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Escherichia coli Hemolysin May Damage Target Cell Membranes by Generating Transmembrane Pores

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***Escherichia coli* hemolysin is secreted as a water-soluble polypeptide of M_r 107,000. After binding to target erythrocytes, the membrane-bound toxin resembled an integral membrane protein in that it was refractory towards extraction with salt solutions of low ionic strength. Toxin-induced hemolysis could be totally inhibited by addition of 30 mM dextran 4 (mean M_r, 4,000; molecular diameter ~3 nm) to the extracellular medium. Uncharged molecules of smaller size (e.g., sucrose, with a molecular diameter of 0.9 nm, or raffinose, with a molecular diameter of 1.2 to 1.3 nm) did not afford such protection. Treatment of erythrocytes suspended in dextran-containing buffer with the toxin induced rapid efflux of cellular K^+ and influx of $^{45}Ca^{2+}$, as well as influx of [^{14}C]mannitol and [^{3}H]sucrose. [^{3}H]inulin only slowly permeated into toxin-treated cells, and [^{3}H]dextran uptake was virtually nil. Membranes lysed with high doses of *E. coli* hemolysin exhibited no recognizable ultrastructural lesions when examined by negative-staining electron microscopy. Sucrose density gradient centrifugation of deoxycholate-solubilized target membranes led to recovery of the toxin exclusively in monomer form. Incubation of toxin-treated cells with trypsin caused limited proteolysis with the generation of membrane-bound, toxin-derived polypeptides of M_r ~80,000 without destroying the functional pore. We suggest that *E. coli* hemolysin may damage cell membranes by partial insertion into the lipid bilayer and generation of a discrete, hydrophilic transmembrane pore with an effective diameter of ~3 nm. In contrast to the structured pores generated by cytolysins of gram-positive bacteria such as staphylococcal α-toxin and streptolysin O, pore formation by *E. coli* hemolysin may be caused by the insertion of toxin monomers into the target lipid bilayers.**

The hemolysin of *Escherichia coli* has attracted much attention in the recent past for two main reasons. First, it appears to represent a significant pathogenic factor (6, 7, 9, 14, 18, 27–31). Second, the toxin represents the only known protein that appears to be genuinely secreted by *E. coli*. Hence, it is an interesting model for the study of protein transport occurring across the inner and outer membrane of gram-negative bacteria (6, 11, 16, 25; N. Mackman, J.-M. Nicaud, L. Gray, and I. B. Holland, Curr. Top. Microbiol. Immunol., in press).

A considerable amount of molecular genetic data have now been accumulated on the nature of the hemolysin system (15; Mackman et al., in press), which have been recently reviewed (Mackman et al., in press). These studies have all concluded that the hemolysin determinant is composed of four genes. These include *hlyC*, which encodes a 20,000-molecular-weight polypeptide that promotes post-translational modification of the protein encoded by the structural gene *hlyA*, rendering this protein hemolytically active. In addition, two genes, *hlyB* and *hlyD*, are essential for secretion of the hemolysin to the medium. The product of *hlyA* is a polypeptide of 107,000 molecular weight (107K polypeptide) which, after activation, is secreted efficiently to the medium (21, 22). This recent recognition contrasts with earlier reports that the *hlyA* product was processed by extensive proteolysis to a 60,000-molecular-weight polypeptide which constituted the secreted, hemolytically active toxin (15). Recent studies have now provided further support that the active hemolysin is indeed the 107K protein (8), and the protein has been extensively purified (12).

Despite these advances, surprisingly little is known regarding the nature of toxin attack on target cells. One recent study dealt with the effect of the toxin on erythrocyte membranes, although the partially purified hemolysin itself was not characterized. This investigation demonstrated a rapid efflux of cellular K^+ and influx of $^{45}Ca^{2+}$ preceding hemolysis (19). Since a similarly rapid influx of sucrose and insulin was not registered, the tentative conclusion was drawn that cell damage and hemolysis might result from selective Ca^{2+} uptake; i.e., the toxin might be acting in a manner similar to Ca ionophores. The previously reported requirement of extracellular Ca^{2+} for induction of hemolysis by the toxin (6, 23) appeared compatible with this concept.

