*Proc. Natl. Acad. Sci. USA*
Vol. 84, pp. 7418–7422, November 1987
Biochemistry

# Conformation of protein secreted across bacterial outer membranes: A study of enterotoxin translocation from *Vibrio cholerae*

(cholera toxin/*Escherichia coli* enterotoxin/protein export/oligomeric proteins)

TIMOTHY R. HIRST*† AND JAN HOLMGREN‡

*Department of Genetics, University of Leicester, Leicester, LE1 7RH, Great Britain; and ‡Department of Medical Microbiology, University of Göteborg, Göteborg, S-413 46, Sweden

*Communicated by Sune Bergström, June 30, 1987*

**ABSTRACT** **The secretion of enterotoxin by *Vibrio cholerae* is punctuated by the transient entry of the toxin subunits into the periplasm. In this paper, we show that the subunits oligomerize into an assembled holotoxin within the periplasm prior to their secretion across the outer membrane. The rate of toxin assembly was studied by pulse-labeling cells with [$^{35}$S]-methionine and then monitoring the turnover of radiolabeled subunits as they assembled within the periplasm. The subunits entered the periplasm as monomers and assembled into oligomers with a half-time of ≈1 min. Since assembly was a rapid event compared to the rate of toxin efflux from the periplasm, which had a half-time of ≈13 min, we conclude that all of the subunits that pass through the periplasm assemble before they traverse the outer membrane. The average concentration of subunit monomers and assembled holotoxin within the periplasm was calculated to be ≈20 and ≈260 μg/ml, respectively. This indicates that the periplasm is a suitably concentrated milieu where spontaneous toxin assembly can occur. Our findings suggest that protein movement across bacterial outer membranes, in apparent contrast to export across other biological membranes, involves translocation of polypeptides that have already folded into tertiary and even quaternary conformations.**

The movement of proteins across membranes is a fundamental characteristic of living cells. It plays an obligatory role in the replenishment and growth of membrane systems and cell envelopes, in organelle biogenesis, and in the secretion of, for example, protein hormones, immunoglobulins, and extracellular enzymes (for reviews, see refs. 1–4). A number of recent reports on protein import into mitochondria, and export of proteins across the cytoplasmic membrane of *Escherichia coli* have implied that nonnative, disordered, or unfolded protein states are needed to ensure successful translocation (5, 6). In addition, several research groups, studying the secretion of proteins into the endoplasmic reticulum (ER) and from the ER to the Golgi complex in eukaryotic cells, have found that protein folding, and in particular subunit oligomerization, are intraluminal processes occurring after translocation across the ER membrane (7–10). Thus, proteins that are translocated across ER and mitochondrial and bacterial cytoplasmic membranes would seem to share a common feature of initiating their export as unfolded polypeptides that develop native tertiary or quaternary conformations only after translocation has occurred (5–12).

Gram-negative bacteria also have, in addition to their capacity to export proteins through cytoplasmic membranes, an ability to secrete selected proteins across their outer membranes (for review, see ref. 13). The mechanisms that facilitate or drive this are unknown. One possibility is that protein secretion across outer membranes is intimately linked to initiation and translocation of proteins across cytoplasmic membranes. A secreted protein therefore might not adopt a native conformation until having successfully translocated beyond the outer membrane. Alternatively, it might first be exported across the cytoplasmic membrane, fold into a native conformation, and then either be translocated in that state or be unfolded to enable passage across the outer membrane.

Investigation of this problem necessitates an amenable system that is open to experimental analysis. We therefore chose to use heat-labile enterotoxin, which is a well-defined diarrheagenic protein that consists of an A subunit (28 kDa) and five identical B subunits (12 kDa each) (14–21). The toxin, which is structurally and functionally related to cholera toxin, is normally produced by enterotoxinogenic strains of *E. coli* (19, 20, 22), but it has also been studied experimentally in *Vibrio cholerae* where it exhibits the property of being efficiently secreted into the extracellular milieu (23–25). In this paper, we present data showing that the heat-labile enterotoxin subunits assemble into a stable quaternary complex before they translocate across the outer membrane of *V. cholerae*. This implies that protein movement across bacterial outer membranes may involve translocation of a folded or even a fully assembled protein by mechanisms that differ fundamentally from polypeptide export across other biological membranes such as the cytoplasmic membrane of bacteria or the envelopes of the ER and other cell organelles.

