Structure and Topology of Diphtheria Toxin R Domain in Lipid Membranes†

ARTICLE *in* BIOCHEMISTRY · DECEMBER 1998

Impact Factor: 3.02 · DOI: 10.1021/bi9818624 · Source: PubMed

Structure and Topology of Diphtheria Toxin R Domain in Lipid Membranes†

Pierre Quertenmont,‡,§ Christian Wolff,‡ Ruddy Wattiez,|| Patrick Vander Borght,‡ Paul Falmagne,|| Jean-Marie Ruysschaert,‡ and Véronique Cabiaux*,‡

Laboratoire de Chimie Physique des Macromolécules aux Interfaces, CP 206/2, Université Libre de Bruxelles, Boulevard du Triomphe, B-1050 Brussels, Belgium, and Service de Chimie Biologique, Université de Mons-Hainaut, Avenue du Champ de Mars 6, 7000 Mons, Belgium

Received August 4, 1998; Revised Manuscript Received October 19, 1998

ABSTRACT: The interaction of the receptor-binding domain (R domain) of diphtheria toxin with a pure lipid membrane has been characterized by several approaches. Using a photoactivatable lipid, the R domain has been shown to deeply insert in the lipid membrane. Three regions of the R domain (residues 380−421, 422−441, and 442 to about 483) are protected by their interaction with the membrane from externally added proteases. At least one of these regions is deeply interacting with the lipid membrane, as evidenced by the location of Cys 461 and 471 determined by fluorescence experiments. Binding of the R domain to the lipid membrane is characterized by the appearance of an α-helical component whose orientation is compatible with a transmembrane orientation.

Bacterial protein toxins are secreted as soluble proteins that need to interact with a target membrane (plasma, endosomal, Golgi..., membranes) to exert their biological activity. Two main strategies are used by toxins to interact with the lipid membrane [for a review, see Cabiaux et al. (*1*)]:

(1) Toxins such as diphtheria toxin or the *E. coli* colicin are characterized by the presence of a bundle of α-helices containing easily identifiable hydrophobic sequences. In response to a modification of the environment, the hydrophobic helices are exposed at the surface of the protein and, then, interact with the lipid membrane.

(2) In toxins such as the α-toxin of *S. aureus* or the aerolysin of *A. hydrophila*, no hydrophobic sequences can be identified. These toxins mainly contain *β*-sheets and have been shown to generate hydrophobicity by oligomerization. In the membrane, each monomer participates in the formation of a *β*-barrel.

Diphtheria toxin (DT) has been crystallized as a monomer and its X-ray structure determined to 2.3 Å resolution (*2*). It has a Y shape in which three domains can be clearly distinguished: (i) the C domain (fragment A), a region containing α-helices and *β*-sheets carrying the enzymatic activity (ADP-ribosylation of elongation factor 2); (ii) the T domain, structurally organized as a bundle of α-helices, essential for the translocation of the A fragment across the endosomal membrane, and (iii) the R domain, the receptor-binding domain, exclusively containing *β*-sheets.

The interaction of the T domain of DT with the lipid membrane has been extensively characterized. It is now believed that, in response to the low-pH environment of the endosomes, two hydrophobic helices (TH8 and TH9) insert into the lipid membrane. They are the only helices required for the channel formation associated with the insertion of DT in artificial membranes (*3*, *4*) or the plasma membrane of Vero cells (*5*, *6*). The role in DT insertion and/or translocation of three other helices of the T domain (TH1, TH6, and TH7) is still a matter of debate (*7*). On the contrary, little attention has been paid to the interaction of the R domain with a lipid membrane since this domain was not believed to play a role in translocation. However, it has been demonstrated that the R domain increases its hydrophobicity below pH 6.0 (*8*). Moreover, when DT is incubated with pure lipid vesicles, the R domain is partially protected by the membrane from externally added proteases (*7*, *9*) and it translocates across that membrane (*10*), as evidenced by antibody binding. This has prompted us to characterize in more detail the interaction of the isolated R domain with a lipid membrane. This fragment can be easily purified and could be used as a model to understand the mechanism of translocation of a *β*-sheet domain across a lipid membrane. In this paper, we combined proteolytic and fluorescence experiments as well as a structural characterization to probe the topology of the R domain in the lipid membrane.

MATERIALS AND METHODS

Materials. Proteinase K, DL-α-dipalmitoylphosphatidylcholine (DPPC),[1] L-α-dipalmitoylphosphatidic acid (DPPA), and asolectin (mixed soybean phospholipid) were from Sigma Chemical Co. (St. Louis, MO). Asolectin was purified according to Kagawa and Racker (*11*). Phenylmethylsulfonyl fluoride (PMSF) was from Serva. PVDF (problott) membranes were obtained from Applied Biosystems, and acrylamide was from Biorad. AMCA-HPDP (*N*-[6-(7-amino-4-methylcoumarin-3-acetamido)hexyl]-3′-(2′-pyridyldithio)pro-

† V.C. is a research associate of the National Fund for Scientific Research (FNRS, Belgium). P.V. and C.W. are fellows of the Fonds pour la recherche dans l'industrie et l'agriculture (FRIA, Belgium).

* To whom correspondence should be addressed. Phone: 32 2 6505365. Fax: 6505382. Email: vcabiaux@ulb.ac.be.

‡ Université Libre de Bruxelles.

§ Present address: ULB Interface, Université Libre de Bruxelles, CP 161, 50 Avenue F. Roosevelt, B-1050 Brussels, Belgium.

|| Université de Mons-Hainaut.