After the development of suitable procedures for recovering the hemolytically active 107K toxin from culture supernatants of *E. coli* in apparently homogeneous form (Mackman et al., in press), we have now performed further studies to delineate its mechanism of action. Here we present data indicating that *E. coli* hemolysin inserts into target lipid bilayers to create hydrophilic transmembrane pores, of approximately 3-nm effective diameter. In contrast to the pore-forming toxins of gram-positive organisms, such as staphylococcal α-toxin and streptolysin-O, which oligomerize in the membrane to form ultrastructurally visible lesions (5, 10), membrane damage by *E. coli* hemolysin may be mediated by the insertion of monomeric protein molecules into the target membrane.

MATERIALS AND METHODS

Preparation of *E. coli* hemolysin. The wild-type *E. coli* strain LE2001, serotype O4, was isolated in Newcastle, England, from a patient with a urinary tract infection. This chromosomal hemolysin determinant was cloned, forming the recombinant plasmid pLG570 (21). One milliliter of an

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overnight *E. coli* culture in tryptic soy broth was inoculated into 1,000 ml of prewarmed Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) containing 10 mM $CaCl_2$ to stabilize the labile toxin (22). After 90 min at 37°C, the culture was transferred to a 30°C water bath and incubation was continued for another 2 to 3 h. The bacteria were then pelleted by centrifugation (Sorvall centrifuge RC 2B rotor type GSA; 16,000 × *g* for 20 min at 4°C). The supernatant was made 3% (vol/vol) in glycerol, and 200 g of solid polyethylene glycol (PEG 4000; Merck Laboratories, Munich, Federal Republic of Germany) was then added. The solution was stirred for approximately 5 min until the PEG had dissolved, after which incubation was continued at 4°C for another 60 min. The PEG precipitate was collected by centrifugation (Sorvall centrifuge RC 2B, GSA rotor; 16,000 × *g* for 20 min at 4°C), the supernatant was discarded, and the pellet was dissolved in approximately 8 ml of saline. Toxin prepared in this fashion retained ≥50% hemolytic activity for 1 week if stored at −20°C.

Assessment of hemolytic titer. Samples (50 μl) of toxin solutions were diluted twofold in saline buffered with 10 mM Tris hydrochloride (pH 7.4) in microtiter plates. A 50-μl volume of a 2.5% rabbit erythrocyte suspension (2.5×10^8 cells per ml) was added to each well, and titers were read visually after 60 min of incubation at 37°C. Titers were expressed as arbitrary hemolytic units (HU) and defined as the last dilution giving >90% hemolysis. Typically, titers of 64 to 128 HU were obtained in the starting culture supernatants, and titers of 8,000 to 16,000 HU were obtained in the dissolved PEG precipitate.

Preparation of toxin-treated erythrocyte membranes. Rabbit erythrocyte suspensions (10%) in saline were lysed by the addition of 1/50 volume of toxin (8,000 to 16,000 HU/ml). After 20 min at 37°C, the membranes were pelleted by centrifugation (Sorvall rotor SS 34; 25,000 × *g* for 10 min at 4°C) and washed three times in 5 mM phosphate buffer (pH 8). To assess the nature of the association between toxin and membrane, the membranes were suspended for 2 h at 37°C in 5 volumes of one of the following solutions: (i) 1 mM EDTA (pH 8); (ii) 1 M NaCl–50 mM sodium phosphate (pH 8); (iii) 5 mM sodium phosphate (pH 8). The membranes were then pelleted by centrifugation (150,000 × *g* for 30 min; Beckmann ultracentrifuge, rotor SW50.1), the supernatants were collected, and the pellets were suspended to their original elution volumes with distilled water. Samples were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the hemolysin protein was detected using immunoblotting with specific antibodies to the toxin.

Sucrose density gradient centrifugation. Toxin-treated membranes were quantitatively solubilized by the addition of solid deoxycholate detergent to a final concentration of 250 mM. Samples (1 ml) of solubilized material were layered onto linear 10 to 50% (wt/vol) sucrose density gradients containing 6.25 mM detergent, and centrifugation was performed at 150,000 × *g* for 16 h at 4°C exactly as described (5). Ten equal fractions were subsequently collected from the bottom of the tubes and analyzed by SDS-PAGE immunoblotting.