## MATERIALS AND METHODS

**Bacterial Strains and Plasmids.** The *V. cholerae* strain used was TRH7000, a thymine auxotroph of the nontoxinogenic strain JBK70, from which the cholera toxin genes have been deleted from the chromosome by site-directed mutagenesis (24, 26). Plasmid pWD600 is a derivative of pBR322 that contains the cistrons for heat-labile enterotoxin cloned from an enterotoxinogenic *E. coli* strain of human origin (19), and was mobilized into *V. cholerae* TRH7000 as described (24).

**Bacterial Growth and Radiolabeling Conditions.** *V. cholerae* TRH7000 was inoculated from frozen stocks onto 1.5% agar plates containing a defined medium of supplemented M9 salts (25, 27). After incubation overnight at 37°C, the cells were inoculated into 20–50 ml of defined medium and the cells were cultured at 37°C on a rotary shaker. When the cell density reached an absorbance at 600 nm of 0.7 (equivalent to $8 \times 10^8$ bacteria per ml), 1–5 ml of culture was radioactively pulse-labeled with [$^{35}$S]methionine (200 μCi/ml; 1000 Ci/mmol; 1 Ci = 37 GBq; from Amersham) for 0.5 min, followed by the addition of a chase of 1 mM L-methionine. Aliquots

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: ER, endoplasmic reticulum.

†To whom reprint requests should be addressed.

(0.5 ml) were sampled at various times into glass vials held on ice and then transferred into ice-cold Eppendorf tubes and centrifuged at 8000 × *g* for 2 min. Medium was immediately removed from the centrifuged samples, and the cell-pellets were kept on ice for subsequent fractionation.

**Cell Fractionation.** The periplasm of *V. cholerae* TRH7000 and TRH7000 (pWD600) was obtained by treatment of the cells with polymixin B as described (25).

**Toxin Assays.** The concentration of heat-labile enterotoxin in medium and periplasmic fractions was determined by a GM1 ELISA, which has been described (28), using monoclonal antibodies against the A subunit (CT-17) or B subunit (LT-39) for detection of bound toxin.

***In Vitro* Reassembly of Cholera Toxin.** A solution of pure cholera toxin (1.0 mg/ml) (List Biologicals, Campbell, CA) was dispensed into Eppendorf tubes and diluted with phosphate-buffered saline (PBS) (pH 7.2) to give a range of toxin concentrations from ≈1 to 400 μg/ml. To each tube, 1.0 mg of bovine serum albumin per ml (Sigma) was added, followed by the addition of hydrochloric acid to a final concentration 20 mM. After 20 min at 25°C, the mixtures were neutralized by the addition of sodium hydroxide and mixed in a Vortex. After a further 60 min, all samples were diluted with PBS/0.1% bovine serum albumin to give a final toxin concentration of 1.0 μg/ml, and then the samples were assayed by GM1 ELISA. The proportion of toxin detectable in the ELISA was determined by direct comparison with a cholera toxin control sample that had been treated as described above, except that distilled water instead of HCl and NaOH was added.

**Determination of Periplasmic Volume.** The volumes of a cell and its compartments were calculated using the method of Stock *et al.* (29).

**Other Techniques.** Electrophoresis and autoradiography were carried out as described (25). Autoradiograms were quantified by densitometric scanning with a Shimadzu CA-910 chromoscan scanner. Equations for calculation of toxin turnover rates and apparent first-order rate constants have been described (25).

## RESULTS

**Location of Enterotoxin Assembly.** The stability of enterotoxin B subunit pentamers in the presence of the ionic detergent $NaDodSO_4$ was utilized to investigate whether subunit assembly occurs prior to toxin secretion through the outer membrane of *V. cholerae*. Assembled B subunits can be distinguished by their electrophoretic mobility on $NaDodSO_4$/polyacrylamide gels. This is exemplified in Fig. 1, where medium from a culture of exponentially growing *V. cholerae* strain TRH7000 (pWD600) was isolated, mixed with $NaDodSO_4$-containing sample buffer, divided into two equal portions, and either boiled or kept at room temperature, and then subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. In the unheated sample, enterotoxin B subunit oligomers with an apparent molecular mass of 45 kDa were present (lane 2). However, upon boiling they were found to dissociate into monomers, which migrated with an approximate molecular mass of 12 kDa (lane 1). This technique therefore permits the oligomeric status of B subunits in any given sample to be determined.

