 (Connaught Laboratories) phase 1 was initially seeded on Bordet-Gengou Agar (Gibco) for 48 h at 37°C and growth was harvested and resuspended in casein-hydrolysate acid (Peptone No. 5; Gibco) 1% w/v. This cell suspension was used to inoculate the liquid medium of Imaizumi *et al.* (1983) without methylated- β -cyclodextrin. Cultures (100 ml) in 250-ml Erlenmeyer flasks were shaken (150 cycles/min) at 37°C for 20 h by which time extinction at 600 nm was 0.2–0.3. Phenethyl alcohol (British Drug Houses Ltd) was added to each culture at final concentrations (% v/v) of 0.01, 0.05, 0.075, 0.1 and 0.2; and incubation was continued. Control cultures without PEA were also set up. Growth was monitored from the extinction at 600 nm of each culture at hourly intervals for 8 h.

In some experiments, PEA was added to final concentrations (% v/v) of 0.05 or 0.075 and cultures were incubated for 2, 4 and 6 h. In a separate experiment, fresh medium was inoculated with a 5% v/v inoculum of organisms grown in the presence of PEA (0.075% v/v) for 4 h and incubated for 24 h. After incubation, all cultures were killed by the addition of a mixture of inhibitors (*vide infra*) and kept on ice at 0°C until centrifugation.

The inhibitors, obtained from Sigma, were: chloramphenicol (to inhibit protein synthesis); carbonyl cyanide-chlorophenyl-hydrazone (to block further export of proteins); and phenylmethanesulphonyl fluoride (PMSF) (to inhibit proteolysis); their final concentrations in the culture medium were 600 μ M, 100 μ M and 200 μ M, respectively. The inhibitors were dissolved initially in isopropanol 30% v/v and stored at –20°C as one stock solution.

Preparation of crude cell envelopes

Bacteria from 100-ml cultures were harvested by centrifugation at 10 000 *g* for 30 min and the cell pellet resuspended in 10 ml of 0.01 M sodium-phosphate buffer (pH 7.4) containing 0.15 M NaCl (PBS). After addition of DNAase (Sigma) to 100 μ g/ml and MgSO₄ to 20 mM, bacteria were disrupted by one passage through a French press (Aminco, SLM Instruments, Urbana, IL, USA) at 2000 bar. Envelope fragments were recovered by centrifugation at 20 000 *g* for 30 min at 4°C, washed once with, and stored frozen in, PBS containing 200 μ M PMSF.

Membranes for fractionation by sucrose density-gradient centrifugation were prepared, as follows. Bacteria were disrupted by three passages through a French press (*vide supra*) and centrifuged at 20 000 *g* for 30 min at 4°C. The pellet, containing large membrane debris, was discarded and the supernate ultracentrifuged at

100 000 *g* for 1 h at 4°C to sediment small envelope fragments. This pellet was resuspended in 0.05 M Tris-HCl buffer (pH 7.5) containing 10 mM EDTA (disodium salt; Sigma) (Tris-EDTA), egg-white lysozyme (Sigma) was added to a final concentration of 80 μ g/ml and the suspension incubated for 1 h at 37°C. The membranes were finally washed twice by centrifugation in Tris-EDTA buffer and stored at 4°C in the same buffer.

Fractionation of membranes by isopycnic sucrose density-gradient centrifugation

This was done as described by Imagawa *et al.* (1979). Crude membranes were layered over discontinuous gradients of sucrose at 40, 45, 50 and 55% w/v in Tris-EDTA buffer. The gradients were ultracentrifuged at 90 000 *g* for 24 h at 4°C. The top and bottom light-scattering bands were collected, diluted 10-fold in Tris-EDTA buffer and concentrated by ultracentrifugation at 100 000 *g* for 1 h at 4°C. The pellets were resuspended in Tris-EDTA buffer and stored frozen at –20°C.

Purified PT

PT was purified as described previously (Perera *et al.*, 1985).

Sodium-dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) and Western-blot analysis

Membrane preparations and purified PT were analysed by SDS-PAGE and Western blotting with mouse-monoclonal antibodies to the S1 subunit of PT as described previously (Perera *et al.*, 1985 and 1986b). Protein standards (Perera *et al.*, 1985) were electrophoretically transferred to nitrocellulose paper and stained with amido black (Sigma) 0.05% w/v in ethanol 50% v/v and acetic acid 10% v/v.

