The Effects of pH on the Interaction of Anthrax Toxin Lethal and Edema Factors with Phospholipid Vesicles†

Sims K. Kochi,‡ Isabelle Martin,§ Giampietro Schiavo,⊥ Michèle Mock,‡ and Véronique Cabiaux*,§

Laboratoire de Génétique Moléculaire des Toxines (URA 557, CNRS), Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris, France, Laboratoire de Chimie Physique des Macromolécules aux Interfaces, Université de Bruxelles, CP 206/2, Boulevard du Triomphe, 1050 Bruxelles, Belgique, and Dipartimento di Scienze Biomediche, Centro CNR Biomembrane, Università di Padova, Via Trieste 75, 35121 Padova, Italy

Received August 12, 1993; Revised Manuscript Received November 24, 1993®

ABSTRACT: *Bacillus anthracis* secretes three distinct proteins which interact in binary combinations to produce two toxins. The two effector moieties, edema factor (EF) and lethal factor (LF), interact competitively with the cell receptor-binding moiety, protective antigen (PA), to produce biologically distinct effects. The passage of the toxins through an acidified endosomal compartment is an essential step in the intoxication process, and it has been shown that low pH triggers the insertion of the activated form of PA, PA_{63}, into model lipid bilayers. In this study, we have examined the effects of pH on the interaction of LF and EF with a model membrane system. Protein labeling by radioactive phospholipid probes indicated that both LF and EF are able to insert into asolectin lipid bilayers in a pH-dependent manner. For LF, the extent of insertion into the bilayer was accompanied in parallel by the release of calcein from preloaded LUV (large unilamellar vesicles). The transition pH for protein insertion, however, was somewhat higher than that for membrane destabilization. The extent of protein radiolabeling and the release of calcein from LUV incubated with EF was similar to that seen with LF; however, the pH dependency was significantly less. Low pH-induced membrane insertion by both proteins was accompanied by only a minimal change in conformation. These results suggest that LF and EF may be actively involved in the process of toxin translocation.

The two toxins produced by the bacterium *Bacillus anthracis*, edema toxin and lethal toxin, are included in a group of bipartite, bacterial protein toxins that are organized structurally into distinct effector (A) and receptor binding (B) domains. The anthrax toxins differ from the prototypical diphtheria toxin, however, since a single B domain, protective antigen (PA) interacts with either of two A domains, edema factor (EF) or lethal factor (LF), to produce biologically distinct effects (for a review on anthrax toxin, see Leppla, 1991).

Neither EF nor LF can bind to cells if PA is not already present on the cell surface (Leppla et al., 1988). Therefore, it is clear that PA plays an essential role in cellular intoxication and that its binding to the cell surface is a critical step in this process. Recently, a putative receptor for PA of approximately 85-90 kDa was identified on CHO-K1 cells (Escuyer & Collier, 1991). It is reasonably well established that PA must be proteolytically "nicked" to a 63-kDa form (PA_{63}) before LF (Novak et al., 1992) or EF are able to bind; it has been proposed that the cellular protease, furin, may be involved in this processing (Klimpel et al., 1992).

Both EF and LF are thought to act on intracellular targets. EF is an adenylate cyclase and requires a cellular component, calmodulin, for activity *in vitro* (Leppla, 1984). It is generally assumed that the pathology associated with EF intoxication is the result of an increase in intracellular levels of cAMP. While no specific mechanism of action has yet been determined for LF, it has been recently shown that there is a Zn^{2+}-binding amino acid consensus sequence (Vallee & Auld, 1990) in the carboxy-terminal portion of the protein (Klimpel et al., 1993). This has led to the proposal that LF, like the clostridial neurotoxins (Schiavo et al., 1992), may be a metalloprotease acting on an intracellular substrate.

There is evidence that edema toxin (PA + EF) or lethal toxin (PA + LF) enter target cells by the process of receptor-mediated endocytosis, and that a pH-sensitive step is required for entry into the cell cytoplasm (Friedlander, 1986; Gordon et al., 1988, 1989). A number of studies with other toxins, most notably diphtheria toxin, have demonstrated that acidification of the endosomal vesicles induces a conformational change in the toxin molecule, exposing previously buried hydrophobic regions that are necessary for the protein to interact with the lipid bilayer (Defrise-Quertain et al., 1989). Therefore, it is possible that the anthrax toxins, like other A–B type toxins, may require an acidic environment to trigger the process of toxin translocation. Recent studies support this model and have shown that PA_{63}, but not unnicked PA, is able to form ion conductive channels in planar asolectin lipid bilayers (Blaustein et al., 1989). Channel formation is both pH- and voltage-dependent. In contrast, neither EF nor LF possess any channel-forming activity. These observations have led to the proposal that EF or LF may be translocated passively from the lumen of the endosome into the cytosol through a PA_{63}-formed channel or pore (Koehler & Collier, 1991; Arora & Leppla, 1993).