In a previous report (25), it was shown that enterotoxin B subunits are exported into the periplasm, where they transiently reside, before being secreted across the outer membrane. We therefore chose to investigate whether the B subunits that pass through the periplasm attain an oligomeric conformation before they translocate across the outer membrane.

*V. cholerae* TRH7000 (pWD600) was radioactively pulse-labeled and chased and then at times ranging from 0.25 to 30 min samples were removed and rapidly chilled. Periplasmic fractions were obtained by treating the cells with polymixin B, using conditions that ensured the release of >90% of the periplasmic and <1% of the cytoplasmic proteins. Periplasmic polypeptides were then analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 2).

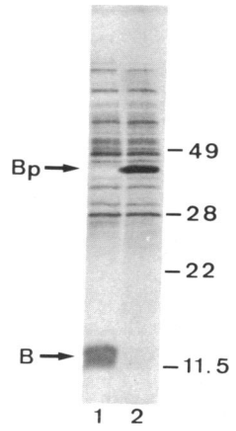


FIG. 1. Stability of B-subunit pentamers in 0.1% $NaDodSO_4$. Medium from a culture of *V. cholerae* TRH7000 (pWD600) was mixed with electrophoresis sample buffer that contained 0.1% $NaDodSO_4$ and was either boiled for 5 min or kept at room temperature prior to subjecting the samples to $NaDodSO_4$/polyacrylamide gel electrophoresis. Lanes: 1, boiled sample; 2, unheated sample. Migration of dissociated B subunits (B) and oligomeric B pentamers ($B_p$) are indicated by the arrows. Numbers on right represent kDa.

When the samples were boiled prior to electrophoresis (Fig. 2, lanes 1–9), the total quantity of B subunits in each fraction migrated as 12-kDa monomers. During the chase, the quantity of labeled B subunits migrating at that position decreased (lanes 1–9) because of their efflux from the periplasm into the extracellular culture medium (25). Measurements of the quantity of labeled B subunits in the periplasm during the first 2 min of the chase (lanes 1–4), performed by densitometric scanning of the autoradiogram, revealed a decrease of ≈12%.

When, however, the same fractions were unheated before electrophoresis, a labeled polypeptide was found that also migrated with an apparent molecular mass of 12 kDa but that rapidly decreased by 76% during the first 2 min of the chase (Fig. 2, lanes 10–13). Experimental controls using a strain of *V. cholerae* TRH7000 that did not harbor an enterotoxin-encoding plasmid, demonstrated that the 12-kDa polypeptide is not endogenous to the periplasm but is plasmid-encoded (data not shown; see ref. 25). We conclude that the 12-kDa polypeptide represents B subunit monomers and that its presence in unheated periplasmic fractions establishes that enterotoxin B subunits are initially released as monomers into the periplasm of *V. cholerae*.

A comparison of the boiled and unheated samples identified a single new protein species in the unheated fractions that had an apparent molecular mass of 45 kDa (Fig. 2, compare lanes 1–9 with lanes 10–13), identical in molecular mass to the extracellular B-subunit pentamer shown in Fig. 1.

Measurements of the quantity of monomers in unheated samples revealed that their rate of decrease followed a simple exponential (first order) decay (Fig. 2). The half-time for turnover of such monomers was calculated to be ≈1 min.

The amount of labeled pentamers in the periplasm was calculated by subtracting the quantity of monomers from the total quantity of labeled B subunits in the boiled fractions. At the earliest chase time of 0.25 min, ≈45% of the B subunits were pentamers and this had risen to ≈83% after 2 min of chase. We therefore conclude (*i*) that the B subunits enter the