SDS-PAGE immunoblotting. SDS-PAGE immunoblotting was performed as described (2, 26). Antisera to *E. coli* hemolysin were raised in rabbits, using either the PEG-purified toxin or toxin-lysed rabbit erythrocyte membranes as antigen. The antisera obtained in both cases exhibited the same monospecificity for *E. coli* hemolysin.

Osmotic protection experiments. Rabbit erythrocyte suspensions (2%) were prepared in 90 mM NaCl–45 mM KCl–12.5 mM phosphate buffer (pH 7.2) containing one of the following substances: (i) 30 mM sucrose; (ii) 30 mM raffinose (Sigma Laboratories, Munich, Federal Republic of Germany); (iii) 30 mM dextran 4 (mean M_r, 4,000; Serva Laboratories, Heidelberg, Federal Republic of Germany). Then 1/200 volume of toxin (8,000 HU/ml) was added, and lysis was recorded after 30 min at 37°C. On the basis of published data, the osmotic protectants were assumed to have the following mean molecular diameters (24): sucrose, 0.9 nm; raffinose, 1.2 to 1.4 nm; dextran 4, 3.0 to 3.5 nm.

Dextran 4 effectively protected the cells against hemolysis (see Results). In these cases, erythrocytes were subsequently pelleted (Eppendorf bench centrifuge), washed three times in dextran-containing buffer to remove nonbound toxin, and subsequently resuspended in saline, and hemolysis was recorded after 15 min at 37°C.

Measurements of K^+ efflux and influx of $^{45}Ca^{2+}$, [^{14}C]mannitol, [^{3}H]sucrose, [^{3}H]inulin, and [^{3}H]dextran into osmotically protected erythrocytes. Measurements of K^+ efflux and radioactive marker influx into erythrocytes basically followed the protocol of Jorgensen et al. (19) with the modification that cells were osmotically protected from lysis by the presence of dextran 4 in the extracellular medium. Rabbit erythrocyte suspensions, (30%) were prepared in the dextran-containing buffer described above. One of the radioactive markers, listed above (all from Amersham Laboratories) was then added to give 5×10^5 to 2×10^6 cpm of radioactivity per ml of supernatant. Samples were warmed to 37°C, and toxin was added to a final concentration of 100 HU/ml. Portions were removed after 1, 5, 10, 15, and 20 min and centrifuged, and the radioactivity in the supernatants was determined. Influx of markers was reflected by a corresponding decrease in radioactivity, and results were expressed as percent change in concentration of the marker in the supernatant as described (19). For K^+ efflux measurements, 20% erythrocyte suspensions in dextran-containing saline were treated with 80 HU of toxin per ml, and K^+ concentrations in the supernatants were determined by flame photometry.

Proteolytic cleavage of membrane-bound toxin. Rabbit erythrocytes suspended (10%) in dextran-containing buffer were treated with toxin (50 HU/ml) for 20 min at 37°C. The cells were then washed in dextran buffer, suspended in the presence of the protectant, and trypsinized (10 μg of trypsin per ml from Serva; 60 min at 37°C). The cells were then washed three times in dextran-buffer and suspended in saline, and hemolysis was recorded. The lysed erythrocyte membranes were finally washed three times in 5 mM phosphate and subjected to SDS-PAGE immunoblotting.

Negative staining and electron microscopy of membranes were performed as described (5).

RESULTS

Properties of native *E. coli* hemolysin. Preparations of hemolysin obtained by PEG precipitation from culture supernatants of strain MC4100(pLG570) exhibited a single protein band of approximately M_r 107,000 during SDS-PAGE in the presence or absence of 5 mM dithiothreitol (Fig. 1). SDS-PAGE immunoblotting showed that antisera raised against this protein reacted with a single identical moiety in unfractionated culture supernatants (Fig. 1, lane b). These results were in accord with previous data that have shown that the 107K protein is the only major secreted product from hemolytic *E. coli* (12, 21; N. Mackman, Ph.D. thesis, University of Leicester, Leicester, England, 1984),

