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# Secondary Structure of Anthrax Lethal Toxin Proteins and Their Interaction with Large Unilamellar Vesicles: A Fourier-Transform Infrared Spectroscopy Approach†

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ABSTRACT: Attenuated total reflection Fourier transform infrared spectroscopy has been used to study the secondary structure of anthrax lethal toxin proteins: protective antigen (PA) and lethal factor (LF), as a function of pH in the absence and in the presence of phospholipid vesicles. We first characterized the binding of LF and PA to the lipid membrane and demonstrated the strong pH dependence of the association of PA and LF to the lipid bilayer as well as the effect of pH neutralization on this binding. Binding of LF to the lipid membrane can be, at least partially, reversed when the pH is brought to neutral whereas in the same conditions PA binding is irreversible. Characterization of the conformational changes undergone by PA and LF upon pH lowering, lipid binding, and, in the case of LF, reversal of binding was carried out (i) by determining the secondary structure of the proteins and (ii) by evaluating their ability to undergo an hydrogen/deuterium exchange.

The two toxins produced by the bacterium *Bacillus anthracis* (Leppla, 1991) 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, 83 kDa), interacts separately with two A domains, edema factor (EF, 89 kDa) and lethal factor (LF, 83 kDa), to produce biologically distinct effects: PA−LF complex, designated as lethal toxin, leads to death in rats and other animals (Smith & Stanley, 1962) whereas PA−EF complex, designated as edema toxin, causes edema when injected intradermally (Harris-Smith et al., 1958). Neither EF nor LF can bind to cells if PA is not already present on the cell surface (Leppla et al., 1988), which suggests that PA plays a key role in cellular intoxication and that its binding to the cell surface is a critical step in this process.

According to the proposed pathways of EF and LF intoxication (Leppla, 1991), PA binds to a specific cell surface receptor of approximately 85−90 kDa, as identified on CHO-K1 cells (Escuyer & Collier, 1991). The bound PA is then submitted to proteolytic cleavage (Singh et al., 1989), probably by furin (Klimpel et al., 1992; Molloy et al., 1992). This cleavage generates two fragments of which the small one, a 20 kDa piece, is released in the outside medium and the larger one, a 63 kDa piece (PA63), remains attached to the receptor. After either EF or LF binding to PA63, the receptor−PA63−LF (or EF) complex is endocytosed by receptor mediated endocytosis. As previously demonstrated for diphtheria toxin (DT) (Draper & Simon, 1980; Sandvig & Olsnes, 1980), an acidic pH is crucial for intoxication, since the cells are completely protected from *B. anthracis* edema toxin and lethal toxin by pretreatment with ammonium chloride and chloroquine, which dissipate intracellular proton gradients and raise the pH of intracellular vesicles. This protection is reversible and is overcome by lowering the pH (Gordon et al., 1988; Friedlander, 1986). Both EF and LF are thought to act on intracellular targets. EF has been shown to be a calcium- and calmodulin-dependent adenylate cyclase, which causes a dramatic elevation of cAMP within cells (Leppla, 1982, 1984; Labruyère et al., 1990). Recent evidences that protease inhibitors prevent the intoxication of macrophages by lethal toxin and that LF is fully inactivated by mutagenesis of a zinc binding site suggest that LF is a metalloprotease (Klimpel et al., 1994) whose target still remains to be identified.

By analogy with DT, where both the A and B domains are directly involved in the interaction with the endosomal membrane in a pH-dependent manner [for a review, see London (1992)], we have previously studied the ability of all three anthrax toxin proteins to interact with a lipid bilayer. All three proteins were able to independently insert into the asolectin lipid bilayer and destabilize the membrane at low pH (Kochi et al., 1994). Moreover, it was demonstrated that PA63 is able to form cation-selective channels in planar phospholipid bilayers (Blaustein et al., 1989), and to induce the release of markers from vesicles and cells at acidic pH (Koehler & Collier, 1991; Milne & Collier, 1993; Milne et

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[1] Abbreviations: PA, protective antigen; LF, lethal factor; EF, edema factor; DT, diphtheria toxin; DTA, diphtheria toxin A fragment; DTB, diphtheria toxin B fragment; LUV, large unilamellar vesicle(s); ATR-FTIR, attenuated total reflection Fourier-transform infrared spectroscopy; CD, circular dichroism; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; SDS, sodium dodecyl sulfate.

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al., 1994). A central question in the anthrax toxin system is how the three proteins interact with the lipid bilayer and how this interaction can lead to the translocation of EF and LF to the cell cytoplasm. In the case of DT, low pH has been shown to induce a conformational change in the protein structure which exposes at its surface hydrophobic domains which were cryptic at neutral pH (Defrise-Quertain et al., 1989). Such hydrophobic domains have never been identified in any of the three anthrax proteins, although lowering the pH results in a slight increase of PA, PA63, and LF hydrophobicity as evidenced by the binding of a hydrophobic fluorescent probe (Koehler & Collier, 1991).

To provide further insight about the mechanism of interaction of the lethal toxin components (PA+LF) with a lipid membrane, we investigated the pH dependence of the binding of PA and LF to a lipid membrane, and the reversibility of this association. Fourier transform infrared spectroscopy (attenuated total reflection method) (FTIR-ATR) was used to study the secondary structure of the components of the lethal toxin (PA and LF) in the absence and in the presence of lipids at different pH. Finally, we studied the accessibility to solvent of the free and the lipid-bound proteins. The data obtained are compared with those already obtained for DT and discussed in terms of the intoxication pathway of the lethal toxin.

## MATERIALS AND METHODS

*Materials.* Asolectin (mixed soybean phospholipids) was obtained from Sigma Chemical Co. (St. Louis, MO), and was purified according to the method of Kagawa and Racker (1971). $D_2O$ was from Merck (Germany). Acrylamide was from Bio-Rad. All other reagents were of the highest purity available.