The enzymatic A domain of diphtheria toxin has been shown to interact with lipid bilayers in a pH-dependent manner, leading to speculation that the effector portion of the toxin may play an active role in translocation (London, 1992). Taking into consideration the similar structural organization of diphtheria and the anthrax toxins, and their apparent utilization of a similar entry pathway, we have examined the

† This work was supported by INSERM Grant CRE910612 and DRET-91048.

* Corresponding author.
‡ France.
§ Belgium.
⊥ Italy.

® Abstract published in *Advance ACS Abstracts,* February 1, 1994.

0006-2960/94/0433-2604$04.50/0

effects of pH on the interaction of LF and EF with a liposome-based model membrane system.

EXPERIMENTAL PROCEDURES

Reagents. Asolectin (mixed soybean phospholipids) was obtained from Sigma Chemical Co. (St. Louis, MO) and was purified according to the procedure of Kagawa and Racker (1971). Calcein (Sigma) was purified by chromatography over a Sephadex LH20 (Pharmacia/LKB) column according to the protocol of Defrise-Quertain et al. (1989). The photoreactive membrane probes PCI and PCII were prepared as previously described (Montecucco, 1988).

Toxin Preparation. Protective antigen (PA), edema factor (EF), and lethal factor (LF) were purified from the culture supernatants of recombinant strains of *B. anthracis* producing only single toxin components (Pezard et al., 1993). Toxin proteins were purified over a MonoQ anion-exchange column (Pharmacia/LKB) as previously described (Blaustein et al., 1989), with minor modifications. Briefly, culture supernatants equilibrated in 20 mM Tris-HCl, pH 8.0, buffer were loaded onto the column equilibrated in the same buffer at a flow rate of 1 mL/min at 4 °C. Proteins were eluted using a 0–400 mM in 30 min NaCl gradient. Fractions containing proteins eluting at approximately 140 (PA), 200 (EF), or 280 mM (LF) NaCl were collected, pooled, and equilibrated against the starting buffer. Samples were reapplied to the column and reeluted using the same salt gradient over 60 min. Peak fractions were again collected, pooled, and equilibrated against the starting buffer. Proteins were greater than 90% pure, as determined on 10% SDS-polyacrylamide gels stained with Coomassie Blue.

The processed form of PA, PA_{63}, was generated by "nicking" purified PA with trypsin (10:1 (w/w) in 20 mM Tris-HCl, pH 8.0; Boehringer Mannheim) for 1 h at 30 °C. Proteolysis was stopped by the addition of soybean trypsin inhibitor (10:1 (w/w); Boehringer Mannheim). The sample was subsequently purified by MonoQ anion-exchange chromatography using the procedure described above, except that an elution gradient of 0–600 mM NaCl in 20 mM ethanolamine–HCl buffer, pH 9.0, over 60 min was employed. PA_{63} eluted at a salt concentration of approximately 385 mM. Using this procedure, greater than 90% of the sample was converted to a form that migrated with an apparent molecular weight of 63 kDa on a SDS-polyacrylamide gel.

Photolabeling. The use of radiolabeled, photoactivatable, phospholipid probes has been previously described (Montecucco et al., 1985). Briefly, ^{3}H-labeled PCI (1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-phospho[^{3}H]choline, 4×10^7 dpm; 2×10^6 dpm/mL), and ^{14}C-labeled PCII (1-myristoyl-2-(12-amino-4-(3-nitro-1-azidophenyl)dodecanoyl)-*sn*-glycero-3-phospho[^{14}C]choline, 2×10^6 dpm; 4×10^5 dpm/mL) were mixed with soybean asolectin vesicles in chloroform (8 mg of total lipid). The suspension was dried under nitrogen gas, resuspended in 10 mM citric acid/10 mM Na_2HPO_4 buffer, pH 7.4, and sonicated in a water bath to optical clarity. All manipulations were performed under a red safety light.

Individual toxin components (5 μg of PA, 9 μg of LF, or 12 μg of EF) were incubated with 0.5 mL of radiolabeled vesicles for 5 min at room temperature. Samples were gently mixed, and the pH was slowly adjusted as desired by the addition of predetermined amounts of 8.5% (v/v) phosphoric acid. Incubation was continued for 10 min. Under these conditions, approximately 50–80% of LF or EF, 80–100% of PA, and 30–50% of nicked PA were bound to the lipid vesicles, as determined by equilibrium centrifugation on a 2–30% sucrose gradient. The proteins and lipids were then cross-linked by exposure to UV light for 10 min. Samples were precipitated with 5% TCA. The precipitate was run on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue. After destaining, protein recovery was quantitated with a Shimadzu CS-930 dual-wavelength scanning densitometer (Shimadzu Instruments Inc., Columbia, MD). Each lane of the gel was sliced (2-mm sections), and the sections were dissolved for a minimum of 12 h in tissue solubilizer (Solvene, Packard Instrument Co., Sheridan, CT). Samples were mixed with scintillation fluid and counted in a Packard Tri-Carb 300C scintillation counter. Radioactivity associated with protein bands was determined as dpm and corrected for differences in protein content by integration of band intensities.