10.1021/bi9818624 CCC: $18.00
Published on Web 12/19/1998

pionamide) was a Pierce product. ^{14}C-PCII (specific radioactivity, 174 Ci/mol) was a gift of Dr. C. Montecucco (University of Padova, Italy), and the plasmid pGEX-2T-DTR was kindly provided by Dr. R. J. Collier (Harvard Medical School, Boston, MA). All other reagents and products were of the highest purity.

R Domain Purification. The R domain was purified from *E. coli* containing the plasmid pGEX-2T-DTR coding for the GST−R domain fusion protein containing a cleavage site for thrombin between the GST and the R domain. The purification was mostly carried out as described in Shen et al. (*12*) with some modifications. The protein expression was induced for 4 h at 30 °C by the addition of 0.1 mM IPTG to a bacterial suspension at $OD_{600} = 1$. The bacteria (*1l*) were then centrifuged 10 min at 5000*g* (18 °C, Sorvall RC 26 Plus), and the pellet was resuspended in 10 mL of a 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, buffer (referred to as buffer A), in the presence of 100 μg/mL lysozyme. The cells were sonicated 3 times for 15 s on ice (40 W, Sonifier B-12, Branson Sonic Power Cy.) and lysed by the addition of 1.5% Triton X-100 (final concentration). The lysate was centrifuged at 10000*g* for 10 min (4 °C). The supernatant was incubated 30 min at room temperature with 2 mL of Sepharose-4B−glutathione under gentle stirring and the unbound material eliminated by filtration in a Poly-Prep column (Bio-Rad). The resin was washed 4 times with 10 mL of buffer A, 1 time with 10 mL of 20 mM Tris-HCl, 500 mM NaCl, pH 7.5, and 5 times with 10 mL of 20 mM Tris-HCl, 150 mM NaCl, 2.5 mM $CaCl_2$, pH 7.5 (buffer B), and suspended in 500 μL of buffer B containing 1% thrombin. After a 1 h incubation at room temperature under gentle stirring, the R domain-containing eluate was collected. To increase the R domain recovery yield, the resin was washed 5 times with buffer B.

For infrared spectroscopy experiments, the R domain was further purified on a cation-exchange Mono S HR 5/5 column (Pharmacia Biotech) equilibrated with a 10 mM Hepes−NaOH, 100 mM NaCl, pH 7.5, buffer. The protein was eluted with a linear salt gradient at a flow rate of 1 mL/min.

pH Dependence of Lipid Binding. A 360 μg aliquot of R domain purified with the Sepharose-4B−glutathione resin was incubated 1 h with 3 mg of asolectin LUV at pH 7.5 (buffer A). Aliquots of this sample were taken and their pH ajusted to 5.0, 5.5, and 6.0. After a further 3 h incubation under the same conditions, the samples were mixed with an equal volume of 80% sucrose at the corresponding pH and run overnight on a 30−2% sucrose gradient (4 °C, 35 000 rpm) to remove the unbound proteins. The proteoliposomes were washed, suspended in water, and spread at the surface of a germanium plate to be analyzed by FTIR (see below). The 0.1 μm asolectin LUV were prepared in buffer A by an extrusion procedure described by Hope et al. (*13*). The concentration of lipids was determined by measuring the lipid phosphorus content (*14*), and the protein concentration, by the Lowry assay (*15*).

Membrane Photolabeling. Asolectin LUV containing the radioactive photoactivatable phospholipid ^{14}C-PCII (a phosphatidylcholine analogue bearing a photoactivatable nitroarylazido group at the methyl terminus of one fatty acyl chain and three ^{14}C at the choline head) (1.03×10^6 dpm/mg of asolectin) (*16*) were prepared as described above. All manipulations were performed under a red safety light; 360 μg of R domain was incubated with 3 mg of LUV for 1 h at 37 °C. When needed, the pH was lowered to 5.0 by addition of a predetermined volume of 1 M sodium acetate−acetic acid, pH 5.0. After 3 h of incubation at 37 °C, the samples were irradiated 20 min with an Osram HQV 125 W UV lamp, and, then, delipidated by a chloroform−methanol extraction (*17*). The protein-containing aqueous phase was analyzed by SDS−PAGE [14%, Laemmli (*18*)]. The gel was stained with Coomassie blue, soaked for 20 min in a fluorographic reagent (Amplify, Amersham), dried at 60 °C for 10 h, and set in the presence of a Biomax MR (Kodak) film (3 weeks, −80 °C) for autoradiography.

To study the reversibility of the R domain membrane insertion, the sample incubated 3 h at pH 5.0 was brought back to pH 7.5 by addition of a predetermined volume of 3 M Tris-HCl, pH 7.7, and incubated for 10 more min in the dark before UV irradiation.