*Purification of Anthrax Lethal Toxin Proteins*. The anthrax lethal toxin proteins (PA and LF) were produced by double mutant strains of *B. anthracis*, in which two of three toxin genes *cya* and *lef*; *cya* and *pag* were respectively inactivated by intragenic deletion. These strains were capable of producing only a single toxin component and were used to avoid the possible contamination from the two other proteins (Pezard et al., 1993). The culture and the purification were carried out as previously described (Pezard et al., 1993). Both PA and LF were eventually dialyzed against a 10 mM Tris/HCl buffer, pH 8.0. The purity of proteins was over 90% as judged by a 10% polyacrylamide−SDS gel stained with Coomassie Blue. In FTIR experiments, the proteins were dialyzed against a 2 mM Hepes buffer at pH 7.2 overnight, and stored at −20 °C.

*Liposome Preparation*. Asolectin was kept as a stock solution in chloroform (100 mg/mL). A film of asolectin was formed on a glass tube, and after an overnight drying under vacuum, the film was rehydrated in a 150 mM NaCl, 10 mM Hepes, pH 7.2, buffer. Large unilamellar vesicles (LUV) were formed by an extrusion procedure (pores: 0.1 $\mu$m diameter) according to Hope et al. (1985). The concentration of lipids was determined by measuring the lipid phosphorus content (Mrsny et al., 1986).

*Association of Protein with LUV*. Forty micrograms of PA or LF was mixed with 300 $\mu$g of LUV and incubated 1 h at 37 °C. The pH was then lowered to the desired pH (4.0, 5.0, or 6.0) by addition of a predetermined volume of 1 mM HCl. After another hour of incubation at 37 °C, the samples were then mixed with an equal volume of 80% sucrose, and overlaid with a 30−2% linear sucrose gradient. The sucrose had been previously dissolved in a 10 mM Hepes, 150 mM NaCl solution at pH 4.0, 5.0, 6.0, and 7.2. The pH was adjusted by NaOH. An overnight centrifugation at 125000*g* at 4 °C in a Beckman L7 ultracentrifuge with an SW60 rotor allowed the complete separation of free protein from lipid-bound protein. The gradients were then fractionated from the bottom to the top of the tube, and the phospholipid and protein distributions were determined respectively by choline dosage (Test combination phospholipids, Boehringer Mannheim Biochemia) and by measuring the Trp fluorescence ($\lambda_{exc}$ = 280 nm and $\lambda_{em}$= 340 nm) using a JY3D (Jobin Yvon) spectrofluorometer. To prepare the samples for the FTIR study, the fractions which contained both lipids and proteins were pooled, centrifuged, and washed twice with a 2 mM Hepes, pH 5.0, which was adjusted with NaOH, in a Centricon-30 tube (Amico, Inc., Beverly, MA) at 5000*g* to remove the sucrose. The protein-containing liposomes were eventually resuspended in 100 $\mu$L of the same buffer. To study the reversibility of the association of proteins with LUV, the initial quantity of proteins and LUV was doubled. After 1 h of incubation at pH 5.0 or pH 6.0, the solution was divided into two samples: one was kept at low pH; the other one was brought back to pH 7.2 by addition of a predetermined volume of 1 mM NaOH. These two samples were then submitted to the sucrose gradient as described above. The pH was checked using a PHG-1 microelectrode (Physitemp Instruments Inc.).

*Infrared Spectroscopy*. Attenuated total reflection infrared spectra (resolution of 4 $cm^{-1}$) were obtained on a Perkin-Elmer 1720X FTIR spectrophotometer as previously described (Goormaghtigh et al., 1994b). Measurements were carried out at room temperature. Thin films were obtained by slowly evaporating a sample under a stream of nitrogen on one side of the ATR plate (Fringeli & Günthard, 1981; Goormaghtigh & Ruysschaert, 1990). The ATR plate was then sealed in a universal sample holder (Perkin-Elmer 186-0354) and deuterated by flushing the sample compartment with $D_2O$-saturated $N_2$ at room temperature for 90 min. The hydrogen/deuterium exchange allows differentiation of the α-helix from the random structure, whose absorption bands shift from about 1655 $cm^{-1}$ to about 1642 $cm^{-1}$ (Rothschild & Clark, 1982; Cortijo et al., 1982). The determination of the secondary structure of proteins was carried out by analysis of the deuterated amide I region as described previously (Goormaghtigh et al., 1990a, 1994a,b; Cabiaux et al., 1989). The frequency limits for the different structures were as follows: 1662−1645 $cm^{-1}$, α-helix; 1689−1682 $cm^{-1}$ and 1637−1613 $cm^{-1}$, $\beta$-sheet; 1644.5−1637 $cm^{-1}$, random; 1682−1662.5 $cm^{-1}$, $\beta$-turns.

*Kinetics of Deuteration*. The experimental procedure was carried out as previously described (Goormaghtigh et al., 1994b). The pH of each sample was checked to ensure that it was equal to 5.0; if it was not, the pH was brought to 5.0 with diluted HCl or NaOH. The samples were spread on a germanium plate as described above. Before starting the deuteration, 10 spectra were recorded in order to verify the stability of the measurements and the reproducibility of the system. At time zero, a $D_2O$-saturated $N_2$ flux was applied to the sample, with a flow rate of 75 mL/min controlled with a Brooks flow meter. The spectrophotometer was driven by a computer program. The spectra at each time point were

the accumulation of 12 scans with a resolution of 4 $cm^{-1}$. The background due to the atmospheric water contribution was computed as described either in Goormaghtigh et al. (1994b) or in Powell et al. (1986) and was subtracted from each spectrum. The two approaches generated very similar subtraction coefficients. The amide I and II band areas were measured between 1702−1596 $cm^{-1}$ and 1585−1502 $cm^{-1}$, respectively. The amide II area was divided by the amide I area for each spectrum in order to take into account any change in the total intensity of the spectra during the deuteration process. This ratio, which was expressed between 0 and 100%, was plotted versus deuteration time. The 100% value is defined by the amide II/amide I ratio obtained before deuteration whereas the 0% value corresponds to a zero absorption in the amide II region. It has been shown previously (de Jongh et al., 1995; de Jongh et al., submitted for publication; Raussens et al., 1996) on a series of proteins which can be fully denaturated state (and therefore fully deuterated in the denaturated state) and then refolded to their original structure, that complete H/D exchange resulted in 0 ± 5% absorption in the amide II region. We are therefore confident that a zero absorbance in the amide II region reflects the full deuteration of the protein.