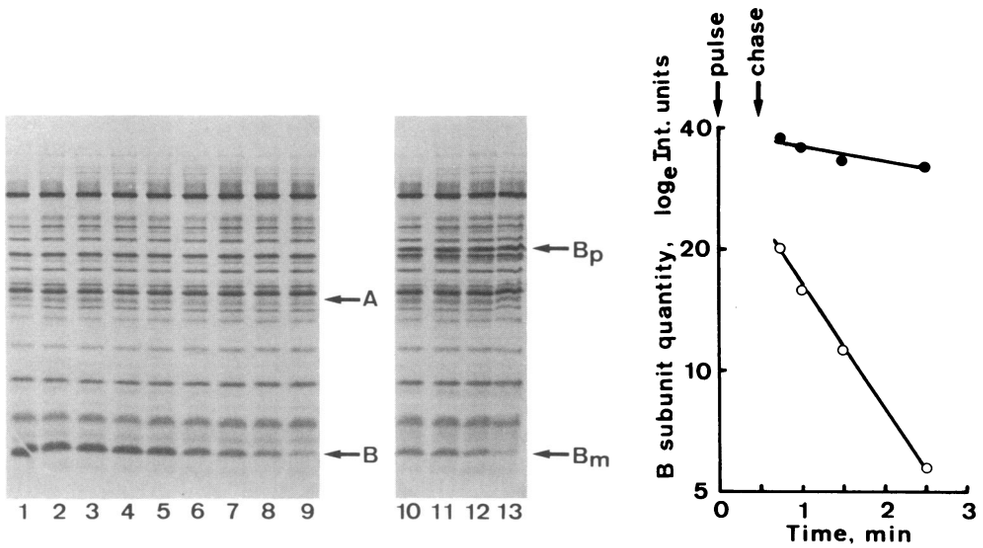


FIG. 2. Kinetics of enterotoxin assembly and secretion. Periplasmic fractions were obtained from a culture of *V. cholerae* TRH7000 (pWD600) that had been radioactively pulse-labeled with [$^{35}$S]methionine for 0.5 min and chased for different lengths of time. Equivalent amounts of each fraction were mixed with $NaDodSO_4$-containing sample buffer and either boiled (lanes 1–9) or kept at 25°C (lanes 10–13) before being analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. (*Left*) Reproductions of the autoradiograms are shown in which the times after the initiation of the pulse were as follows: 0.75 min (lanes 1 and 10), 1 min (lanes 2 and 11), 1.5 min (lanes 3 and 12), 2.5 min (lanes 4 and 13), 5 min (lane 5), 10 min (lane 6), 15 min (lane 7), 20 min (lane 8), 30 min (lane 9). The migration positions of heat-labile enterotoxin A- and B-subunit standards (A and B) and of the B-subunit monomers ($B_m$) and assembled pentamers ($B_p$) are indicated by arrows. (*Right*) The quantity of labeled B subunits migrating in the B-subunit position (B) in lanes 1–4 (●) and in the B monomer position ($B_m$) in lanes 10–13 (○) were determined by densitometric scanning of the autoradiogram. The amounts given are in arbitrary integration (Int.) units and are plotted against the time at which the samples were taken after the initiation of the pulse.

periplasm as monomers, (*ii*) that they rapidly assemble, and (*iii*) that they attain a $NaDodSO_4$-resistant stable quaternary structure before their efflux across the outer membrane.

Confirmation that B subunits enter the extracellular milieu as assembled oligomers was obtained by analyzing media taken throughout the pulse–chase. All labeled B subunits in the extracellular fractions were found to migrate as 45-kDa oligomers (data not shown); there was no evidence for a transient pool of extracellular monomers.

The above assessment of enterotoxin oligomeric status, using B-subunit stability in $NaDodSO_4$, limits the study to the B subunits alone since B-subunit–A-subunit interaction is destabilized by $NaDodSO_4$. Therefore, we chose to use a GM1-based ELISA to investigate whether periplasmic B subunits are associated with A subunits. The assay depends on the ability of B subunits to bind to GM1 ganglioside (15, 28) and on the availability of monospecific antibodies that recognize the A subunit (30). Analyses of periplasmic fractions using a GM1 ELISA revealed the presence of GM1-bound A subunits that could be detected with an anti-A-subunit monoclonal antibody (Table 1). Therefore, since A subunits bind to GM1 only via their intimate stable association to B subunits, we conclude that the periplasm of *V. cholerae* contains fully assembled holotoxin.

**Concentration Dependence of Subunit Oligomerization.** A rationale for enterotoxin assembly taking place in the periplasm of *V. cholerae* before translocation across the outer membrane would be that the periplasm represents a concentrated milieu in comparison to the environment beyond the outer membrane and that such a milieu, with its correspondingly high concentration of enterotoxin subunits, is necessary for native holotoxin to form.