Proteolysis Experiments. Domain R proteoliposomes were prepared at pH 5.0 as described above and run on a 30−2% sucrose gradient. For the proteolysis experiment, 2% (w/w) proteinase K (1 mg/mL) was added to a proteoliposome suspension in 20 mM sodium acetate−acetic acid, 150 mM NaCl, pH 5.0, buffer (final volume: 200 μL). The proteolysis was performed for 2 h at 37 °C, and stopped with 8 μL of PMSF (20 mg/mL in ethanol). After another centrifugation in a sucrose gradient to remove the unbound proteolyzed peptides and removal of the sucrose by washing in the low-pH buffer, the vesicles were suspended in 400 μL of the same buffer, and the phospholipids were extracted according to Bligh and Dyer (*17*). The aqueous phase (including the interface) was concentrated and run on a Tris−Tricine−SDS−polyacrylamide (16.5% T, 6% C) electrophoresis gel (*19*). After migration, the protein material was electrotransferred onto a PVDF membrane (2 h, 24 V) and subsequently stained with Coomassie blue. The peptide bands were cut off the membrane, and the peptide's N-terminal sequences were determined by automated Edman degradation using a Beckman LF3400 protein−peptide microsequencer equipped with an on-line Gold 126 microgradient HPLC system and a Model 168 Diode Array detector (Beckman Instruments, Inc., Fullerton, CA). All samples were sequenced using standard Beckman sequencer procedure 4. The phenylthiohydantoin amino acid derivatives were quantitatively identified by reverse phase HPLC on an ODS spherogel micro column (3 μm diameter particles, 2 × 150 mm, Beckman Instruments). All sequencing reagents were from Beckman.

Cysteine Labeling. Binding of the fluorescent probe *N*-[6-(7-amino-4-methylcoumarin-3-acetamido)hexyl]-3′-(2′-pyridyldithio)propionamide (AMCA-HPDP, 1 mg/mL in DMSO) was performed on the R domain in solution and the R domain reconstituted in lipid vesicles, before and after proteolysis. Before addition of AMCA-HPDP, the protein had to be reduced to generate the free thiols. The reduction step was

[1] Abbreviations: AMCA-HPDP, *N*-[6-(7-amino-4-methylcoumarin-3-acetamido)hexyl]-3′-(2′-pyridyldithio)propionamide; ATR-FTIR, attenuated total reflection Fourier transform infrared spectroscopy; DPPC, DL-α-dipalmitoylphosphatidylcholine; DPPA, L-α-dipalmitoylphosphatidic acid; LUV, large unilamellar vesicles; PVDF, poly(vinylidene difluoride); PMSF, phenylmethylsulfonyl fluoride; IPTG, isopropyl β-D-thiogalactoside; GST, glutathione *S*-transferase; PCII, 2-{12-[*N*-(4-azido-2-nitrophenyl)]aminododecanoyl}-1-myristoyl-*sn*-glycero-3-phospho[^{14}C]choline.

carried out for 2 h at room temperature in the presence of a 100-fold molar excess of dithiothreitol (DTT) in a 20 mM Tris, 150 mM NaCl, 2 mM EDTA, pH 7.0, buffer in the absence or presence of 6 M guanidine hydrochloride. The reducing agent was removed by centrifugation of the samples through a G50 mini column. The labeling step of the protein was carried out for 1 h at room temperature in the presence of a 20-fold molar excess of AMCA-HPDP and, when proteoliposomes were involved, in the absence or presence of 0.15% Triton X-100. The unreacted AMCA-HPDP was eliminated by gel filtration on a G50 mini column equilibrated with 0.1% SDS in water. The fluorescence associated with the R domain was measured with an SLM 8000 spectrofluometer (λ_{ex} = 345 nm; λ_{em} = 450 nm), and the protein concentration was determined by Trp fluorescence using a standard curve obtained with the R domain in 0.1% SDS.

Fluorescence Spectroscopy. The fluorescence of the R domain reconstituted in proteoliposomes (3×10^{-6} M protein, 20 mM sodium-acetate−acetic acid, 150 mM NaCl, pH 5.0, buffer) before and after proteolysis was determined at 37 °C and under stirring with an SLM 8000 spectrofluometer (λ_{ex}: 280 nm).

Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR). Attenuated total reflection infrared spectra (resolution of 4 cm^{-1}) were obtained with a Perkin-Elmer 1720X FTIR spectrophotometer as previously described (*20*). Measurements and sample deuteration (1 h) were carried out as described in Wang et al. (*21*): hydrogen/deuterium exchange allows differentiation of the α-helix from the random structure, whose absorbance bands shift from about 1655 cm^{-1} to about 1642 cm^{-1} (*22*, *23*). The determination of the secondary structure of proteins was carried out by analysis of the deuterated amide I region as described previously (*24*). The frequency limits for the different structures were as follows: 1662−1645 cm^{-1}, α-helix; 1689−1682 and 1637−1613 cm^{-1}, *β*-sheet; 1645−1637 cm^{-1}, random coil; and 1682−1662 cm^{-1}, *β*-turns. The control spectra of the 2 mM Hepes buffer, pH 7.2, and asolectin vesicles at both pH 7.2 and pH 5.0 showed no absorbance between 1700 and 1600 cm^{-1} (data not shown). The determination of molecular orientations in membranes by infrared ATR spectroscopy was performed as described by Goormaghtigh and Ruysschaert (*25*). Spectra are recorded with two orthogonally polarized lights, and a preferential orientation of the C═O dipole will be reflected by a maximum of absorption at one of the two polarizations.

Sample Preparation. The R domain obtained after HPLC purification was concentrated (Microcon-3, Amicon), and the buffer was replaced by a 5 mM Hepes, 50 mM NaCl, pH 7.0, buffer. The R domain proteoliposomes prepared as described above were washed and suspended in 50 *μ*L of water acidified at pH 5.0 with HCl. All samples were spread at the surface of a Germanium plate and dried under N_2 flux.