## RESULTS

*pH-Dependent Lipid Association of Anthrax Lethal Toxin Proteins.* The anthrax lethal toxin belongs to the group of the A−B-type toxins which, after internalization by the target cells, are believed to interact with the lipid membrane of an endocytotic compartment and to cross that membrane to reach their target in the cell cytoplasm. To characterize this interaction, we have studied the pH-dependent association of the components of *B. anthracis* lethal toxin with lipid vesicles. PA or LF was incubated with asolectin LUV at different pHs (pH 7.2, 6.0, 5.0, and 4.0) as described under Materials and Methods. The samples were run on a sucrose gradient, and the amount of proteins and lipids was determined in each fraction.

At pH 7.2, there is no association of either PA or LF to the lipid membrane (Figure 1, A1 and B1). The free proteins remain at the bottom of the tube whereas the "empty" liposomes are found between fractions 7 and 14. These protein and lipid distributions are characteristic of the proteins or the lipid vesicles alone, whatever the pH (not shown). On the contrary, when the pH is lowered to 6.0 (Figure 1, A2 and B2) or 5.0 (Figure 1, A3 and B3), both proteins are associated to the lipid vesicles. Because the quantum yield of both PA and LF is strongly dependent upon pH and the presence of lipids (Kochi et al., 1994), the Trp fluorescence was of no use for determining the proportion of the lipid-bound protein. We therefore made use of the FTIR technique to build a calibration curve as described in Goormaghtigh et al. (1990a). Briefly, increasing quantities of LF were mixed with a fixed quantity of asolectin LUV, and the FTIR spectra of these samples were recorded. The calibration curve was established by plotting log (area amide I/area $\nu_{C=O}$ lipids) versus log (protein/lipid w/w). The gradient fractions containing both lipids and proteins were then collected (Figure 1, A2, A3, B2, and B3) (pH 4.0 not shown) and studied by FTIR. The protein/lipid ratio in these samples was determined by measuring the area amide I/area $\nu_{C=O}$ lipids and by reporting this value on the calibration curve.

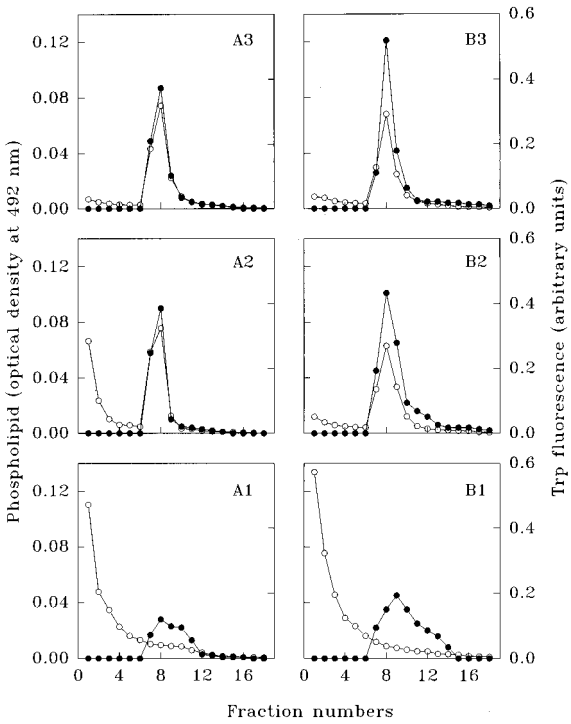


FIGURE 1: Sucrose gradient profiles of PA and LF incubated with liposomes at different pHs. 40 $\mu$g of PA or LF was mixed with 300 $\mu$g of asolectin LUV, and the sample was prepared as described under Materials and Methods. Eighteen fractions were collected from the bottom to the top of the sucrose gradient and measured for protein (○) and lipid (●) contents. Panel A is PA incubated with liposomes at pH 7.2 (A1), 6.0 (A2), and 5.0 (A3). Panel B is LF incubated with liposomes at pH 7.2 (B1), 6.0 (B2), and 5.0 (B3).

The percentage of association of PA to the lipid membrane (Figure 1, A) is pH-dependent, and has been determined as described above using the calibration curve: 64% at pH 6.0, 96% at pH 5.0, and 65% at pH 4.0. For PA, the maximum association was observed at pH 5.0 whereas it diminished at pH 4.0, suggesting that as the pH is lowered there is a competition between protein self aggregation and binding to the lipid vesicles. For LF, we found that 100% of LF which was added to the lipid suspension is lipid-associated when the incubation was performed at pH 6.0 or below (Figure 1, B).

By analogy with the DT system where the B fragment binding to a lipid membrane at low pH is mostly irreversible (60−70%; Montecucco et al., 1985), we have also tested the reversibility of association of PA with the lipid membrane. To test this property, PA was mixed with asolectin vesicles at pH 5.0 and 6.0 as previously described. After an hour of incubation, the mixtures were divided in two: half was kept at pH 5.0 and 6.0; another half was brought back to pH 7.2. The two samples were then run on a sucrose gradient as described under Materials and Methods. According to the determination of the protein/lipid ratio by FTIR, 96% and 64% of PA was associated to the lipid fractions when the pH was kept at pH 5.0 and 6.0, respectively. When the pH was brought back to 7.2, in both cases, 100% of the bound protein (which corresponds to 64% of the total protein quantity when the incubation was carried out at pH 6.0) was still associated to the lipid vesicles after the pH rise (Figure 2, A), suggesting the irreversibility of PA binding to the lipid vesicles.