Evidence that toxin assembly is indeed dependent on subunit concentration was obtained by analyzing the *in vitro* dissociation and reassociation of the subunits of purified cholera toxin (Fig. 3). A GM1 ELISA was used for measuring the extent of cholera toxin subunit reassociation. The assay

Table 1. GM1 ELISA analysis of periplasmic enterotoxin from *V. cholerae* TRH7000 (pWD600)

| Monoclonal antibody | Antibody specificity | Toxin concentration, ng/ml ± SEM |
|---|---|---|
| LT-39 | B subunit | 61 ± 14 |
| CT-17 | A subunit | 88 ± 4 |

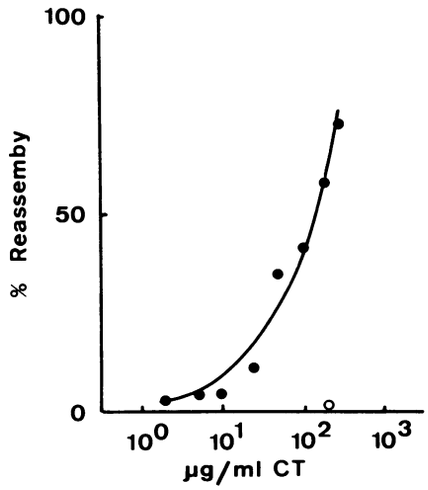


FIG. 3. Concentration-dependent reassembly of cholera toxin (CT) *in vitro*. Different concentrations of cholera toxin were acidified with HCl and then neutralized with NaOH (●) as described. The concentration of toxin subunits at neutralization is shown along the abscissa. Percentage subunit reassembly was calculated from the proportion of neutralized toxin detected with monoclonal antibody LT-39 using a GM1 ELISA. The efficacy of HCl in dissociating toxin into subunit monomers was verified in an experiment in which cholera toxin (200 μg/ml) was treated with HCl, diluted with PBS to 1.0 μg/ml, and then assayed for the percent that was able to react with LT-39 (○).

was based on the finding that only B pentamers but not B-subunit monomers reacted with monoclonal antibody LT-39 that was used to detect bound toxin. When pure cholera toxin was acidified with hydrochloric acid and then neutralized, the amount of toxin subunits detectable in the GM1 ELISA was critically dependent on the concentration of subunits at the moment of neutralization. The same results were obtained when an anti-A-subunit monoclonal antibody (CT-17) was used for the GM1 ELISA. We therefore conclude that the formation of assembled holotoxin *in vitro* is critically dependent on subunit concentration.

**Periplasmic Toxin Concentration.** The concentration of assembled enterotoxin in the periplasm of *V. cholerae* TRH7000 (pWD600) was determined to be ≈260 μg/ml (Table 2). This was calculated from estimates of the volume of the periplasm and the amount (in μg) of periplasmic toxin. The volume of periplasm in a suspension of $8 \times 10^8$ bacteria was determined to be 0.29 μl and the volume of all aqueous cell compartments (i.e., the cytoplasm and periplasm) was calculated to occupy 2.64 μl. Therefore, an "average cell" within the suspension had a periplasmic volume of $3.6 \times 10^{-10}$ μl. The total amount of periplasmic toxin in the suspension was determined by using a GM1 ELISA to be 0.076 μg. Therefore, the average cell had a periplasmic enterotoxin concentration of 262 μg/ml, which represents an 1800-fold higher concentration than found in the extracellular milieu (Table 2).