RESULTS

The R domain was purified on the basis of the protocol described by Shen et al. (*12*). The procedure was modified as described under Materials and Methods, leading to a yield of 1.5 mg of purified R domain per liter of cell culture. The SDS−PAGE diagram of the purified R domain is shown in Figure 1A, and the protein has been estimated by N-terminal microsequence analysis to be 90% pure. This sample was used for all experiments except the determination of secondary structure and orientation in the lipid membrane by FTIR which requires a higher degree of purity. The sample shown Figure 1A was loaded on a Mono S HR5/5 cation exchange column and eluted with a NaCl gradient. The R domain eluted as a single peak at a salt concentration of about 450 mM NaCl and gave a single band in SDS−PAGE (Figure 1B).

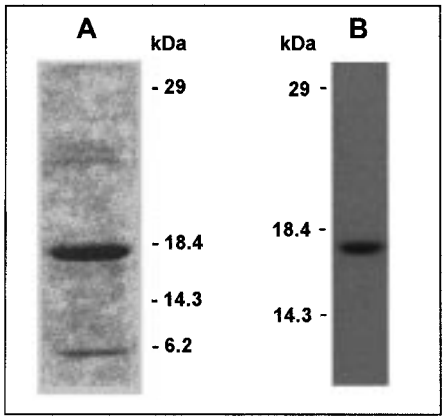

FIGURE 1: Purification of the R domain. 15% SDS−PAGE gel electrophoresis of samples from (A) Sepharose 4B−glutathione chromatography and (B) Mono S chromatography (cation exchange) of the sample shown in (A).

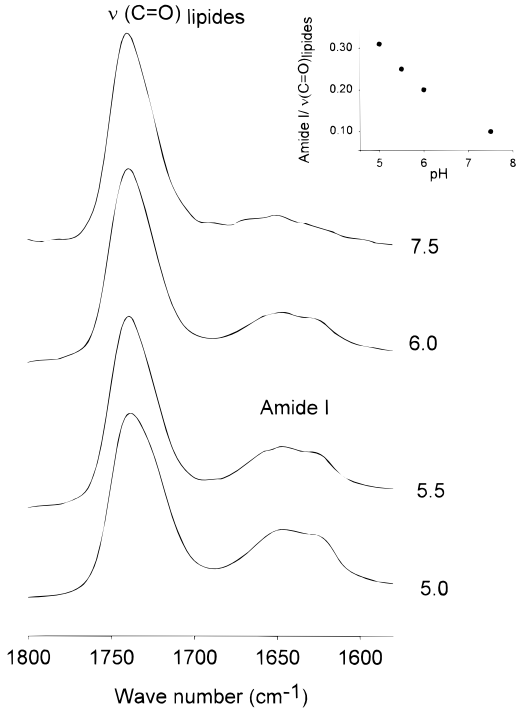

FIGURE 2: FTIR spectra of proteoliposomes of the R domain prepared at different pHs. Insert: Ratio of the areas of $\nu C{=}O_{lipids}$ and $\nu C{=}O_{proteins}$ as a function of pH.

Figure 2 shows the FTIR spectra of proteoliposomes containing the R domain, prepared at different pHs and run on a sucrose gradient to remove the free proteins. The infrared absorption of the $\nu C{=}O_{lipids}$ and $\nu C{=}O_{proteins}$ (amide I) is dependent on the respective concentrations of lipids and proteins. The ratio of the areas of these two absorption peaks therefore allows the evaluation of the lipid/protein ratio of

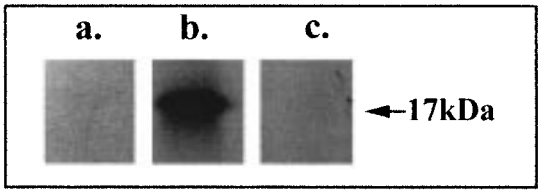

FIGURE 3: R domain incubated with proteoliposomes containing ^{14}C-PCII. Autoradiography of a 14% SDS−PAGE gel. (a) pH 7.5. (b) pH 5.0. (c) pH 7.5 after prior acidification at pH 5.0.

the samples. The R domain binding was clearly pH-dependent, with little binding at pH 7.2 and 100% binding at pH 5.0 (insert Figure 2). The 100% binding was obtained by direct lipid and protein dosages of the sample prepared at pH 5.0, and the other values were expressed from the areas of the $\nu C{=}O_{lipids}$ and $\nu C{=}O_{proteins}$.

The ability of the isolated R domain to deeply interact with a lipid membrane was investigated by incubating the R domain at pH 7.5 and 5.0 with liposomes containing a radioactive and photoactivatable lipid [^{14}C-PCII (*16*)]. This lipid contains a nitroarylazido group at the extremity of one of its two acyl chains. Under illumination, the azide is converted to a highly reactive nitrene that forms covalent bonds with the lipid and protein molecules of its environment. Autoradiography revealed that the R domain is labeled by PCII only upon incubation at pH 5.0 (Figure 3b), which demonstrates its deep insertion into the lipid membrane. No labeling is observed at pH 7.5 (Figure 3a), which indicates that the low level of binding observed at neutral pH (Figure 2) does not lead to a deep membrane insertion. Interestingly, when the pH of the sample was raised to 7.2 before illumination, no labeling of the R domain could be detected (Figure 3c), suggesting that its insertion in the lipid membrane is reversible.