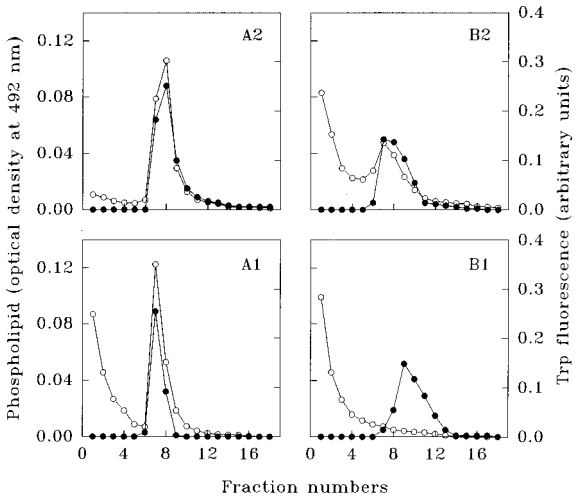


FIGURE 2: Reversibility of the association of PA and LF with lipid vesicles. 80 $\mu$g of PA (A) or LF (B) was mixed with 600 $\mu$g of asolectin LUV, and the sample was prepared as described under Materials and Methods. All samples were run on a sucrose gradient. The protein (○) and lipid (●) distributions are shown at pH 7.2 after pH reversal from pH 6.0 (A1 and B1) and pH 5.0 (A2 and B2).

Since LF is considered as the A moiety possessing the enzymatic activity, it is probably able to pass through the lipid membrane and enter into the cytosol where it exhibits its activity in the environment of neutral pH. In this model, its association to liposomes should be reversible, as the association of diphtheria toxin A fragment has been demonstrated to be fully reversible (Montecucco et al., 1985). An experiment in which LF was associated to liposomes at either pH 5.0 or pH 6.0 and then the pH was brought back to 7.2 has been carried out in the conditions as described above. We found that 100% of LF was associated to the lipid fractions when the pH was kept at 5.0 and 6.0 (not shown). When the pH was brought back to 7.2, the pattern of association of LF to the lipid vesicles was depended on the initial pH of incubation. Indeed, when the sample had been prepared at pH 6.0, increasing the pH back to 7.2 resulted in a complete release of the proteins from the lipid vesicles (Figure 2, B1). This release was only partial (42%) when the starting pH was 5.0 (Figure 2, B2), suggesting two different types of association of LF to the lipid vesicles as a function of pH.

*Effect of pH and the Presence of Lipids on the Structure of Anthrax Lethal Toxin Proteins.* One of the main questions to be solved when studying the mechanism of intoxication of bacterial toxins is dealing with the understanding of the factors that allow the protein to move from a soluble form in the outside medium to a membrane-associated form. For *B. anthracis* lethal toxin, pH is a key factor in mediating the interaction with the lipid membrane. To provide further insight into the mechanism by which this interaction is taking place, we have studied the effect of pH and the presence of lipids on the structure of PA and LF.

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) has been successfully used to investigate the structure of soluble and membrane proteins (Goormaghtigh & Ruysschaert, 1990; Cabiaux et al., 1989; Goormaghtigh et al., 1987, 1990a,b, 1993; Sonveaux et al., 1994; Vandenbussche et al., 1992; Challou et al., 1994). This method is based on the analysis of the vibration bands of protein and particularly the amide I band, $\nu$(C═O), whose

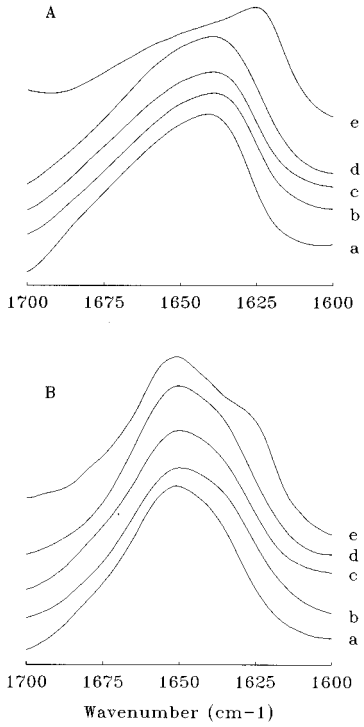


FIGURE 3: Deuterated infrared spectra of PA and LF as a function of pH and the presence of a lipid membrane. 40 $\mu$g of either PA (A) or LF (B) was used for each sample whose pH was adjusted to 7.2 (a), 6.0 (b), 5.2 (c), and 4.0 (d). Each sample was incubated for 30 min at the desired pH before being spread at the surface of a germanium plate. (e) PA and LF were incubated at pH 5.0 in the presence of asolectin LUV (see Materials and Methods). All spectra have been rescaled in the region between 1700 and 1600 cm$^{-1}$ to the same amplitude and smoothed with a 5 cm$^{-1}$ wide Gaussian line shape.

frequency of absorption is dependent upon the secondary structure.

Figure 3A represents the FTIR-ATR deuterated spectra of PA recorded at different pHs (pH 7.2, 6.0, 5.2, and 4.0). All four deuterated spectra look similar which suggests that no significant secondary structure change is taking place upon lowering the pH. A similar conclusion was drawn from CD data (data not shown). Whatever the pH, the main absorption band within the amide I band (1637−1635 cm$^{-1}$) is located in a region associated to the $\beta$-sheet structure, suggesting that this structure is predominant in PA. As a comparison, the maximum of absorption of the *E. coli* porin, a mainly $\beta$-sheet protein, is located at 1634 cm$^{-1}$ (Goormaghtigh et al., 1990a). A curve-fitting procedure performed as previously described (Cabiaux et al., 1989) gives percentages of 28 ± 5% and 38 ± 5%, respectively, for the α-helical and $\beta$-sheet structures of PA, whatever the pH.

The deuterated FTIR-ATR spectra of LF as a function of pH are shown in Figure 3B. The shape of the deuterated amide I of four spectra was very similar, indicating that LF secondary structure is not dependent upon the pH. The main absorbance (1652 cm$^{-1}$) is assigned to the α-helical structure, and the curve-fitting procedure gives percentages of 39 ± 5% and 18 ± 5%, respectively, for the α-helical and $\beta$-sheet structures. Therefore, PA and LF seemed to contain both α-helical and $\beta$-sheet structures but the $\beta$-sheet structure is predominant in PA whereas LF is characterized by a high content of α-helical structure.