This value, however, does not include the concentration of toxin monomers in the periplasm since the GM1 ELISA technique (as shown above) only detects oligomers. Nevertheless, it is possible to calculate the concentration of toxin monomers in the periplasm by making the following assumptions. First, that the flux of monomers through the periplasm is a result of both their influx, due to biosynthesis and export, and their assembly into holotoxin. Second, that the quantity of subunits synthesized, exported, assembled, and then secreted at any given time is in a steady state such that the relative pool sizes of monomers and assembled subunits in the periplasm are constant. And third, that the A and B subunits are synthesized in their stoichiometric ratio of 1:5. From the kinetic data presented in Fig. 2, it is clear that radiolabeled B monomers turn over with a half-time of 1 min. The apparent first-order rate constant, therefore, is 0.69 $min^{-1}$ and the proportion of B monomers that assemble per minute from the monomer pool is equivalent to 0.69. If we then use the assumption that a steady state exists, an equivalent amount of assembled toxin must leave per minute from the assembled toxin pool as a result of efflux across the outer membrane. We have previously shown that the rate of B-subunit efflux from *V. cholerae* TRH7000 (pWD600) is equivalent to the secretion of 34 B pentamers per minute per cell (see ref. 25). Therefore, the size of the B-monomer pool in an average cell can be calculated to be ≈246 molecules. A 1:5 ratio of A and B subunits suggests that an average cell contains ≈50 A subunits and 246 B subunits in the monomeric state at any given time, which is equivalent to a concentration of ≈20 μg/ml. This value is consistent with the view that the periplasm engenders a very concentrated milieu where subunit assembly can occur spontaneously and before translocation across the outer membrane.

Table 2. Distribution and relative concentration of enterotoxin in the medium and periplasm of *V. cholerae* TRH7000 (pWD600)

| Location | Compartment vol, ml | Toxin amount, μg | Toxin concentration, μg/ml | Relative toxin concentration |
|---|---|---|---|---|
| Periplasmic | 0.00029* | 0.076 | 262 | 1800 |
| Extracellular | 1.00 | 0.144 | 0.144 | 1 |

*Determined by the method of Stock *et al.* (29).

## DISCUSSION

Proteins that are secreted by Gram-negative bacteria into the extracellular milieu are confronted with the tantalizing problem of having to traverse a cell envelope, consisting of two distinct membranes, interspersed by a protein-filled periplasmic space (for reviews, see refs. 2 and 13). Perhaps not surprisingly, several different mechanisms appear to have evolved that are able to accomplish this feat. Examples include the mechanism of α-hemolysin (HlyA) secretion from *E. coli*, in which the carboxyl-terminal domain of HlyA and two gene products, HlyB and HlyD, are requisites for translocation of the hemolysin molecule across the cell envelope (31–33); the secretion of colicins from Gram-negative bacteria where "lysis" proteins play a crucial role in stimulating changes in membrane composition and in facilitating colicin release (34, 35); the mechanism of IgA protease release from *Neisseria gonorrhoeae*, which appears to involve sequential proteolytic cleavage of the polypeptide (36); and the two-step translocation mechanism proposed for enterotoxin secretion from *V. cholerae*, in which the toxin subunits are exported across the inner membrane by a signal sequence-dependent process, released into the periplasm, and then secreted across the outer membrane by a separate and independent translocating mechanism (24, 25).

One recent development in the understanding of protein movement across biological membranes has been the emergence of a new paradigm; that proteins are in a nonnative state (i.e., an unfolded, or only partially folded conformation) prior to translocation (1, 5, 6, 11, 12). This concept was elegantly demonstrated in studies of protein import into mitochondria and protein export in bacteria. Eilers and Schatz showed that a chimeric protein consisting of the leader sequence of cytochrome oxidase and dihydrofolate reductase could not be imported into mitochondria in the presence of a specific ligand, methotrexate (5). The methotrexate trapped the protein in a folded conformation, which prevented the molecule from being translocated across the mitochondrial envelope. Randall and Hardy demonstrated that the export of newly synthesized chains of maltose-binding protein into the periplasm of *E. coli* was inversely correlated with the extent of maltose-binding protein folding, so that only polypeptide chains that were in a protease-sensitive (i.e., nonnative) conformation were able to be translocated (6).

In this paper, we have investigated whether conformational constraints of this kind are important for the secretion of proteins across the outer membranes of Gram-negative bacteria. To address this, we studied the conformational status of the subunits of heat-labile enterotoxin during their secretion across the cell envelope of *V. cholerae*. We had previously demonstrated that the secretion of toxin subunits across the cytoplasmic and outer membranes were separate events punctuated by the transient entry of the subunits into the periplasm (25).