Liposome Preparation. Large unilamellar vesicles (LUV) used in calcein release and spectrofluorometric studies were prepared by extrusion according to a procedure described elsewhere (Hope et al., 1985). The osmolarities of all buffers and solutions were preadjusted to 295 ± 5 mosmol/kg of H_2O using a Fiske osmometer. Dry lipid films were rehydrated to a concentration of 20 mg/mL in 10 mM Hepes pH 7.2 buffer containing 62 mM calcein and submitted to five freeze/thaw cycles. The resulting preparation was extruded 10 times through two stacked 0.1-μm-pore size polycarbonate filters (Nucleopore Corp., Pleasanton, CA) with dry nitrogen gas (100–200 lbs/in.^{2}). Unencapsulated dye was removed by passing the liposome preparation over a Sephadex G50 gel filtration column, equilibrated with 10 mM Hepes, 150 mM NaCl, 1 mM EDTA buffer, pH 7.2. Liposome concentration was estimated by measuring lipid phosphorus content (Mrsny et al., 1986).

Calcein Fluorescence. Release of the fluorescent dye calcein from preloaded LUV was monitored using a SLM 8000 fluorometer (SLM Instruments Inc., Urbana, IL). Experiments were conducted in a 1-mL stirred cuvette, with right angle illumination. Temperature was kept constant at 37 °C. Excitation and emission wavelengths were set at 430 and 520 nm, respectively, employing a slit width of 4 nm. Toxin components were added to liposomes (0.05 mg/mL) pre-equilibrated at the desired pH value. The addition of Triton X-100 to a final concentration of 0.1% (v/v) was used to determine maximal release. The percentage of total fluorescence was defined as

$$\%F_t = \frac{I_t - I_0}{I_f - I_0} \times 100$$

where I_0 is the initial fluorescence, I_f is the total fluorescence observed after addition of Triton X-100, and I_t is the fluorescence after addition of toxin at $t = 740$ s, corrected for dilution. The following buffers were used to achieve the desired reaction vessel pH: pH 4.5–5.5, 10 mM acetic acid/acetate buffer; pH 5.5–6.6, 10 mM MES/NaOH buffer; pH 7.2, 10 mM Hepes/KOH buffer. In addition, all buffers contained 150 mM NaCl and 1 mM EDTA.

Tryptophan Fluorescence. Fluorescence spectra were recorded as a function of the emission wavelength (excitation wavelength, 280 nm). Large unilamellar asolectin vesicles were prepared as described above. The intrinsic fluorescence of proteins alone (2.4×10^{-7} M PA; 4.4×10^{-7} M LF; 5×10^{-7} M EF) or in the presence of LUV (0.1 mg/mL in 10 mM Hepes, 0.15 M NaCl, pH 7.2 buffer) were recorded as a function of pH which was adjusted by the addition of predetermined amounts of 1 M acetic acid/acetate buffer, pH 4.5. No fluorescence was observed for the lipid suspension

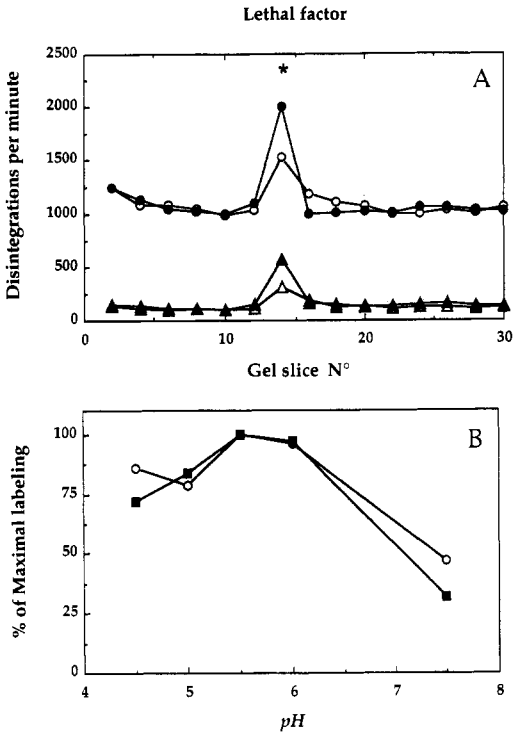

FIGURE 1: Radiolabeling of LF by the photoreactive phospholipids [^{3}H]PCI (circles) or [^{14}C]PCII (triangles). A. LF labeling profiles in the presence of asolectin SUVs in SDS-polyacrylamide gel slices at pH 7.50 (open symbols) or 4.50 (closed symbols). The gel was overrun to remove unbound radioactive lipids that run near the tracking dye. The asterisk indicates the position of LF on the Coomassie Blue-stained gel. B. PCI (○)/PCII (■) labeling of LF as a function of buffer pH. Radioactivity data are expressed as a percentage of maximal labeling, with the following normalized values (in dpm) taken as 100%: [^{3}H]PCI, 1163; [^{14}C]PCII, 686.

alone (0.1 mg/mL asolectin in 10 mM Hepes, 0.15 M NaCl, pH 7.2 buffer), at any pH tested.