To study the effect of the presence of a lipid membrane on the secondary structure of PA and LF, the two proteins

were incubated with asolectin LUV at pH 5.0, a pH at which most of the protein is lipid-bound (Figure 1). The membrane-bound proteins were separated from the free proteins by centrifugation on a sucrose gradient. In the presence of lipids, for both PA and LF, a shoulder was identified at 1626 $cm^{-1}$ (Figure 3e). The significance of this shoulder will be discussed later. Evaluation of the secondary structures gives a content of α-helical and $\beta$-sheet structures of respectively 38 + 5% and 51 + 5% for PA and 38 + 5% and 34 + 5% for LF. Similar results were obtained when the experiment was carried out at pH 6.0 (data not shown). To rule out a possible artifact due to lipid absorption, we have recorded the spectra of asolectin vesicles at both pH 7.2 and pH 5.0 as well as the spectra of PA and LF mixed with asolectin vesicles at pH 7.2. Whatever the pH, asolectin showed no absorption between 1700 and 1600 $cm^{-1}$, and the spectra of PA and LF mixed with lipid vesicles in conditions in which there is no interaction with the lipid membrane did not display the 1626 $cm^{-1}$ shoulder (data not shown).

The above structure determination shows that pH has little effect on the secondary structure of PA and LF whereas binding to the lipid membrane is characterized in both cases by an increase of $\beta$-sheet and/or α-helical content. Moreover, lowering the pH has been demonstrated to increase the hydrophobicity of both PA and LF and to induce the oligomerization of PA (Koehler & Collier, 1991; Milne et al., 1994). This suggests that upon pH lowering, some region of the proteins might be reorganized (without any secondary structure modification) in order to provide the right conformation to interact with the lipid bilayer which, in turn, will induce a modification of the secondary structure. To further characterize these conformational changes, we have followed the kinetics of deuteration of PA and LF in the presence or absence of a lipid membrane. Indeed, with constant experimental conditions (pH, secondary structure, and temperature), the rate of hydrogen/deuterium exchange is related to the solvent accessibility of the NH amide groups of the protein, and consequently to the tertiary structure of the proteins and to the stability of the secondary structures. Peptide hydrogen exchange of the proteins was followed by monitoring the amide II absorption peak [$\delta$(N−H) maximum in the 1596−1502 $cm^{-1}$ region] decrease because of its shift to the 1460 $cm^{-1}$ region [amide II′, $\delta$(N−D)] upon deuteration (Figure 4). We measured, therefore, the kinetics of H/D exchange of the two components of the lethal toxin with and without lipids at pH 5.0 (Figure 4A−D).

Figure 5 represents the percentage of deuteration of PA and LF in the presence or absence of lipids, calculated from the ratio of amide II/amide I (from Figure 4) as described under Materials and Methods. In PA and LF at pH 5.0, about 60% of the residues are exchanged upon deuteration. The presence of liposomes does not significantly modify the extent of H/D exchange undergone by PA (Figure 5A), indicating that the accessibility to solvent is not modified by the interaction with a lipid bilayer. This suggests either that a very low number of residues are protected by the lipid membrane, in addition to those which are buried in the protein, or that a similar number of residues that were protected in the free protein because of the folding are now protected from the solvent because of their insertion in the lipid membrane. The lipid-bound LF is undergoing a slightly faster exchange than free LF (Figure 5B), which suggests that the membrane form has a structure more accessible to solvent than the protein in solution.

As demonstrated above, most of the lipid-bound LF was released from the membrane when the pH was brought back to neutral. To study the structure of the released LF, a sample in which the pH had been reversed to 7.2 was centrifuged on a sucrose gradient as described under Materials and Methods. The fractions containing the released LF (fraction numbers 1−3 in Figure 2, B2) were collected, and sucrose was eliminated by centrifugation. Clearly, the passage from lipid-bound (Figure 6, a) to lipid-released protein (Figure 6, b) was accompanied by the disappearance of the shoulder at 1626 $cm^{-1}$. The shape of the spectrum of dissociated LF (Figure 6, b) and that of native LF at pH 7.2 (Figure 6, c) were very similar, suggesting an identity in the secondary structure. However, the kinetics of H/D exchange of dissociated LF at pH 7.2 are slower than the kinetics of exchange of the native protein at the same pH (Figure 7). Moreover, the standard deviation of the set of experiments shown in Figure 7 is higher than the standard deviation characteristic of the other experiments (Figure 5), suggesting that samples in which the pH has been reversed to pH 7.2 are less homogeneous than other samples (see Discussion).

## DISCUSSION

The mechanism by which bacterial toxins cross their target membrane to reach the cytosol is far from being molecularly described. The system for which more information is available is the translocation of diphtheria toxin across the endosomal membrane. DT is the classical representation of the family of so-called "A-B type toxins" [for a review, see Olsnes et al. (1990)]. These toxins have two moieties, usually linked by a disulfide bridge: an effector moiety carrying the biological activity (A) and a moiety (B) involved in cell binding and in the translocation of the A moiety across the target membrane. *B. anthracis* toxins are more complex systems because there are one B moiety (PA) and two effectors (LF and EF) and these three proteins are secreted independently from each other. Nevertheless, previous studies have suggested that their mode of internalization was closely related to that of the other "A-B type" toxins: a receptor-mediated endocytosis followed by translocation across the membrane of an acidic organelle in response to the acid environment [for a review, see Leppla (1991)]. In this paper, we have characterized the mechanism of association of the two components of *B. anthracis* lethal toxin PA and LF to a lipid membrane by studying the pH dependence of binding as well as the effect of pH and of the presence of lipids on the secondary structure and accessibility to the solvent of the two proteins.

In a previous paper, we had demonstrated that PA and LF were both able to deeply insert in a lipid bilayer and to induce the release of an encapsulated fluorescent dye in the lipid vesicles. Both phenomena were pH-dependent, with PA requiring a slightly lower pH (about 0.5 pH unit) than LF to interact with the lipid bilayer (Kochi et al., 1994). Accordingly, we have demonstrated here that PA required pH 5.0 to be fully bound to the lipid membrane whereas LF already shows 100% of association at pH 6.0.