Negative staining for electronmicroscopy

Membranes were negatively stained with aqueous ammonium molybdate 2% w/v and examined in a Phillips EM300 transmission electronmicroscope operated at 60 kV.

Protein assay

Protein was estimated by a modified Lowry method (Peterson, 1977).

Results

Effect of PEA on growth

The rate of growth of *B. pertussis* was not appreciably affected by PEA 0.01 or 0.05% v/v but was progressively inhibited at concentrations above 0.075% (results not shown). Accordingly, the effect

of PEA 0.05 and 0.075% v/v on the synthesis of subunit S1 of PT was examined.

Western-blot analysis of crude cell envelopes

When cell envelopes obtained from cells grown without PEA were examined by Western blotting with S1-specific monoclonal antibodies, two peptides were detected: the S1 subunit of mol. wt 28×10^3 and a second peptide (pS1) of mol. wt 32×10^3 (fig. 1a, lane 2). When bacteria were grown in the presence of PEA 0.05% v/v, the relative amount of pS1 increased as judged by the intensity of the labelling (fig. 1a, lanes 3 and 4). In experiments with PEA 0.075% v/v, subunit S1 was not detected; instead, the peptide pS1 was labelled (fig. 1b, lanes 1, 2 and 3) and accumulated in the cell envelopes during the 6-h period of observation. This effect of PEA was reversible because the background levels of both pS1 and mature S1 normally present in control cultures were restored following transfer of PEA-treated bacteria to fresh, PEA-free medium (results not shown).

Sucrose density-gradient centrifugation

Crude cell envelopes, obtained from bacteria grown in the presence of PEA 0.075% v/v for 4 h,

were fractionated by isopycnic sucrose density-gradient centrifugation. Most of the cell debris that appeared after disruption could not be fractionated because large aggregates formed. Thus, small particulate vesicles only were fractionated and two fractions, corresponding to buoyant densities of 1.18 and 1.22 g/cm³, were obtained. Because these buoyant densities were similar to those described for the membranes of *Salmonella typhimurium* (Osborn *et al.*, 1972), the fractions were regarded as being cytoplasmic and outer membranes, respectively.

Analysis of fractionated membranes

The SDS-PAGE patterns of outer membranes and cytoplasmic membranes are shown in fig. 2 (lanes 1 and 2, respectively). The cytoplasmic-membrane fraction contained some proteins (lane 2) not present in the outer-membrane fraction. As well as qualitative differences, quantitative differences in the two protein profiles were also obvious.

Examination of the membranes by electron microscopy revealed that the cytoplasmic membranes were relatively homogeneous, small fragments with a granular surface (fig. 3a). This appearance was in contrast to the larger, smoother and more hetero-

Fig. 1. Western-blot analysis with monoclonal antibodies to subunit S1. (a) Lane 1, purified PT 10 μ g; 2, normal cell envelopes 80 μ g; 3 and 4, cell envelopes (80 μ g) of bacteria grown in the presence of PEA 0.05% v/v for 2 and 4 h, respectively. (b) Lanes 1–3, cell envelopes (100 μ g) of bacteria grown in the presence of PEA 0.075% v/v for 2, 4 and 6 h, respectively; 4, purified PT 10 μ g. Positions of pS1 and S1 are shown.

Fig. 2. The SDS-PAGE pattern of membranes. Lane 1, outer membranes 60 µg; 2, inner membranes 60 µg; 3, protein standards with mol. wts (10^3) indicated on the right. Arrows show peptides absent from outer membranes. The gel (acrylamide 12% w/v) was stained with Coomassie brilliant blue 0.025% w/v.

geneous particles present in the outer-membrane preparation (fig. 3b).

Western-blot analysis of membranes grown in the presence of PEA revealed that pS1 was located in the cytoplasmic membranes but not in the outer-membrane fractions (fig. 4, lanes 2 and 1, respectively).