RESULTS

Liposome-based model systems have been widely used to study the interaction of lipids and protein toxins and have provided information useful for understanding processes of toxin translocation. In this study, membrane insertion was determined in hydrophobic photolabeling experiments using asolectin SUV doped with [^{3}H]PCI, whose photoreactive group is restricted to the bilayer surface, and [^{14}C]PCII in which the reactive group is located in the hydrophobic acyl chain domain (for structures, see Montecucco, 1988). The distribution of radioactivity associated with LF after interaction with lipid vesicles at neutral and low pH is shown in Figure 1A. For both probes, a single radioactive peak was observed and corresponded to the position of the full length protein on a SDS-polyacrylamide gel. LF was covalently labeled by both probes, indicating an interaction with the surface of the vesicles and buried domains. While some labeling occurred at neutral pH, a marked and reproducible pH-dependence was observed (Figure 1B). Maximal labeling of LF by either probe was seen beginning at pH 6.0. A small decrease in the amount of labeled LF was observed at lower pH values, due perhaps to protein aggregation in solution. This effect does not appear to be the result of a change in the tracer lipid itself because of the pH drop (Harshman et al., 1989).

We studied the effect of pH on LF-induced membrane destabilization by following the release of the dye, calcein,

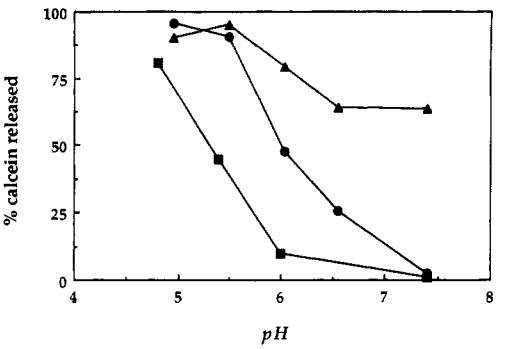

FIGURE 2: pH-induced calcein release by individual anthrax toxin components. LF, EF, or PA were incubated with asolectin LUVs (0.05 mg/mL) at the pH indicated, and calcein release was monitored as described under Experimental Procedures. Symbols: LF (●), EF (▲), PA (■).

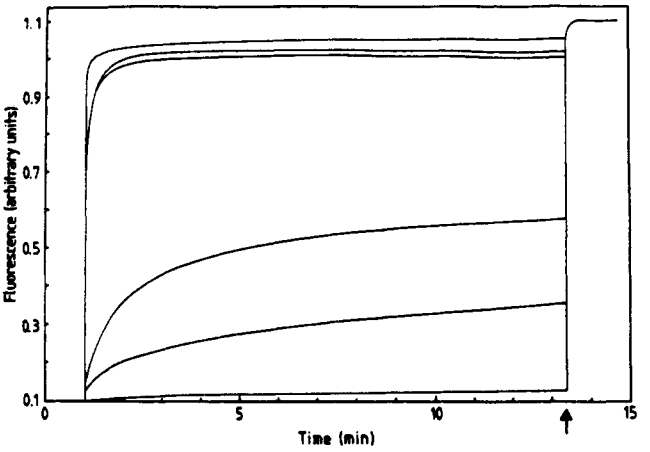

FIGURE 3: Kinetics of LF-induced liposome destabilization as a function of buffer pH. Calcein release was monitored as described under Experimental Procedures and maximum release determined by the addition of 0.1% Triton X-100 (arrow). pH values (top to bottom): 4.95, 5.05, 5.50, 6.05, 6.55, 7.20. All spectra were normalized against fluorescence values obtained at pH 7.2.

encapsulated in asolectin vesicles. No leakage of calcein was observed at neutral pH, after addition of the protein to the vesicles (Figure 2). However, a progressive enhancement of LF-induced calcein release was observed as the pH was lowered: LF induced an almost immediate release of calcein from vesicles, and fluorescence rapidly reached its maximum value (Figure 3). The pH values for 50% and maximal calcein release (pH 6.0 and 5.0–5.5, respectively) (Figure 2) were 0.5–1 pH unit lower than that observed at the transition pH[1] for LF labeling by the radiolabeled membrane probes. The extent of LF-induced calcein release was concentration dependent, and 50% calcein release was observed at a LF concentration of approximately 3.5×10^{-9} M (Figure 4) at pH 5.5, the pH at which maximum release was observed.

Conformational changes induced in LF by pH were studied by examining intrinsic fluorescence in the presence or absence of asolectin vesicles. Tryptophan-containing domains in LF did not appear to undergo significant structural changes as the buffer pH was lowered, and the λ_{max} of fluorescence of LF alone (336 nm) remained unchanged (data not presented). When incubated in the presence of asolectin vesicles, however, a blue shift to 331 nm was observed between the pH values 6.3 and 5.8 (Figure 5), within the range encompassing the threshold pH observed for LF-induced calcein release. We

[1] The transition pH is defined as the midpoint of the pH titration curve.