We have studied the secondary structure of both PA and LF as a function of pH. The shape of the spectra of both proteins is characteristic of an α/$\beta$-type protein although the

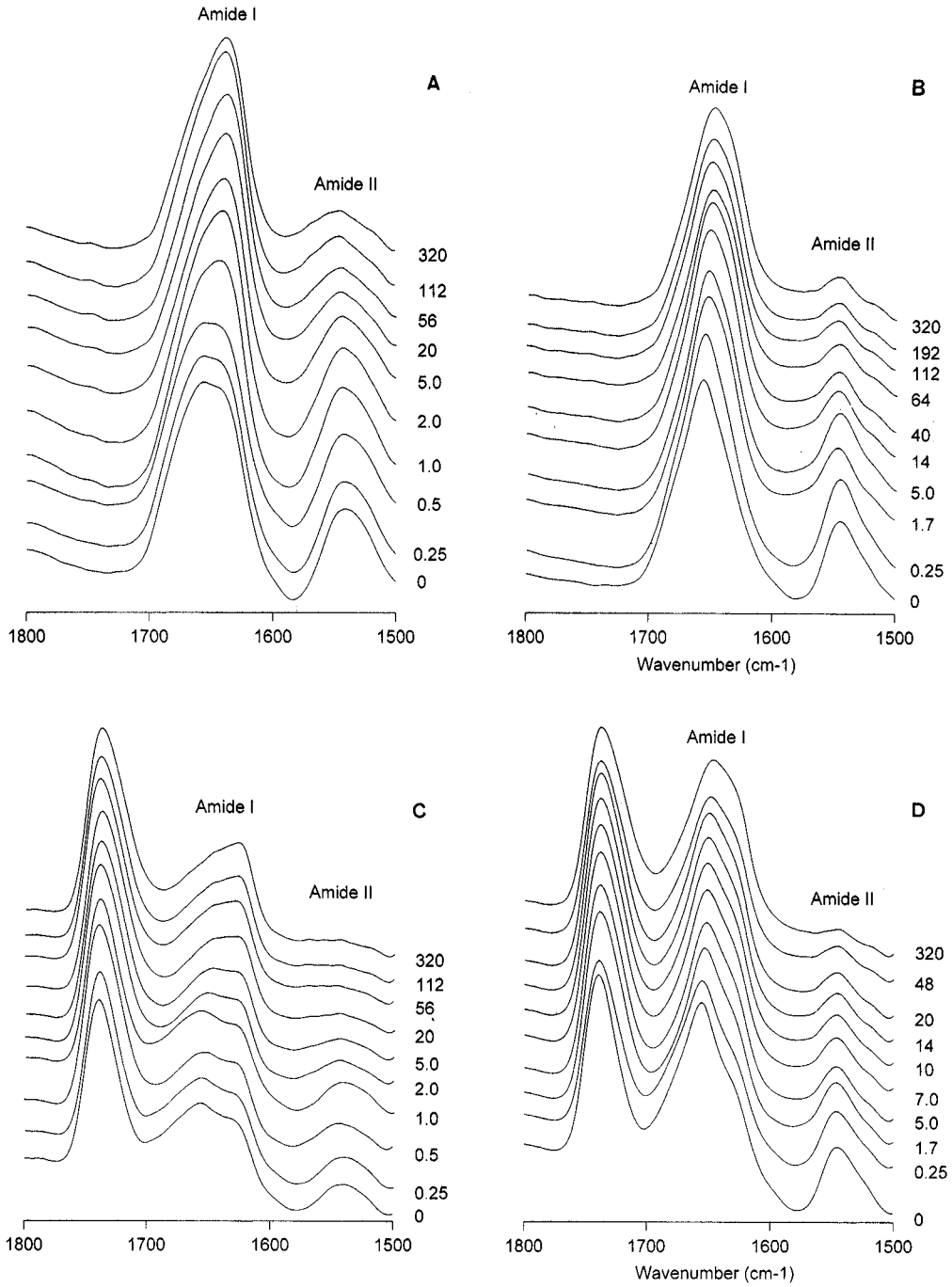


FIGURE 4: Infrared spectra between 1800 and 1400 $cm^{-1}$ of free and lipid-associated PA and LF (protein:lipid ratio of 1:7.5 w/w) at pH 5.0. Spectra were recorded as a function of time of exposure to $D_2O$-saturated $N_2$ flow, which is indicated in minutes at the right side of the figure. (A) PA; (B) LF; (C) lipid-associated PA; and (D) lipid-associated LF. Each group of kinetic spectra is from one of three (for panels A, C, and D) or four (for panel B) independent experiments.

shape of the PA spectrum reveals the presence of a predominant $\beta$-sheet structure whereas the shape of the LF spectrum indicates that LF contains a higher amount of $\alpha$-helical structure than $\beta$-sheet structure. The secondary structure of both PA and LF is not modified when the protein is incubated at pH 6.0 or below. However, in both cases, incubation with lipid vesicles at low pH resulted in the appearance of a component at 1626 $cm^{-1}$. Such a component has been attributed to $\beta$-sheet structure. The low-frequency absorption suggests that the $\beta$-strands are forming strong hydrogen bonds which may arise from intermolecular bonds formed between aggregated or oligomerized proteins (Jackson & Mantsch, 1995). Since this component is not observed in the absence of lipids and since we have removed the free proteins from the lipid-containing samples, our data suggest that a conformational change is taking place upon binding of PA and LF to the lipid membrane. This conformational change could involve the formation of intermolecular bonds between oligomerized proteins at the surface or in the lipid membrane. The hypothesis of the PA and LF oligomerization is in agreement with previous fluorescence studies which have demonstrated that the Trp fluorescence of these two proteins was quenched when the proteins were incubated at low pH in the presence of a lipid bilayer (Kochi et al., 1994). Moreover, it has been shown that PA63 exists as a heptamer when incubated at low pH or when associated to CHO-K1 cells (Milne et al., 1994).