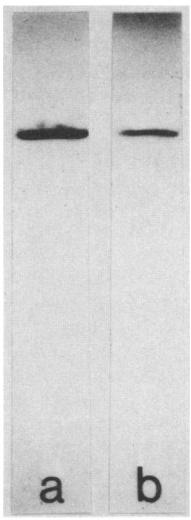

FIG. 1. SDS-PAGE of *E. coli* hemolysin. (a) Coomassie-stained gel loaded with purified toxin (5 μg); (b) immunoblot of unfractionated culture supernatant (80 μl) developed with an antiserum raised against the purified protein (gel a). Native *E. coli* hemolysin is secreted as a homogeneous protein of M_r 107,000.

that hemolytic activity coincided with the presence of this protein during ammonium sulfate fractionation and glycerol density gradient centrifugation, and that antibodies raised against this protein neutralized hemolytic toxin activity (Mackman, thesis). Due to spontaneous inactivation of the toxin, it has not yet been possible to obtain reliable estimates of specific hemolytic activity in the toxin preparations.

Although freshly prepared toxin exhibited satisfactory hemolytic titers, activity was rapidly lost upon aging (several hours at 37°C, or overnight at 4°C). Activity could best be preserved by storage at −20°C. We were also unable to recover appreciable activity after either sucrose density gradient centrifugation, Sephacryl S-300 chromatography, or ion-exchange chromatography of the toxin. SDS-PAGE revealed that freshly inactivated toxin still gave rise to the 107K band, whereas prolonged aging or incubation at 37°C caused progressive appearance of closely banding polypeptides in the M_r 60,000 region with concomitant reduction of the native 107K band (data not shown). SDS-PAGE of inactive toxin fractions recovered from sucrose density gradients showed that the toxin sedimented to cover a broad region covering 5 to 35S (not shown). Therefore, we conclude that initial toxin inactivation derived from aggregation of the molecule, which was followed by a second step of proteolytic degradation.

Osmotic protection experiments. Erythrocytes were suspended in buffers containing various osmotic protectants. We found that neither sucrose (effective molecular diameter, 0.9 nm; 24) nor raffinose (molecular diameter, 1.2 to 1.4 nm) inhibited toxin-dependent hemolysis. However, cells suspended in dextran 4 (molecular diameter, ~3 nm) exhibited virtually no hemolysis after 45 min of incubation with the toxin. When these cells were subsequently washed in dextran-containing buffer and suspended in saline, rapid hemolysis ensued that was complete within 5 min at 37°C. These results indicated that dextran did not primarily inhibit the binding of *E. coli* hemolysin to erythrocytes, but acted as an osmotic protectant by counterbalancing the intracellular osmotic pressure of hemoglobin.

Measurements of K^+ efflux and radioactive marker influx into erythrocytes. The contention that *E. coli* hemolysin generated small pores in the erythrocyte membrane was supported by the following experiments. Erythrocytes suspended in dextran-containing buffer were treated with toxin, and in accord with previous data (19), we recorded a rapid release of cellular K^+ that was complete within 1 to 2 min (Fig. 2). Analogous experiments designed to measure the influx of radioactive markers showed that extracellular $^{45}Ca^{2+}$ rapidly permeated the toxin-induced membrane lesions (Fig. 3), and these results again were identical to those previously reported by Jorgensen et al. (19). In addition, we found a rapid influx of [^{14}C]mannitol and a progressively slower but clearly measurable influx of [^{3}H]sucrose and

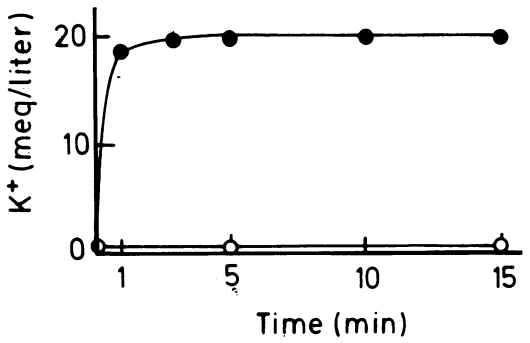

FIG. 2. Efflux of K^+ from rabbit erythrocytes induced by *E. coli* hemolysin. Erythrocytes were suspended in saline containing 30 mM dextran 4 (2×10^9 cells per ml). Toxin treatment induced a rapid efflux of K^+ that was essentially complete within 1 min. Cell lysis was totally inhibited by the dextran throughout the duration of the experiment. Symbols: ●, toxin-treated cells; ○, control cells.