Here we have shown that the subunits of heat-labile enterotoxin assemble into oligomers within the periplasm prior to their secretion across the outer membrane. Subunit flux through the periplasm was monitored by pulse-labeling cells with radioactive methionine and then isolating periplasmic fractions at different time intervals after the cessation of radiolabel uptake. The B subunits were found to be released into the periplasm as monomers, where they exhibited a characteristic rate of turnover corresponding to a half-time of ≈1 min. Concomitantly, assembled toxin could be detected within the periplasm, whose B-pentamer component migrated on $NaDodSO_4$/polyacrylamide gels as 45-kDa oligomers. Since the half-time for efflux of labeled B subunits from the periplasm was slow ($t_{1/2}$ = 13 min) compared to the rate of B monomer turnover ($t_{1/2}$ = 1 min), we conclude that all of the

B subunits that enter the periplasm assemble prior to their secretion across the outer membrane.

The presence in the periplasm of associated A and B subunits (i.e., holotoxin) was established by a GM1 ELISA using a monoclonal antibody that reacted with the A subunit (Table 1). In the periplasmic fractions obtained during the pulse–chase, no assembly intermediates (e.g., B-subunit dimers) were detected, which suggests that after the initiation of oligomerization, stable holotoxin is very rapidly formed. We therefore propose that the major toxin pools in the periplasm are A- and B-subunit monomers and fully assembled holotoxin.

Based on this assumption, we calculated the concentration of monomers and assembled toxin in the periplasm of an average cell from the amount of toxin detected by a GM1 ELISA and from the rates of subunit turnover. The concentration of A and B monomers in the periplasm was estimated to be $\approx$20 $\mu$g/ml, and the concentration of assembled toxin was estimated to be $\approx$260 $\mu$g/ml. These high concentrations would clearly favor the spontaneous formation of holotoxin from subunit monomers. It is also conceivable that the high concentration of fully assembled toxin in the periplasm is important in facilitating movement of the molecule across the outer membrane. We found a massive 1800-fold difference in toxin concentration between the cis (periplasmic) and trans (external) sides of the outer membrane (Table 2), which may give rise to apparent unidirectional translocation as a result of the partitioning of the toxin into and across the membrane. If this is the case, it remains to be established what influence membrane lipids, specific outer membrane translocator proteins, and intragenic toxin domains have on such partitioning mechanisms.

The findings documented in this paper show that the subunits of heat-labile enterotoxin fold and assemble into holotoxin before they are translocated across the outer membrane. This implies that the conformation of the toxin during its traversal across the outer membrane retains its quaternary structure. It would seem to us inconceivable that the molecule would dissociate into its component subunits and unfold and then reassemble on the trans side of the membrane, especially since the interactions between the B subunits are very stable, as shown by their resistance to disruption by $NaDodSO_4$. This suggests that the physicochemical principles governing toxin translocation across the outer membrane of *V. cholerae* differ markedly from the subdomain export mechanisms envisaged for transfer of proteins across ER, organelle, and bacterial inner membranes (1, 5, 6, 12). We propose that proteins, which are synthesized with amino-terminal signal sequences, and which are subsequently secreted from Gram-negative bacteria—e.g., oligomeric proteins such as cholera toxin and pertussis toxin (37), complex surface adhesins such as the components of P-fimbriae (38), and numerous enzymes including proteases and cellulases (39–41)—fold into tertiary or quaternary conformations before they are translocated across a bacterial outer membrane.

We thank Susanne Johansson for her well-executed technical skills. T.R.H. is a Wellcome Trust Senior Research Fellow in Basic Biomedical Sciences. This work was supported by grants from The Wellcome Trust (Project 15213/1.4P), The Swedish Board for Technical Development (Project 3089), and The Medical Research Council of Sweden (Project 16X-03382-17B).