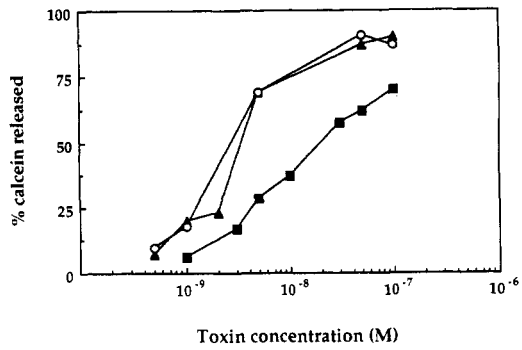

FIGURE 4: Concentration-dependent calcein release by anthrax toxin components. Increasing concentrations of LF, EF, or PA were incubated with asolectin LUVs at a pH which had been previously determined to give maximal release (see text). Calcein release was monitored as described under Experimental Procedures. Symbols: LF, pH 5.50 (○); EF, pH 5.50 (▲); PA, pH 4.95 (■).

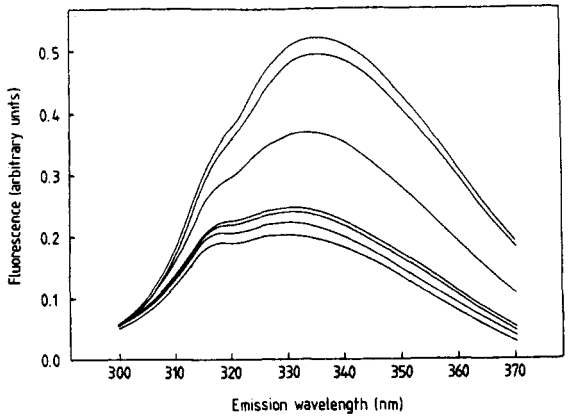

FIGURE 5: Fluorescence emission spectra of LF (4.4×10^{-7} M) incubated with asolectin LUV (0.1 mg/mL) at various buffer pHs (37 °C). Curves from top to bottom: pH 7.20, 6.80, 6.30, 5.80, 5.30, 4.80, 4.50.

were unable to determine the depth of tryptophan insertion using spin-labeled lipids (doxylpalmitoylstearoylphosphatidylcholine; Chattopadhyay & London, 1987), however, because of the quenching observed in the presence of unlabeled lipids (Figure 5).

Figure 6 shows the results when photolabeling experiments were performed with EF. We noted only a minimal dependence on pH for EF interaction with either the tritiated surface probe or the ^{14}C-labeled deep probe, and labeling by both markers was considerable even at neutral pH. The kinetics of calcein release were rapid and reached maximum value after only a few seconds of incubation (data not presented). EF-induced calcein release was also only minimally pH-dependent, and greater than 60% release was observed at neutral pH. The transition pH for EF was observed at approximately 6.0 (Figure 2). Calcein release induced by EF was concentration dependent but was apparently no more efficient than LF at optimal pH, since the transition concentration for both proteins was essentially the same (Figure 4).

Acidic pH did not appear to induce significant conformational changes in EF, and a λ_{max} value of 336 nm was observed at all pH values tested. As in the case of LF, however, λ_{max} blue shifted to 330 nm in the presence of asolectin LUV in the pH range 5.8–6.3 (data not presented).

Previous results have indicated that the proteolytically nicked form of PA, PA_{63}, undergoes a global change in hydrophobicity at low pH, allowing the protein to insert into lipid bilayers and forming ion-conducting channels. Channel

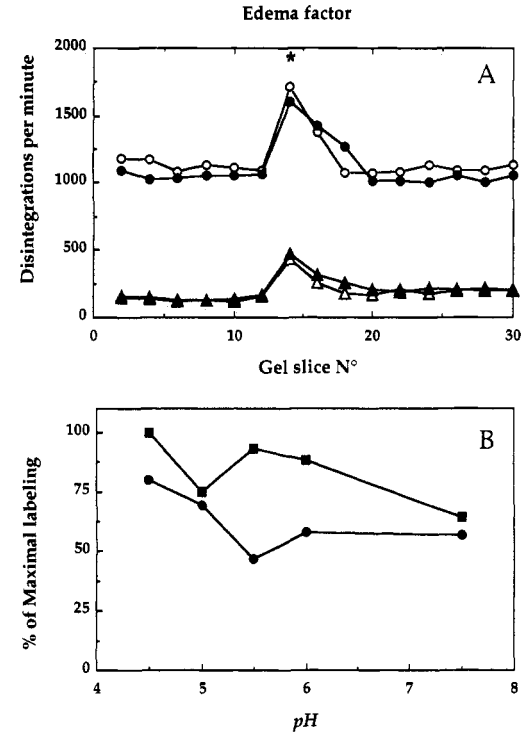

FIGURE 6: Radiolabeling of EF by the photoreactive phospholipids [^3H]PCI (circles) or [^{14}C]PCII (triangles). A. EF labeling profiles in the presence of asolectin SUVs in SDS-polyacrylamide gel slices at pH 7.50 (open symbols) or 4.50 (closed symbols). The asterisk indicates the position of EF on the Coomassie Blue-stained gel. B. PCI (●)/PCII (■) labeling of EF as a function of buffer pH. Radioactivity data are expressed as a percentage of maximal labeling, with the following normalized values (in dpm) taken as 100%: [^3H]-PCI, 1492; [^{14}C]PCII, 695.