The location of the R domain regions involved in the interaction with the membrane was investigated by proteolysis experiments. R domain-containing liposomes prepared as described under Materials and Methods were treated with 2% proteinase K (w/w). Incubation times were from 0 to 24 h. The samples were then analyzed by electrophoresis on a Tris−Tricine gel (16.5% T, 6% C), and a 2 h digestion was found sufficient to obtain a stable pattern of digestion (data not shown). A sample obtained after 2 h of digestion was then run on an electrophoresis gel as described above (Figure 4). Seven bands, with molecular masses ranging from 17 kDa to about 6 kDa, were identified. Bands E, F, and G are not clearly apparent on the figure, but they were unambiguously identified on the gel. The 17 kDa band (A) corresponds to the full-length protein indicating that proteolysis was not complete. The bands were transferred onto a PVDF membrane, excised from the membrane, and microsequenced for N-terminal amino acid sequence determination (Table 1). Some bands contained more than one peptide. Three N-terminal moieties have been clearly identified: Gly380 (B1, F1, and G), Ala 422 (B2, D1, and F2), and Asp442 (B3, C, D2, E, and F3); Thr 421 and Leu 441 could respectively be the C-terminal amino acids of G and F1, on the basis of the apparent molecular masses of these peptides.[2] Other cleavage points should be located probably between Val 483 and Ser 535 (the C-terminal ends of the other peptides).[2] This shows that there are three regions with no cleavage site: residues 380−421, residues 422−441, and residues 442 to around 483.

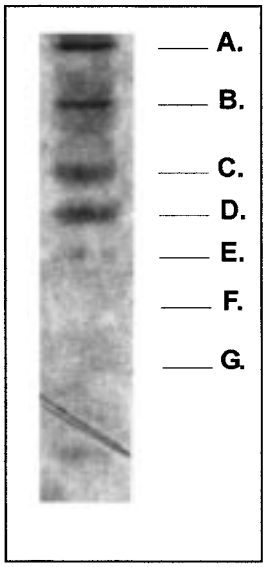

FIGURE 4: Silver staining of a R domain-proteolyzed sample [2 h, 2% proteinase K (w/w)] run on a 16.5% T, 6% C Tris−Tricine−polyacrylamide gel.

Table 1: N-Terminal Sequences of the Protected Peptides

molecular mass (kDa)	band	N-terminal sequence
17	A	380Gly-Ser-Pro-Gly-His-Lys-Thr
13.5	B1	380Gly-Ser-Pro-Gly-His
	B2	422Ala-Glu-Asn-Thr-Pro-Leu
	B3	442Asp-Val-Asn-Lys-Ser-Lys
10.8	C	442Asp-Val-Asn-Lys-Ser-Lys
8.8	D1	422Ala-Glu-Asn-Thr-Pro-Leu
	D2	442Asp-Val-Asn-Lys-Ser-Lys
about 8	E	442Asp-Val-Asn-Lys-Ser-Lys
about 7	F1	380Gly-Ser-Pro-Gly-His-Lys-Thr
	F2	422Ala-Glu-Asn-Thr-Pro-Leu
	F3	442Asp-Val-Asn-Lys-Ser-Lys
about 6	G	380Gly-Ser-Pro-Gly-His-Lys-Thr

The Trp fluorescence of the R domain was decreased by about 70% after proteolysis but was still measurable, and the λ_{max} of fluorescence was shifted from 331 to 326 nm (data not shown). Whether the remaining fluorescence is due to the protected peptide or to the nonproteolyzed full-length protein cannot be assessed. Nevertheless, the fluorescence shift suggests that the remaining Trp is located in a hydrophobic environment.

Advantage was taken from the presence of a disulfide bridge in the 442−483 peptide (Cys 461−Cys 471). Indeed, a fluorescent probe like AMCA-HPDP binds to free thiols generated by reduction of a disulfide bridge. The amount of bound AMCA-HPDP was determined by the protein fluorescence (λ_{ex}, 345 nm; and λ_{em}, 450 nm) after removal of the unbound probe. Binding of this probe was measured for

[2] The C-terminal residues have been determined from the apparent molecular mass of the peptides, as evaluated from the distance of migration on the gel. They should therefore only be considered as indicative. A good example is given by peptide B3 which starts at residue 442. This peptide is running on the gel with an apparent molecular mass of 13.5 kDa. Extending at most until the end of the R domain (residue 535), its actual molecular mass should only be 10−11 kDa.