Discussion

Previous studies have shown that PT was released into the culture supernate during mid-exponential

Fig. 3. Negatively stained preparations of (a) cytoplasmic membranes and (b) outer membranes. Bars = 100 nm.

growth of *B. pertussis* and that the kinetics of toxin release closely paralleled that of growth (Perera *et al.*, 1986a). *B. pertussis* grows slowly and has a mean generation-time of 6 h and an initial lag-phase of c. 11 h in the conditions used (unpublished results). Thus, it was assumed that the intracellular turnover of PT would be slow compared with the rapid synthesis and release of proteins such as enterotoxin LT of *E. coli* (Hofstra *et al.*, 1984) or exotoxin A of *Pseudomonas aeruginosa* (Lory *et al.*, 1983). Cell-envelope fractions from PEA-treated cells were examined, therefore, for the presence of a precursor

Fig. 4. Western-blot analysis with monoclonal antibodies to subunit S1. Lane 1, outer membranes 80 μ g; 2, cytoplasmic membranes 80 μ g; 3, purified PT 10 μ g. Positions of pS1 and S1 are shown.

of S1 subunit of PT at 2-h intervals for 6 h. PEA, a membrane-perturbing agent that depolarises membranes by increasing the permeability of cell membranes to protons (Silver and Wendt, 1967), was added to the growth medium to block processing of precursors to mature proteins.

When membranes from bacteria grown in the presence of concentrations of PEA that partially blocked processing of precursor proteins to mature proteins were examined by Western blotting, some mature S1 peptide was detected, but the main peptide labelled was one of mol. wt 32×10^3 , which co-labelled with mature S1. This finding suggested that the two peptides were antigenically related. The peptide of mol. wt 32×10^3 , therefore, was considered to be the precursor (pS1) of subunit S1. On the other hand, with concentrations of PEA $\geq 0.075\%$ that completely blocked processing, pS1 only accumulated in the cell envelopes. The observation that the accumulated pool of pS1 could be chased to mature S1 with the transfer of bacteria to fresh medium further substantiated the view that the two peptides were related. The finding that

there was a critical concentration of PEA required to block completely the processing was similar to the concentration effects shown for the action of uncouplers such as 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenyl-hydrazone on mini-cells of *E. coli* strain K12 (Palva *et al.*, 1981). The difference of c. 4 in the mol. wts (10^3) of pS1 and S1 probably represents the signal sequence. Thus, subunit S1 appears to be exported in a signal peptide-dependent manner. This finding is substantiated by the recent report by Nicosia *et al.* (1986) who have demonstrated signal peptides in all five subunits of PT. The length of the signal sequence of the S1 subunit was reported to be 34 amino-acid residues (the mol. wt of which can be estimated to be c. 4.4×10^3).

In an attempt to determine the location of pS1, cell envelopes were fractionated into cytoplasmic and outer membranes by density-gradient centrifugation. The outer membranes obtained were similar to the extracellular outer-membrane projections of *B. pertussis* reported by Morse and Morse (1970) and resembled outer-membrane vesicles of gram-negative bacteria reported by others (Work *et al.*, 1966; Perera *et al.*, 1982). The localisation of pS1 in the cytoplasmic membranes conformed to the concept that most bacterial proteins destined for either the periplasm or the outer membrane are synthesised as precursors in the cytoplasmic membrane (Randall *et al.*, 1978; Hall and Silhavy, 1981; Pagès and Lazdunski, 1981). An exception seems to be the synthesis of exotoxin A of *P. aeruginosa*, a precursor of which accumulated in the outer membrane when the membranes were perturbed with ethanol 10% v/v (Lory *et al.*, 1983). Precursor was not detected in the absence of perturbant and it has been suggested that exotoxin A is rapidly exported into the outer membrane *via* Bayer's junctions and co-translationally secreted into the medium. Since some pS1 was demonstrated in the absence of perturbant (fig. 1a, lane 2), the export of subunit S1 appeared to be less rapid than that of exotoxin A of *P. aeruginosa*.

This study has demonstrated the existence of a precursor form of S1 subunit of PT in the cytoplasmic membrane. Little, however, is known about the mechanism whereby PT is released into the culture medium. It is not known whether the five different subunits of PT are assembled in the periplasm, like the enterotoxin LT of *E. coli* (Hirst *et al.*, 1983 and 1984; Hofstra *et al.*, 1984), or in the outer membrane. However, unlike enterotoxin LT of *E. coli*, there does not appear to be a periplasmic pool of toxin (unpublished results) suggesting that the mature subunits are transported directly to the

outer membrane where they may accumulate before release into the culture medium. The localisation of the histamine-sensitising activity (attributed to PT by Wardlaw and Parton, 1982) mainly in the outer membrane of *B. pertussis* strain Tohama (Imagawa *et al.*, 1979) may substantiate this view. Further work is in progress to determine how other subunits

of PT are synthesised and to elucidate the way in which PT is released into the culture medium.

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