formation is not observed with unnicked PA (Koehler & Collier, 1991; Blaustein et al., 1989). Figure 7 shows that unnicked PA was labeled by both radiolabeled probes, suggesting an interaction with both the surface of the vesicles and the acyl chain domain. While PA interacted with both probes to a minimal extent at neutral pH, the degree of insertion indicated a strong pH dependence. Maximum PA labeling by either probe was observed at pH 5.0, a value approximately 0.5–1 pH unit lower than that observed for LF. A strong pH dependence was observed for PA-induced calcein release from asolectin vesicles that paralleled the profile of membrane photolabeling (Figure 2). Covalent labeling of PA_{63} by both radiolabeled probes indicated a similar pH dependence to that of unnicked PA (maximum at pH 5.0), and membrane destabilization induced by PA_{63} produced a similar pH response curve (data not presented). PA- and PA_{63}-induced calcein release were dependent on protein concentration but were apparently less efficient in destabilizing the vesicles than either LF or EF, since 5–10 times more protein was required to produce a similar level of effect (Figure 4). The time course of calcein release induced by unnicked PA or PA_{63} was rapid and occurred on a similar time scale as that previously described for LF and EF (data not presented).

PA was characterized by a λ_{max} that varied from 331 to 333 nm, between pH 7.2 and 4.8. In the presence of lipid vesicles, λ_{max} remained unchanged regardless of the pH of the buffer (data not presented).

DISCUSSION

The mechanisms by which bacterial toxins enter sensitive eucaryotic cells are poorly understood. The most studied

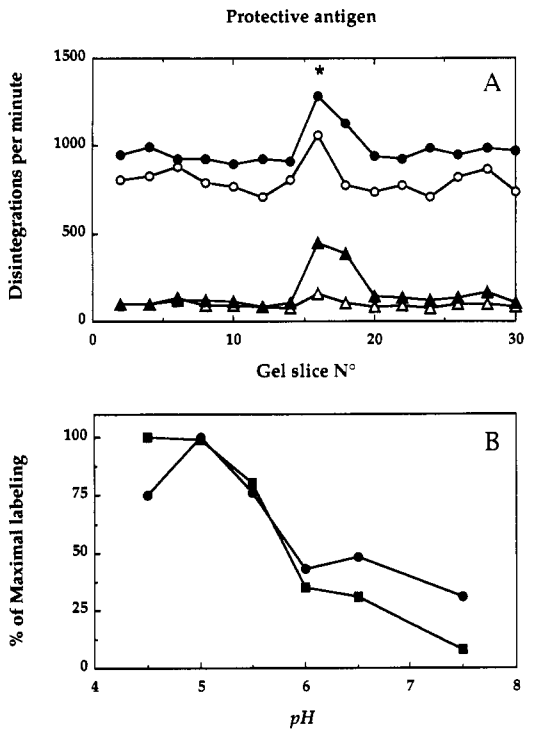

FIGURE 7: Radiolabeling of PA by the photoreactive phospholipids [^{3}H]PCI (circles) or [^{14}C]PCII (triangles). A. PA labeling profiles in the presence of asolectin SUVs in SDS-polyacrylamide gel slices at pH 7.50 (open symbols) or 4.50 (closed symbols). The asterisk indicates the position of PA on the Coomassie Blue-stained gel. B. PCI (●)/PCII (■) labeling of PA as a function of buffer pH. Radioactivity data are expressed as a percentage of maximal labeling, with the following normalized values (in dpm) taken as 100%: [^{3}H]-PCI, 862; [^{14}C]PCII, 664.

example is diphtheria toxin, the prototypical A–B type toxin. Diphtheria toxin enters cells through receptor-mediated endocytosis and is subsequently translocated across the endosomal membrane into the cytoplasm by a process requiring low pH. It has been suggested that the enzymic portion of the molecule plays a key role in toxin translocation (London, 1992). Recently, a number of studies have provided evidence which suggest that entry of the anthrax toxins may occur through the same or a similar pathway as diphtheria toxin. This led us to examine the contribution of LF and EF to the low pH-mediated translocation of lethal and edema toxin, using a model membrane system. Our results suggest that LF or EF may play an active role in translocation, much like the A fragment of diphtheria toxin (Montecucco et al., 1985), rather than being passively transferred into cytoplasm, as has been previously suggested.

LF is able to insert into and destabilize lipid membranes in the absence of PA, and both phenomena are optimized at acidic pH. The transition pH at which calcein release occurs, however, is about 1 pH unit lower than that observed for labeling by the membrane probes. This suggests that LF may undergo more than one pH-induced conformational change: one in which the protein interacts with the membrane and another in which the protein is competent to destabilize the lipid membrane. A similar phenomenon whereby membrane damage is composed of two separate events, membrane insertion and perturbation, has been described for staphylococcal α-toxin (Harshman et al., 1989). In this case, the transition pH for photoreactive radiolabeling differs from that of K^+ release and changes in global hydrophobicity by 1–1.5 pH units.