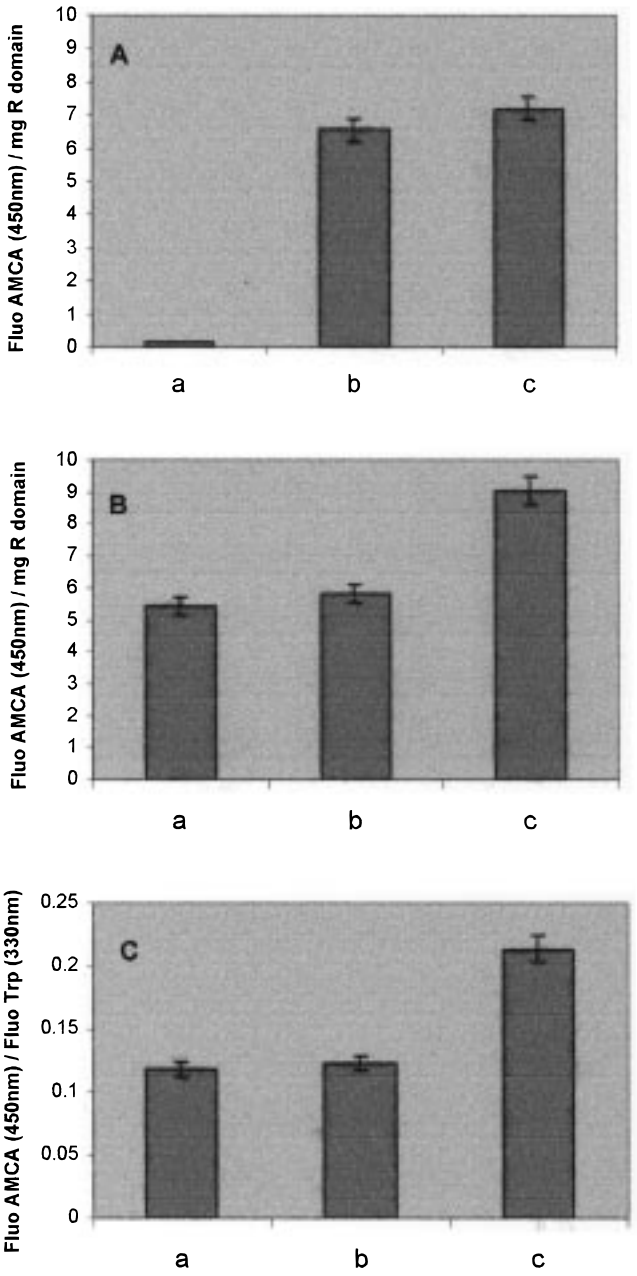

FIGURE 5: Binding of AMCA-HPDP to soluble (A) or membrane-associated R domain without (B) or with (C) proteolysis. (A and B) the concentration of R domain was obtained as described under Materials and Methods. (a) Reduction with DTT; (b) reduction with DTT in the presence of 6 M guanidine chloride. (c) Reduction with DTT in the presence of 6 M guanidine chloride and 0.15% TX100. All fluorescence measurements were performed in 0.1% SDS (see Materials and Methods), and the background fluorescence in the absence of reducing agent was subtracted. The values are the average of 3 measurements, and the error bars represent the standard deviation.

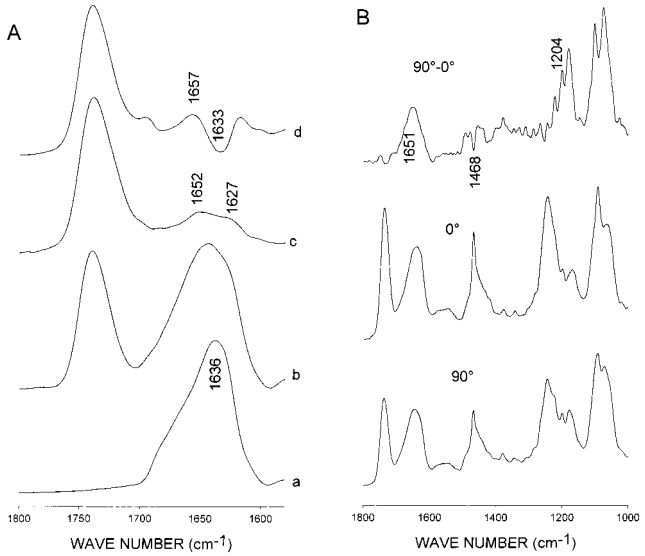

FIGURE 6: (A) Infrared spectra of deuterated R domain. (a) R domain in solution; (b) lipid-bound R domain; (c) lipid-bound R domain treated with proteinase K; (d) difference between the spectra of lipid-bound R domain and R domain in solution (b − a). The subtraction factor was chosen in order to zero the ν(C═O) proteins between 1600 and 1700 cm^{-1}. (B) Infrared spectra of deuterated lipid-bound R domain recorded with incident light polarized perpendicular (90°) and parallel (0°) to the plane of the membrane. The difference spectrum (90° − 0°) was obtained by subtracting the 0° polarized spectrum from the 90° polarized one. The subtraction factor was 1.3 and was chosen in order to zero the ν-(C═O) lipids. The difference spectrum has been enlarged 3.5 times compared to the 90° spectrum.

the R domain in solution (Figure 5A), the R domain associated with lipid vesicles (Figure 5B), and the proteolyzed lipid-bound R domain (Figure 5C). The disulfide bridge was hidden in the R domain in solution, as it has been clearly shown by X-ray crystallography. Binding of AMCA-HPDP therefore required the denaturation of the R domain to expose the disulfide bridge and efficiently promote its reduction (Figure 5Ab). Interestingly, when bound to the lipid membrane, the R domain exposed its disulfide bridge to the solvent since the fluorescence of the protein was similar in the absence or presence of the denaturing agent (Figure 5B, a−b). Addition of Triton X-100, which leads to disruption of the liposomes, resulted in a significant increase of fluorescence, suggesting that some of the Cys residues were hidden by the lipid membrane. This fluorescence increase was not due to the detergent itself since addition of a similar amount of TX100 to the free R domain resulted only in a slight increase of fluorescence (Figure 5Ac). Binding of AMCA-HPDP to a proteolyzed sample was very similar to what was observed with the lipid-bound intact R domain: it did not depend on the presence of the denaturing agent and was increased by about 30% in the presence of detergent (Figure 5C).