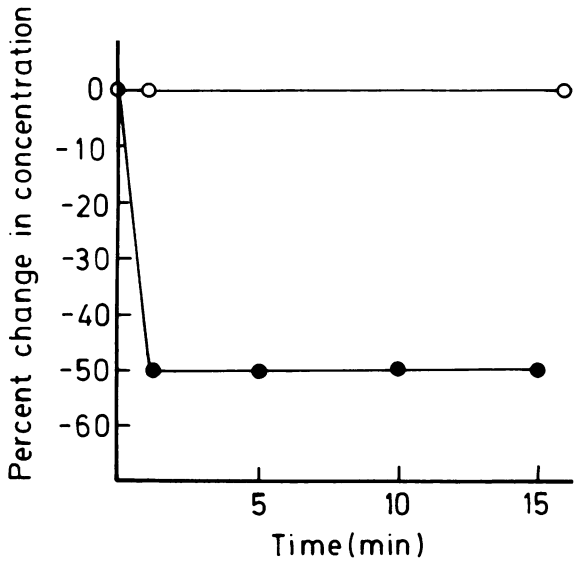

FIG. 3. Toxin-induced influx of ^{45}Ca into dextran-protected erythrocytes. A suspension of rabbit erythrocytes in dextran-containing buffer (3×10^9 cells per ml) was treated with ^{45}Ca (10^6 cpm of radioactivity per ml of supernatant). After toxin treatment, samples were removed and radioactivity in the supernatants was measured. Influx of ^{45}Ca was reflected by a decrease in radioactivity, expressed as percent change in ^{45}Ca concentration. Intracellular accumulation of ^{45}Ca was enhanced due to Ca-binding proteins in the cytoplasm. Symbols: ●, toxin-treated cells; ○, control cells.

[^{3}H]inulin (Fig. 4). The extracellular concentration of dextran 4, in contrast, remained relatively constant over the observed time period; hence, this marker did not permeate into the toxin-treated cells. No change in the extracellular concentration of any marker was observed in non-toxin-treated controls.

From these results, we concluded that *E. coli* hemolysin generates circumscribed functional transmembrane pores in erythrocyte membranes that permit rapid transmembrane passage of cations and small uncharged molecules such as mannitol. There is slower passage of uncharged molecules of effective diameter of 1 to 2 nm, and the erythrocytes remain impermeable to molecules whose diameter exceeds 3 nm.

Properties of membrane-bound toxin. Next, the nature of the association of the toxin with erythrocyte membranes was examined. First, we determined whether the bound toxin formed oligomeric complexes that were stable in detergent. Toxin-treated membranes were solubilized with deoxycholate and centrifuged through a sucrose density gradient. Figure 5 (upper panel) depicts a Coomassie blue-stained SDS-polyacrylamide gel loaded with toxin-treated rabbit erythrocyte membranes (lane b) and samples of fractions 1 through 10 recovered from the gradient. The SDS-PAGE pattern of toxin-treated membranes did not differ from that of untreated controls (not shown), and the bulk of membrane proteins were recovered in the top fractions of the gradient, as previously reported (5). The lower panel of Fig. 5 depicts a parallel analysis in which SDS-PAGE of the same samples was followed by immunoblotting with a specific antiserum to the hemolysin. Lane a was loaded with the native toxin preparation; in this experiment, we utilized a preparation that contained some 60,000-molecular-weight degradation products to demonstrate that only the 107K moiety and not the degradation product subsequently bound to cell membranes (lane b, lower panel). Analysis of the sucrose density gradient fractions revealed that the membrane-bound toxin was recovered exclusively in monomer form in the three top gradient fractions. Hence, *E. coli* hemolysin did not form detergent-stable oligomeric aggregates after binding to the membrane. Some toxin degradation was observed after aging of the membranes, and these proteolytic products were detected in the immunoblots (Fig. 5, lower panel, fractions 8 through 10).