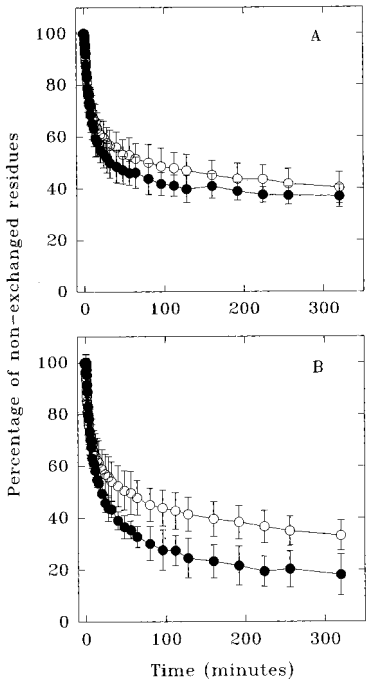


FIGURE 5: Evolution of the proportion of non-exchanged residues as a function of the deuteration time. PA (panel A) and LF (panel B) were studied by kinetics of H/D exchange of FTIR with (●) and without (○) lipids at pH 5.0. The film of the samples spread on a germanium plate was exposed to a $D_2O$-saturated $N_2$ flux, and the spectra were recorded automatically by increasing time as described under Materials and Methods. The amide II/I area ratio for each spectrum which was expressed between 0 and 100% was reported as a function of deuterated time. Each curve is the average of three or four experiments, and the error bars represent the standard deviation.

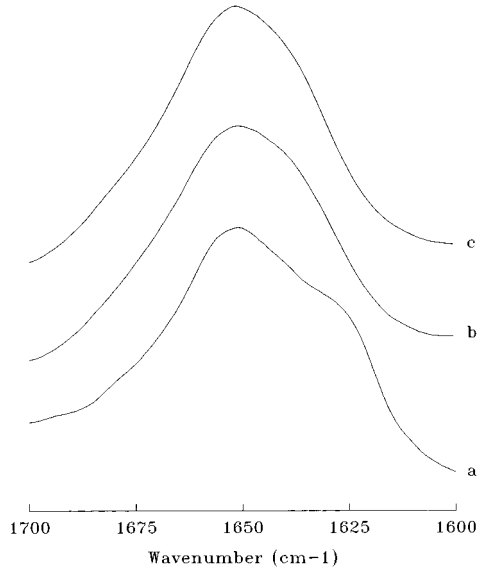


FIGURE 6: Deuterated infrared spectra of LF dissociated from liposomes. (a) Lipid-bound LF at pH 5.0 (from Figure 4B, b); (b) dissociated LF from the first three fractions at the bottom of the sucrose gradient (Figure 2, B2); (c) LF at pH 7.2 (from Figure 3B, a).

LF has been proposed to be a metalloprotease whose site of action is still unknown. By analogy with DT, where the enzymatic fragment has to be released in the cytoplasm after its translocation across the endosomal membrane, we have studied the reversibility of the association of LF and PA to the lipid membrane when the pH was brought back to 7.2. Interestingly, at both pH 6.0 and 5.0, PA was irreversibly

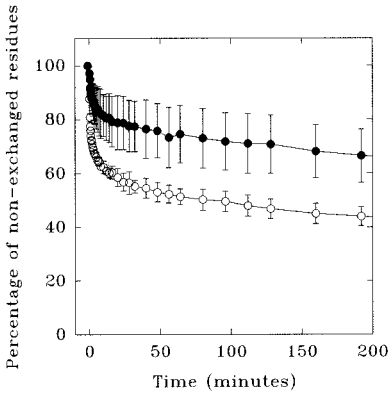


FIGURE 7: Evolution of the proportion of non-exchanged residues as a function of the deuteration time. Native (○) and dissociated (●) LF at pH 7.2. Each curve is the average of three independent experiments, and the error bars represent the standard deviation.

bound to the lipid membrane whereas LF was released in the outside medium. Such a behavior had been previously observed for DTB and DTA respectively, which reinforces the parallel made between the two systems. The secondary structure of released LF was found to be identical to the structure of LF at pH 7.2, suggesting not only the reversibility of lipid binding but also the reversibility of secondary structure modification observed upon lipid binding. The H/D exchange of released LF at pH 7.2 is slower than the exchange of the native protein at the same pH, suggesting that the tertiary (or quaternary) structure adopted by LF when bound to the lipid membrane at low pH cannot be reversed. Whether the H/D exchange of released LF at pH 7.2 is characteristic of a tertiary structure that would exist at pH 5.0 cannot be inferred from our data since the exchange by itself is strongly pH-dependent (Rosenberg, 1986). Interestingly, although the binding of LF to a lipid membrane reaches 100% at pH 6.0 and 5.0, the reversibility of binding is strongly dependent upon the pH at which incubation has been performed, suggesting that the interaction with the lipid membrane is different at pH 6.0 and 5.0. This is in agreement with the previous finding that although LF was fully labeled by membrane-inserted probes at pH 6.0 its maximum efficiency in inducing calcein release from large unilamellar vesicles was observed at pH 5.0 (Kochi et al., 1994). Since the structure of lipid-bound LF is identical at pH 6.0 and 5.0, we can rule out the possibility of a secondary structure conformational change being responsible for the observed effect. At pH 6.0, only the histidine residues are protonated, whereas at pH 5.0 protonation of acidic residues can occur. This protonation could increase the hydrophobicity of LF, making the protein more efficient (through an oligomerization process?) in its interaction with the lipid membrane. As an example, protonation of glutamic acid and aspartic acid residues is required for the correct insertion of DT into a lipid membrane (Silverman et al., 1994). Whether or not the LF-competent form for translocation across the endosomal membrane is the pH 6.0 or 5.0 form remains to be determined.

The data provided here as well as the comparisons with the mechanism of intoxication of DT are providing some insight about the biological activity of the lethal toxin of *B. anthracis*. It has been demonstrated that the first step of this mechanism is the binding of PA to cell surface receptors, followed by a cleavage which generates the nicked form of PA, PA63 (Leppla, 1991). The cleavage unmasks a binding

site for either LF or EF. The complex (PA/LF or PA/EF) is then endocytosed by receptor-mediated endocytosis (Leppla, 1991). The question of the routing of these complexes is of importance. The ability of LF to efficiently interact with a lipid membrane in the absence of PA (Kochi et al., 1994, and this report) supports the hypothesis of the direct involvement of LF in its routing, by interacting with the lipid bilayer which does not exclude an interaction with a component of the endocytotic pathway. This interaction could involve a conformational change of LF and/or an oligomerization of the protein. Once the complex has reached the right compartment, PA would help LF to cross the membrane by a mechanism which is, so far, poorly understood. However, the slight increase in the H/D exchange ability in the presence of the lipid membrane indicates that LF may have the capability of partially unfolding in order to cross the membrane. Protein unfolding has been previously proposed as a key step in the translocation of the endosomal membrane by diphtheria toxin A fragment (London, 1992). After translocation and because of the neutral pH of the cytoplasm, LF would be released from the membrane, having a structure which is characteristic of its structure at pH 7.2, before the pH pulse. On the contrary, PA would remain associated to the lipid membrane.