1. Zimmermann, R. & Meyer, D. I. (1986) *Trends Biochem. Sci.* **11**, 512–515.
2. Randall, L. L., Hardy, S. J. S. & Thom, J. R. (1987) *Annu. Rev. Microbiol.*, in press.
3. Douglas, M. G., McCammon, M. T. & Vassorotti, A. (1986) *Microbiol. Rev.* **50**, 166–178.
4. Wickner, W. T. & Lodish, H. F. (1985) *Science* **230**, 400–407.
5. Eilers, M. & Schatz, G. (1986) *Nature (London)* **322**, 228–232.
6. Randall, L. L. & Hardy, S. J. S. (1986) *Cell* **46**, 921–928.
7. Kvist, S., Wiman, K., Claesson, L., Peterson, P. A. & Dobberstein, B. (1982) *Cell* **29**, 61–69.
8. Gething, M.-J., McCammon, K. & Sambrook, J. (1986) *Cell* **46**, 939–950.
9. Kreis, T. E. & Lodish, H. F. (1986) *Cell* **46**, 929–937.
10. Proia, R. L., d'Azzo, A. & Neufeld, E. F. (1984) *J. Biol. Chem.* **259**, 3350–3354.
11. Rothman, J. E. & Kornberg, R. D. (1986) *Nature (London)* **322**, 209–210.
12. Singer, S. J., Maher, P. A. & Yaffe, M. P. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1015–1019.
13. Pugsley, A. P. & Schwartz, M. (1985) *FEMS Microbiol. Rev.* **32**, 3–38.
14. Gill, D. M., Clements, J. D., Robertson, D. C. & Finkelstein, R. A. (1981) *Infect. Immun.* **33**, 677–682.
15. Holmgren, J. (1973) *Infect. Immun.* **8**, 851–859.
16. Holmgren, J. (1981) *Nature (London)* **292**, 413–417.
17. Moss, J. & Richardson, S. H. (1978) *J. Clin. Invest.* **62**, 281–285.
18. Palva, E. T., Hirst, T. R., Hardy, S. J. S., Holmgren, J. & Randall, L. L. (1981) *J. Bacteriol.* **146**, 325–330.
19. Dallas, W. S. (1983) *Infect. Immun.* **40**, 647–652.
20. Leong, J., Vinal, A. C. & Dallas, W. S. (1985) *Infect. Immun.* **48**, 73–77.
21. Yamamoto, T., Gojobori, T. & Yokota, T. (1987) *J. Bacteriol.* **169**, 1352–1357.
22. Sack, R. B. (1975) *Annu. Rev. Microbiol.* **29**, 333–353.
23. Neill, R. J., Ivins, B. E. & Holmes, R. K. (1983) *Science* **221**, 289–291.
24. Hirst, T. R., Sanchez, J., Kaper, J. B., Hardy, S. J. S. & Holmgren, J. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2645–2649.
25. Hirst, T. R. & Holmgren, J. (1987) *J. Bacteriol.* **169**, 1037–1045.
26. Kaper, J. B., Lockman, H., Baldini, M. M. & Levine, M. M. (1984) *Nature (London)* **308**, 655–658.
27. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
28. Svennerholm, A.-M. & Holmgren, J. (1978) *Curr. Microbiol.* **1**, 19–27.
29. Stock, J. B., Rauch, B. & Roseman, S. (1977) *J. Biol. Chem.* **252**, 7850–7861.
30. Svennerholm, A.-M., Wickström, M., Lindblad, M. & Holmgren, J. (1986) *Med. Biol.* **64**, 23–30.
31. Wagner, W., Vogel, M. & Goebel, W. (1982) *J. Bacteriol.* **154**, 200–210.
32. Gray, L., Mackman, N., Nicaud, J.-M. & Holland, I. B. (1986) *Mol. Gen. Genet.* **205**, 127–133.
33. Felmlee, T., Pellett, S. & Welch, R. A. (1985) *J. Bacteriol.* **163**, 94–105.
34. Pugsley, A. P. & Schwartz, M. (1984) *EMBO J.* **3**, 2393–2397.
35. Cavard, D., Baty, D., Howard, S. P., Verheij, H. M. & Lazdunski, C. (1987) *J. Bacteriol.* **169**, 2187–2194.
36. Pohlner, J., Halter, R., Beyreuther, K. & Meyer, T. F. (1987) *Nature (London)* **325**, 458–462.
37. Nicosia, A., Perugini, M., Franzini, C., Casagli, M. C., Borri, M. G., Antoni, G., Almoni, M., Neri, P., Ratti, G. & Rappuoli, R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4631–4635.
38. Uhlin, B. E., Båga, M., Göransson, M., Lindberg, F. B., Lund, B., Norgren, M. & Normark, S. (1985) *Curr. Top. Microbiol. Immunol.* **118**, 163–178.
40. Andro, T., Chambst, J.-P., Kotoujansky, A., Cattaneo, J., Bertheau, Y., Barras, F., van Gijsegem, F. & Coleno, A. (1984) *J. Bacteriol.* **160**, 1199–1203.
41. Thurn, K. K. & Chatterjee, A. K. (1985) *Appl. Environ. Microbiol.* **50**, 894–898.