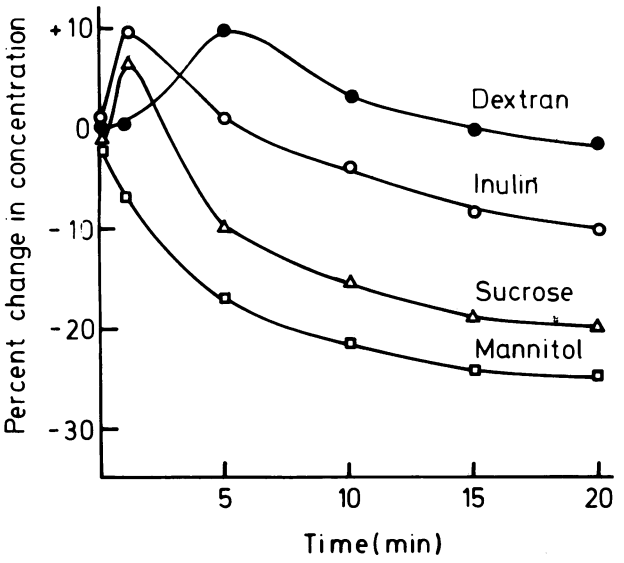

FIG. 4. Influx of radioactive markers into toxin-treated erythrocytes. The experimental design was as described for Fig. 3 (for details, see text and reference 19). Toxin treatment of rabbit erythrocytes suspended in dextran buffer caused a rapid influx of mannitol and a progressively slower influx of sucrose and inulin, whereas dextran uptake was virtually nil. Slight increases in marker concentrations registered after 1 min may have been due to initial cellular swelling (see reference 19).

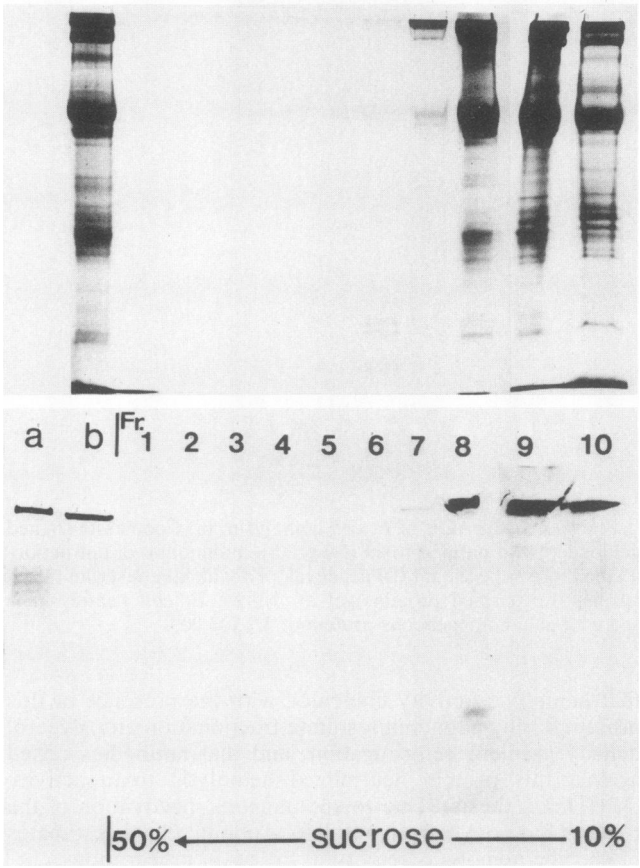

FIG. 5. SDS-PAGE of rabbit erythrocyte membranes lysed with *E. coli* hemolysin (lane b) and of fractions recovered after sucrose density gradient centrifugation of deoxycholate-solubilized membranes (fractions 1 through 10; direction of sedimentation is from right to left as indicated). Upper panel, Gel stained with Coomassie blue; lower panel, immunoblot developed with antibodies to the hemolysin. A sample of *E. coli* hemolysin that was used in the experiment was additionally applied in the SDS-PAGE immunoblot analysis (lane a, lower panel). Note the binding of the 107K hemolysin to the erythrocyte membranes and the presence of membrane-derived, detergent-treated toxin exclusively in monomer form in the three top fractions of the gradient.