This paper provides new and important information about the structure of PA and LF in relation with their biological activity. It should provide a basis for future studies on the mechanism of translocation of *B. anthracis* lethal toxin across the endosomal membrane.

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## REFERENCES

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

Cabiaux, V., Brasseur, R., Wattiez, R., Falmagne, P., Ruysschaert, J. M., & Goormaghtigh, E. (1989) *J. Biol. Chem. 264*, 4928−4938.

Challou, N., Goormaghtigh, E., Cabiaux, V., Conrath, K., & Ruysschaert, J. M. (1994) *Biochemistry 33*, 6902−6910.

Cortijo, M., Alonso, A., Gomez-Fernandez, J. C., & Chapman, D. (1982) *J. Mol. Biol. 157*, 597−618.

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

de Jongh, H. H. J., Goormaghtigh, E., & Ruysschaert, J. M. (1995) *Biochemistry 34*, 172−179.

Draper, R. K., & Simon, M. I. (1980) *J. Cell Biol. 87*, 849−854.

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

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

Fringeli, U. P., & Günthard, H. H. (1981) *Mol. Biol. Biochem. Biophys. 31*, 270−332.

Goormaghtigh, E., & Ruysschaert, J. M. (1990) in *Molecular Description of Biological Components by Computer Aided Conformational Analysis* (Brasseur, R., Eds.) pp 285−329, CRC Press, Boca Raton, FL.

Goormaghtigh, E., Brasseur, R., Huart, P., & Ruysschaert, J. M. (1987) *Biochemistry 26*, 1789−1794.

Goormaghtigh, E., Cabiaux, V., & Ruysschaert, J. M. (1990a) *Eur. J. Biochem. 193*, 409−420.

Goormaghtigh, E., De Meutter, J., Cabiaux, V., Szoka, F., & Ruysschaert, J. M. (1990b) *Eur. J. Biochem. 195*, 421−429.

Goormaghtigh, E., Cabiaux, V., De Meutter, J., Rosseneu, M. Y., & Ruysschaert, J. M. (1993) *Biochemistry 32*, 6104−6110.

Goormaghtigh, E., Cabiaux, V., & Ruysschaert, J. M. (1994a) *Subcell. Biochem. 23*, 329−450.

Goormaghtigh, E., Vigneron, L., Scarborough, G. A., & Ruysschaert, J. M. (1994b) *J. Biol. Chem. 269*, 27409−27413.

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

Harris-Smith, P. W., Smith, H. & Keppie, J. (1958) *J. Gen. Microbiol. 19*, 91−103.

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

Jackson, M., & Mantsch, H. H. (1995) *Crit. Rev. Biochem. Mol. Biol. 30*, 95−120.

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. (1994) *Mol. Microbiol. 13*, 1093−1100.

Kochi, S. K., Martin, I., Schiavo, G., Mock, M., & Cabiaux, V. (1994) *Biochemistry 33*, 2604−2609.

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

Labruyère, E., Mock, M., Ladant, D., Michelson, S., Gilles, A. M., Laoide, B., & Bârzu, O. (1990) *Biochemistry 29*, 4922−4928.

Leppla, S. H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3162−3166.

Leppla, S. H. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res. 17*, 189−198.

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. M. (1988) in *Bacterial Protein Toxins* (Fehrenbach, F., Alouf, J. E., Falmagne, P., Goebel, W., Jeljaszewicz, J., Jurgen, D., & Rappuoli, R., Eds.) pp 111-112, Gustav Fischer, New York.

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

Milne, J. C., & Collier, R. J. (1993) *Mol. Microbiol. 10*, 647−653.

Milne, J. C., Furlong, D., Hanna, P. C., Wall, J. S., & Collier, R. J. (1994) *J. Biol. Chem. 269*, 20607−20612.

Molloy, S. S., Bresnahan, P. A., Leppla, S. H., Klimpel, K. R., & Thomas, G. (1992) *J. Biol. Chem. 267*, 16396−16402.

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.

Olsnes, S., Stenmark, H., Moskaug, J. O., McGill, S., Madshus, I. H., & Sandvig, K. (1990) *Microbiol. Pathogen. 8*, 163−168.

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

Powell, J. R., Wasacz, F. M., & Jacobsen, R. J. (1986) *Appl. Spectrosc. 40*, 339−344.

Raussens, V., Narayanaswami, V., Goormaghtigh, E., Ryan, R. O., & Ruysschaert, J. M. (1996) *J. Biol. Chem. 271*, 23089−23095.

Rosenberg, A. (1986) *Methods Enzymol. 127*, 630−648.

Rothschild, K. J., & Clark, N. (1982) *Methods Enzymol. 88*, 696−714.

Sandvig, K., & Olsnes, S. (1980) *J. Cell Biol. 87*, 828−832.

Silverman, J. A., Mindell, J. A., Finkelstein, A., Shen, W. H., & Collier, R. J. (1994) *J. Biol. Chem. 269*, 22524−22532.

Singh, Y., Chaudhary, V. K., & Leppla, S. H. (1989) *J. Biol. Chem. 264*, 19103−19107.

Smith, H., & Stanley, J. L. (1962) *J. Gen. Microbiol. 29*, 517−521.

Sonveaux, N., Conrath, K., Capiau, C., Brasseur, R., Goormaghtigh, E., & Ruysschaert, J. M. (1994) *J. Biol. Chem. 269*, 25637−25645.

Vandenbussche, G., Clercx, A., Clercx, M., Curstedt, T., Johansson, J., Jörnvall, H., & Ruysschaert, J. M. (1992) *Biochemistry 31*, 9169−9176.

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