The FTIR technique relies on the analysis of the vibration bands of proteins and particularly on the amide I band ($\nu_{C=O}$ of the peptidic bond) whose frequency of absorption is dependent upon the secondary structure. In agreement with the X-ray data, the free R domain (HPLC purified, see Materials and Methods) showed a major absorption band at 1636 cm^{-1}, which is characteristic of the presence of a large amount of β-sheets (Figure 6Aa). No spectrum could be recorded at low pH due to R domain precipitation. The amide I band of the lipid-bound protein was much broader than the amide I band of the soluble protein (Figure 6Ab). An easy way to detect a conformational change is to subtract the two spectra that are compared to each other. Figure 6Ad shows that subtraction of the free R domain from the lipid−bound R domain resulted in a spectrum with a positive deviation at 1657 cm^{-1} and a negative deviation at 1633 cm^{-1}. This indicates the appearance of an α-helical structure at the expense of the β-sheet structure on binding to the lipid membrane. The α-helical component was also clearly

identifiable after proteolysis (1652 cm^{-1}, Figure 6Ac), which suggests it may belong to a membrane-associated domain. The ATR-FTIR technique can also be used to determine the orientation of a given secondary structure in a lipid membrane. For that purpose, spectra are recorded with incident light polarized perpendicular (90°) or parallel (0°) to the plane of the membrane. A dipole absorbing at 90° will be mainly oriented perpendicularly to the membrane plane. The orientation of a protein domain is strongly dependent on the orientation of the lipid bilayer. Experimentally, this orientation can be easily characterized when saturated lipids are used, and we therefore inserted the R domain into DPPC/DPPA (9/1 w/w) LUV at pH 5.0 using the protocol described for asolectin under Materials and Methods. The dichroic spectra as well as the difference spectrum 90° − 0° of the R domain inserted into DPPC/DPPA vesicles are given in Figure 6B. The orientation of the lipids was assessed both from the strong 90° polarization at 1204 cm^{-1} [phospholipid $\gamma_w(CH_2)$] and from the 0° polarization at 1468 cm^{-1} [phospholipid $\delta(CH_2)$] indicating that the all-trans hydrocarbon chains of DPPC and DPPA are oriented nearly normal to the germanium surface. The positive deviation observed at 1651 cm^{-1} provides evidence that the C═O dipole of the α-helical component of the R domain was perpendicular to the membrane surface, which is compatible with a transmembrane orientation of the α-helices.

The R domain associated with the DPPC/DPPA LUV was proteinase K treated, and similar patterns of digestion were found for DPPC/DPPA and asolectin (data not shown). However, no orientation of any of the structure left in the lipid membrane was detected by FTIR (data not shown). This suggests that if the protected helices really do adopt a transmembrane orientation, extramembrane domains are required for their stabilization in the lipid membrane.

DISCUSSION

Treatment of a membrane-bound protein with proteases and identification of the protected peptides have been widely used to elaborate hypotheses about the protein topology in the membrane. Our data suggest that an important part of the R domain of diphtheria toxin is protected by the interaction with the lipid membrane from the activity of an externally added protease. Indeed, the R domain extends from residues 380 to 535, and residues 380−421, 422−441, and 442 to about 483 are protected against proteolysis. Three types of interaction with the lipid membrane can account for the protein protection: (i) a close interaction with the membrane surface; (ii) insertion in the lipid membrane; and (iii) translocation across the lipid membrane. Labeling of the R domain with a membrane probe (the PCII lipid) demonstrated the presence of at least one transmembrane domain. The 442−483 region is likely to contain such a domain. Indeed, both before and after proteolysis, we observed binding of AMCA-HPDP to the R domain-containing lipid vesicles. However, the full accessibility of cysteines 461 and 471 to the fluorescent probe required a detergent solubilization of the lipid vesicles. Since this experiment was performed on samples from which the free protein had been removed and the results were similar before and after proteolysis, our data suggest the presence of two populations: one would be tightly associated to the surface of the lipid membrane (exposing the disulfide bridge), and another would interact deeply with the lipid membrane in a way that the cysteines are accessible only after disruption of the lipid vesicles. This last situation could result either from an insertion in the lipid membrane or from the translocation across that lipid membrane. Translocation of R domain has been suggested by Tortorella et al. (*10*) using monoclonal antibodies to probe the exposure of the three domains of DT (C, T, and R domains; see the introduction) on the cis or the trans sides of an artificial membrane (liposomes). As far as the R domain is involved, at low pH and at both 30 and 37 °C, about 30% of the DT molecules would expose region(s) of the R domain on the trans side of the membrane. No final conclusion can be drawn about a transmembrane insertion of peptide 380−421 even if the fluorescence shift from 331 to 326 nm upon proteolysis suggests that Trp 398 is in a hydrophobic environment. A membrane location of this Trp would be in agreement with previous results obtained with CB1, a cyanogen bromide peptide of DTB (residues 339−459), and showing that this Trp was quenched by lipid-bound quenchers located at the level of the 6−7th carbon of the lipid acyl chain (V. Cabiaux, unpublished results).

The question of the secondary structure of the membrane-embedded peptides is a puzzling one. In solution and according to X-ray crystallography, the R domain contains only β-sheets (*2*). Interestingly, our FTIR data showed that binding of the R domain to the lipid membrane resulted in the appearance of an α-helical component with an orientation compatible with a transmembrane association. This component was still present after proteolysis which suggests that it is indeed membrane-associated. A sequence analysis with the Shirmer and Cowan algorithm (*26*) has revealed the presence, in the protected peptides, of several amphipatic β-sheets having the characteristics of the bacterial porin β-sheets: residues ^{394}YAVSWNT, ^{416}HDIKITA, ^{448}THIGVNG, ^{457}IRMRCRA, and ^{483}VHANLHV. The presence of an α-helical component with a putative transmembrane orientation as well as amphipathic β-strands in the peptides protected from proteolysis suggests that the interaction of the R domain with the lipid membrane may involve these two types of structure in a complex structural organization. Involvement of both α-helix and β-sheet in membrane insertion has been described for the acetylcholine receptor (*27*).