Second, SDS-PAGE immunoblotting was used to assess the strength of binding of toxin to target membranes. These experiments were undertaken because peripheral membrane proteins invariably are partially extractable from membranes by elution with buffers of low ionic strength such as 1 mM EDTA (pH 8) (1, 3), whereas integral membrane proteins typically remain membrane bound. Toxin-lysed membranes were extracted with 1 mM EDTA (pH 8), 1 M NaCl, or 5 mM phosphate (pH 8), and the supernatants were examined for the presence of toxin. The toxin entirely resisted elution in every case and was quantitatively recovered in the membrane pellets (Fig. 6). Note the appearance of toxin-derived

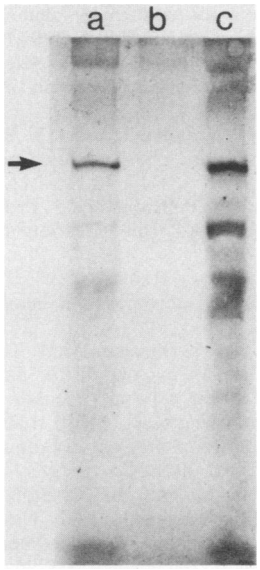

FIG. 6. Refractoriness of membrane-bound hemolysin towards extraction with 1 mM EDTA (pH 8). Erythrocyte membranes lysed with *E. coli* hemolysin were suspended in 5 vol of 1 mM EDTA (pH 8) (lane a). After 2 h of incubation at 37°C, the supernatant (lane b) and membrane pellet (lane c) were analyzed for the presence of toxin by SDS-PAGE immunoblotting. Note the entire absence of toxin in the EDTA supernatant. Lower M_r bands represent degradation products of the toxin.

degradation products typically appearing after the incubations at 37°C.

Stability of the toxin pore towards tryptic digestion. Erythrocytes were treated with toxin under conditions of dextran 4 protection. The cells were then washed, suspended in dextran 4 buffer, and trypsinized at 37°C for 60 min. No hemolysis was observed at this stage. When the trypsinized cells were washed to remove trypsin and finally resuspended in saline, rapid hemolysis ensued. Hence, once generated in a target membrane, the toxin pores could not be removed or destroyed by trypsin. When membranes from cells treated with trypsin in this way were analyzed by SDS-PAGE immunoblotting, the membrane-bound toxin was detected primarily in degraded form as closely banding polypeptides of molecular weight about 80,000 (Fig. 7).

Absence of ultrastructural lesions on toxin-treated membranes. Negative-staining electron microscopy revealed no detectable alterations in the structure of toxin-treated erythrocyte membranes compared to controls obtained by hypotonic lysis. In particular, no indications for the presence of open or closed circular lesions on the membranes could be obtained.

DISCUSSION

The 107K *E. coli* hemolysin is secreted as a water-soluble molecule of M_r 107,000 which binds tightly to target erythrocyte membranes. Thus, the toxin was not extracted from the membranes with aqueous solutions of low or high ionic strength. From this and the relative resistance of bound toxin to trypsin attack, we suggest that the toxin might undergo a hydrophilic-amphiphilic transition at the membrane surface, resulting in partial insertion of the polypeptide into the hydrophobic membrane domain.

The process of membrane binding can be dissociated from the lysis step promoted by the toxin if the erythrocytes are suspended in buffer containing dextran 4. This finding is analogous to the protection of cells towards hemolysis by the pore-forming staphylococcal α-toxin (2) and C5b-9 complement complexes (3, 13). Hemolysis in all these cases is inhibited because the protectant molecule which is too large to pass the transmembrane pore remains in the extracellular space and counteracts the colloid-osmotic pressure of intracellular hemoglobin. Since osmotic protection was observed with dextran 4 (molecular diameter, ~3 nm) but not with sucrose (0.9 nm in diameter) or raffinose (1.2 to 1.4 nm in diameter), we conclude that the functional diameter of the *E. coli* hemolysin pore is around 2 to 3 nm. This contention was corroborated by measurements of influx of marker molecules into toxin-treated erythrocytes. We used the same experimental approach described by Jorgensen et al. (19), with the modification that all experiments were performed under dextran protection to suppress hemolysis. A very rapid influx of Ca^{2+} (essentially complete within 1 min) occurred, paralleled by an equally rapid efflux of cellular K^+. We also registered a rapid influx of [^{14}C]mannitol and a progressively slower influx of [^{3}H]sucrose and [^{3}H]inulin. In contrast, dextran uptake was nil. These results indicated that the effective diameter of the toxin pore was approximately the size of inulin (molecular diameter, 2.8 nm; 24). One point which we interpret differently from Jorgensen et al. relates to the sucrose influx. These authors also registered slow influx of this marker, but attributed their finding to an unspecific effect. They emphasized the rapid Ca^{2+} influx, proposing that hemolysis occurred as a secondary result of a Ca-ionophore-like action of the toxin and the intracellular accumulation of Ca^{2+} ions. We favor the concept of a more nonspecific toxin pore because there is rapid influx of mannitol and efflux of K^+. The slower influx of sucrose and inulin would be expected since the size of these markers approaches the proposed dimensions of the toxin lesion.