EF exhibits somewhat different behavior than LF or PA, and we observe only a minimal, but reproducible, pH dependence for either membrane destabilization or protein radiolabeling. These two events are maximal below pH 6.0. This suggests that the conformational change which takes place in the same pH range, as evidenced by a shift in EF fluorescence emission wavelength, is essential for maximal interaction of EF with the lipid vesicles. However, at pH 7.4, EF can efficiently insert into and destabilize the lipid vesicles, although no change in its fluorescence emission spectra can be detected. This suggests that the interaction of EF with lipid membrane may also proceed in two steps. In the first, EF may insert into and destabilize the membrane at neutral pH without undergoing conformational changes which would expose Trp residues to the membrane environment. This is supported by the fact that the maximum emission wavelength of EF in solution (336 nm) is characteristic of solvent-shielded Trp (Eftink & Ghiron, 1976). In the second step, occurring at a pH below 6.0, EF would adopt a conformation which allowed maximal destabilization of the membrane, and that was characterized by the exposure of EF Trp to the hydrophobic membrane environment.

Our observation that EF interacts with a lipid membrane in the absence of PA suggests that at some point in its evolution EF may have had the ability to interact with eucaryotic membranes in the absence of a ligand. In support of this hypothesis we have identified a truncated form of EF lacking the PA binding domain but that is capable of entering CHO-K1 cells (E. Labruyère, personal communication). These findings favor the hypothesis that this protein could be the fusion product of two genes, one coding for an adenylate cyclase and the other a PA-binding domain (Mock & Ullmann, 1993).

The intrinsic fluorescence spectra in solution of LF, EF, and PA indicate that λ_{max} is not strongly pH dependent and suggests that either low pH does not promote significant protein unfolding or that unfolding does not change significantly the environment of tryptophan residues. However, preliminary results from Fourier transformed infrared spectroscopy studies suggest that secondary structure is largely conserved (V. Cabiaux, unpublished observations). Since the intrinsic tryptophan fluorescence of all three proteins is quenched when the pH is lowered, low pH may induce oligomerization, as has been proposed for PA_{63} (Leppla, 1991; Milne et al., 1993). In the presence of lipids, all three proteins appear to undergo some pH-dependent structural changes, but again, secondary structure appears to be largely conserved (V. Cabiaux, unpublished observations). This suggests that insertion of these proteins may occur through the formation of a molten globule-folding intermediate, a transition state in which the elements of secondary structure remain largely conserved in spite of changes in protein tertiary structure (Ewbank & Creighton, 1991). It has been proposed that the bactericidal protein, colicin A, undergoes such a transition during the process of membrane insertion (van der Goot et al., 1991).

No significant membrane spanning domains have been identified in PA, EF, or LF (Leppla, 1991), allowing only speculation as to how these proteins are able to insert into membranes. The initial interaction between the toxin components and the membrane may be electrostatic, since each protein component interacts maximally at a transition pH close to its isoelectric point (Leppla, 1991). The effect of lipid binding may then serve to induce or increase toxin oligomerization, exposing localized hydrophobic surfaces that facilitate insertion as has been shown for the bacterial toxin aerolysin (Buckley, 1992; van der Goot et al., 1993).

Alternatively, lipid binding, by changing protein conformation, may increase the affinity of the anthrax toxin components for membrane receptors, as has been suggested for several bacterial protein toxins, including DT and the clostridial neurotoxins (Papini et al., 1987; Montecucco, 1986).

The results presented here would suggest that EF and LF have the capacity to interact independently with cellular membranes. However, the pH required to acid pulse EF (Gordon et al., 1988, 1989) or LF (Friedlander, 1986) into cells treated with chloroquine is characteristic of PA and not of LF or EF. This indicates that the translocation of EF and LF into the cytosol is dependent more on the interaction of PA with cellular components than with EF or LF. Thus, PA may play an important role at several stages in the intoxication process. For instance, it is well established that LF and EF interact with the cell surface only after binding to receptor-bound PA. In addition to fixing these two components on the cell surface, this interaction may also be required to relieve steric constraints imposed on the interaction between LF or EF and the membrane by any of a number of surface structures. This limitation would not be present in an artificial membrane system. The acidic pH values that are required for EF and LF to interact maximally with asolectin membranes are consistent with the model that these proteins pass through an acidic compartment at some point in the intoxication process. Therefore, at a subsequent stage, PA may also be involved in routing LF or EF through intracellular compartments. If this is the case, the pH-dependency differences that we observe may indicate that translocation of EF and LF to the cytoplasm occurs in different compartments. Finally, while EF or LF alone can destabilize membranes, the presence of PA seems to be essential for their translocation. This suggests that efficient insertion of PA into the membrane may require some local destabilization, perhaps mediated by EF or LF, that subsequently facilitates the translocation of the toxin complex across the endosomal membrane. This hypothesis is supported by the previous finding that PA_{63} is able to induce pore formation in black lipid bilayers, while LF and EF cannot.