Translocation of the R domain has been observed across the plasma membrane of Vero cells (*28*) which suggests that our data do not represent an artifact linked to the use of artificial vesicles. Whether the insertion/translocation of the R domain is involved in the A fragment translocation remains an open question. But nevertheless, our data demonstrated that an important part of the R domain has the ability to associate/insert or to translocate across a lipid membrane, although the R domain does not contain typical hydrophobic membrane-interacting sequence, and that this process requires no membrane proteins. The only requirement is a pH drop that is probably needed to generate hydrophobicity. This process may therefore involve the oligomerization of the R domain as has been observed with the α-toxin of *S. aureus* (*29*) or the protective antigen of *B. anthracis* (*30*).

DT has been clearly identified as a toxin interacting with the lipid membrane through well-identified hydrophobic helices (T domain). We provide here evidence that it also contains a domain (R domain) sharing some of the charac-

teristics of the toxins interacting with the lipid membrane through amphipathic β-sheets. Characterization of the respective roles of the T and R domains in the insertion/translocation processes should open new horizons in the study of translocation of proteins across the lipid membrane.

ACKNOWLEDGMENT

We thank Dr. R. J. Collier (Harvard Medical School) for providing us with the pGEX-2T-DTR plasmid, Dr. C. Montecucco (University of Padova) for the gift of PCII, and C. Degeyter for her help in R domain HPLC purification.

REFERENCES

1. Cabiaux, V., Wolff, C., and Ruysschaert, J. M. (1997) *Int. J. Biol. Macromol. 21*, 285−298.
2. Bennett, M. J., and Eisenberg, D. (1994) *Protein Sci. 3*, 1464−1475.
3. Donovan, J. J., Simon, M. I., Draper, K. K., and Montal, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 172−176.
4. Silverman, J. A., Mindell, J. A., Zhan, H., Finkelstein, A., and Collier, R. J. (1994) *J. Membr. Biol. 137*, 17−28.
5. Papini, E., Sandona, D., Rappuoli, R., and Montecucco, C. (1988*) EMBO J. 7*, 3353−3359.
6. Eriksen, S., Olsnes, S., Sanvig, K., and Sand, O. (1994) *EMBO J. 13*, 4433−4439.
7. Quertenmont, P., Wattiez, R., Falmagne, P., Ruysschaert, J. M., and Cabiaux, V. (1996) *Mol. Microbiol. 21*, 1283−1296.
8. Esbensen, Q. Y., Falnes, P. O., Olsnes, S., and Mahdsus I. H. (1993) *Biochem. J. 294*, 663−666.
9. Cabiaux, V., Quertenmont, P., Conrath, K., Brasseur, R., Capiau, C., and Ruysschaert, J. M. (1994) *Mol. Microbiol. 11*, 43−50.
10. Tortorella, D., Sesardic, D., Dawes, C. S., and London, E. (1995) *J. Biol. Chem. 270*, 27446−27452.
11. Kagawa, Y., and Raker, E. (1971) *J. Biol. Chem. 246*, 5477−5487.
12. Shen, W. H., Choe, S., Eisenberg, D., and Collier, R. J. (1994) *J. Biol. Chem. 269*, 29077−29084.
13. Hope, M. J., Bally, M. B., Webb, G., and Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55−65.
14. Mrsny, R. J., Volwerk, J. J., and Griffith, O. H. (1986) *Chem. Phys. Lipids 39*, 185−191.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
16. Montecucco, C., Schiavo, G., and Tomasi, M. (1985) *Biochem. J. 231*, 123−128.
17. Blight, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 87*, 911−917.
18. Laemmli, U. K. (1970) *Nature 227*, 680−685.
19. Schägger, H., and von Jagow, G. (1987) *Anal. Biochem. 166*, 368−379.
20. Goormaghtigh, E., Cabiaux, V., and Ruysschaert, J. M. (1994) *Subcell. Biochem. 23*, 329−450.
21. Wang, X. M., Wattiez, R., Mock, M., Falmagne, P., Ruysschaert, J. M., and Cabiaux, V. (1997) *Biochemistry 36*, 14906−14913.
22. Rothschild, K. J., Sanches, R., and Clark, N. (1982) *Methods Enzymol. 88*, 696−714.
23. Cortijo, M., Alonso, A., Gomez-Fernandez, J. C., and Chapman, D. (1982) *J. Mol. Biol. 157*, 597−618.
24. Cabiaux, V., Brasseur, R., Wattiez, R., Falmagne, P., Ruysschaert, J. M., and Goormaghtigh, E. (1989) *J. Biol. Chem. 264*, 4928−4938.
25. Goormaghtigh and Ruysschaert (1990) in *Molecular description of biological components by computer aided conformational analysis* (Brasseur, R., Ed) pp 285−392, CRC Press, Boca Raton, FL.
26. Schirmer, T., and Cowan, S. W. (1993) *Protein Sci. 2*, 1361−1363.
27. Unwin, N. (1995) *Nature 373*, 37−43.
28. Moskaug, J. Ø., Stenmark, H., and Olsnes, S. (1991) *J. Biol. Chem. 266*, 2652−2659.
29. Song, L., Hobaugh, M. R., Shustak, C., Cheley, S., Bayley, H., and Gouaux, J. E. (1996) *Science 274*, 1859−1866.
30. Petosa, C., Collier, R. J., Klimpel, K. R., Leppla, S. H., and Liddington, R. C. (1997) *Nature 385*, 833−838.

BI9818624