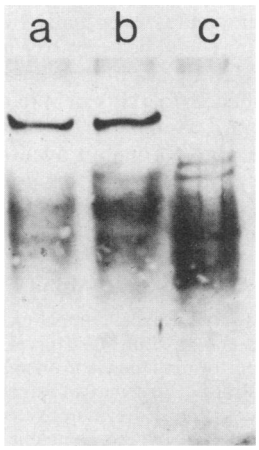

FIG. 7. Limited proteolysis of membrane-bound toxin. Erythrocytes were treated with *E. coli* hemolysin in the absence (lane a) or presence (lane b) of dextran 4 as an osmotic protectant. After incubation with the toxin in dextran, the intact erythrocytes were washed in dextran to remove unbound toxin, suspended in dextran buffer, and exposed to 10 μg of trypsin per ml for 60 min at 37°C. Thereafter, cells were washed in saline and the lysed membranes were subjected to SDS-PAGE (lane c). Immunoblotting with antihemolysin antiserum was used to detect the toxin. Note the limited proteolysis of membrane-bound toxin to polypeptides of approximately 80,000 molecular weight. Tryptic attack did not destroy the functional pore (see text).

Moreover, we have found that hemolysis can be induced in the presence of 5 mM EDTA and thus does not exhibit an absolute requirement for Ca^{2+} (data not shown). Extracellular Ca^{2+} does appear to exert some enhancing effect on hemolysis, but the mechanism and significance of this process are unclear and require further study.

Experiments in which toxin-treated cells were proteolysed under dextran protection showed that, once formed, the toxin pore could not be removed by tryptic attack. SDS-PAGE immunoblotting revealed limited proteolysis of the toxin with the generation of major degradation products of M_r around 80,000. Further studies will be required to examine whether the protease-resistant part of the molecule may carry the membrane-altering, pore-forming regions. The residual part of the molecule may be involved in the primary membrane-binding step. Whether or not the latter process involves specific membrane binder molecules has not been addressed in the present study. Interpretation of any toxin-binding experiments must take the unusual lability of the toxin into consideration. Toxin inactivation, occurring presumably through aggregation of the protein, occurs parallel to the event of membrane binding, so that a major portion of the inactive toxin molecules is recovered in lysis supernatants (data not shown).

Electron microscopic examination of target membranes revealed no visible circular "lesions" as have been documented for the staphylococcal α-toxin (10), streptolysin O (5), and complement pores (3, 4). Oligomerized toxin structures could also not be recovered from solubilized membranes; all membrane-derived toxin was present in monomer form in deoxycholate detergent solution. Thus, in contrast to the structured pores formed through oligomerization of protein molecules in the previously studied cases, the fairly large functional pores generated by *E. coli* hemolysin may derive from the insertion of single toxin molecules into the lipid bilayer. Of course, we cannot exclude that the pores may consist of toxin oligomers that are dissociated by deoxycholate. If, however, the concept of pore formation by monomeric toxin molecules eventually turns out to be correct, this principle may well be operative in the case of other pore-formers produced by gram-negative bacteria, including the recently described cytolysins of *Aeromonas hydrophila* (17) and *Pseudomonas aeruginosa* (20). The molecular basis for transmembrane pore formation awaits resolution in the future; the primary structure alone does not reveal the existence of apolar domains that might form the membrane-penetrating parts of the toxin molecule (8).

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