ACKNOWLEDGMENT

We thank C. Montecucco for invaluable discussion. We are also grateful to J. M. Ruysschaert and C. Montecucco for critical reading of the manuscript. S.K.K. was supported by fellowships from the National Institutes of Health/INSERM Fogarty International program, and the Fondation pour la Recherche Médicale.

REFERENCES

Arora, N., & Leppla, S. H. (1993) *J. Biol. Chem. 268*, 3334–3341.

Blaustein, R. O., Koehler, T. M., Collier, R. J., & Finkelstein, A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2209–2213.

Buckley, J. T. (1992) *FEBS Lett. 307*, 30–33.

Chattopadhyay, A., & London, E. (1987) *Biochemistry 26*, 39–45.

Defrise-Quertain, F., Cabiaux, V., Vandenbranden, M., Wattiez, R., Falmagne, P., & Ruysschaert, J.-M. (1989) *Biochemistry 28*, 3406–3412.

Eftink, M. R., & Ghiron, C. A. (1976) *Biochemistry 15*, 672–680.

Escuyer, V., & Collier, R. J. (1991) *Infect. Immun. 59*, 3381–3386.

Ewbank, J. J., & Creighton, T. E. (1991) *Nature 350*, 518–520.

Ezzell, J. W., & Abshire, T. G. (1992) *J. Gen. Microbiol. 138*, 543–549.

Friedlander, A. M. (1986) *J. Biol. Chem. 261*, 7123–7126.

Gordon, V. M., Leppla, S. H., & Hewlett, E. L. (1988) *Infect. Immun. 56*, 1066–1069.

Gordon, V. M., Young, W. W., Jr., Lecher, S. M., Gray, M. C., Leppla, S. H., & Hewlett, E. L. (1989) *J. Biol. Chem. 264*, 14792–14796.

Harshman, S., Boquet, P., Duflot, E., Alouf, J. E., Montecucco, C., & Papini, E. (1989) *J. Biol. Chem. 264*, 14978–14984.

Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55–65.

Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477–5487.

Klimpel, K. R., Molloy, S. S., Thomas, G., & Leppla, S. H. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10277–10281.

Klimpel, K. R., Arora, N., & Leppla, S. H. (1993) Abstracts of the 93rd General Meeting of the American Society for Microbiology, Atlanta, GA, No. B-111.

Koehler, T. M., & Collier, R. J. (1991) *Mol. Microbiol. 5*, 1501–1506.

London, E. (1992) *Biochim. Biophys. Acta 1113*, 25–51.

Leppla, S. H. (1984) in *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*, (Greengard, P., Ed.) Vol. 17, pp 189–198, Raven Press, New York.

Leppla, S. H. (1991) in *Sourcebook of Bacterial Protein Toxins* (Alouf, J. E., & Freer, J. H., Eds.) pp 277–302, Academic Press, London.

Leppla, S. H., Friedlander, A. M., & Cora, E. (1988) in *Bacterial Protein Toxins* (Fehrenbach, F., Alouf, J. E., Falmagne, P., Goebel, W., Jeljaszewicz, J., Jurgen, D., & Rappuoli, R., Eds.), pp 111–112.

Milne, J. C., Hanna, P. C., Furlong, D., & Collier, R. J. (1993) Abstracts of the Second Workshop on pore-forming toxins, Mainz, Germany, *Medical Microbiol. Immunol. 182.*

Mock, M., & Ullmann, A. (1993) *Trends Microbiol. 1*, 187–192.

Montecucco, C. (1986) *Trends Biochem. Sci. 11*, 314–317.

Montecucco, C. (1988) *Methods Enzymol. 165*, 347–357.

Montecucco, C., Schiavo, G., & Tomasi, M. (1985) *Biochem. J. 231*, 123–128.

Mrsny, R. J., Volwerk, J. J., & Griffith, O. H. (1986) *Chem. Phys. Lipids 39*, 185–191.

Novak, J. M., Stein, M. P., Little, S. F., Leppla, S. H., & Friedlander, A. M. (1992) *J. Biol. Chem. 267*, 17186–17193.

Papini, E., Colonna, R., Schiavo, G., Cusinato, F., Tomasi, M., Rappuoli, R., & Montecucco, C. (1987) *FEBS Lett. 215*, 73–78.

Pezard, C., Duflot, E., & Mock, M. (1993) *J. Gen. Microbiol. 139*, 2459–2463.

Schiavo, G., Rossetto, O., Santucci, A., Dasgupta, B., & Montecucco, C. (1992) *J. Biol. Chem. 267*, 23479–23483.

Vallee, B. L., & Auld, D. S. (1990) *Biochemistry 29*, 5647–5659.

van der Goot, F. G., Gonzalez-Manas, J. M., Lakey, J. H., & Pattus, F. (1991) *Nature 354*, 408–410.

van der Goot, F. G., Pattus, F., Wong, K. R., & Buckley, J. T. (1993) *Biochemistry 32*, 2636–2642.