Insertion of Diphtheria Toxin in Lipid Bilayers Studied by Spin Label ESR†

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ABSTRACT: The pH dependence of the insertion of diphtheria toxin into bilayers of dioleoylphosphatidylglycerol (DOPG) has been studied by using ESR spectroscopy of spin-labeled phosphatidylglycerol with the reporter group at either the 5-position or the 14-position of the *sn*-2 chain (5-PGSL and 14-PGSL, respectively). At neutral pH, addition of diphtheria toxin has little effect on the ESR spectra of either spin label in large unilamellar vesicles of DOPG. At acidic pH, the outer hyperfine splitting of the 5-PGSL label is increased, and a second component corresponding to lipids whose chain motion is selectively restricted appears in the spectra of the 14-PGSL label, in the presence of diphtheria toxin. The motionally restricted component of 14-PGSL has a large outer hyperfine splitting ($2A_{max} \approx 61$ G) and corresponds to spin-labeled lipids the chains of which are in direct contact with the membrane-penetrant part of the inserted toxin. This restricted component is present, although to a lesser extent, in vesicles containing 90% of the zwitterionic lipid dioleoylphosphatidylcholine and displays a limited selectivity for negatively charged relative to zwitterionic spin-labeled phospholipids. The fraction of lipids which are motionally restricted by the toxin increases with decreasing pH, titrating in DOPG vesicles with an apparent pK_a of approximately 6.1. The outer hyperfine splitting of the 5-PGSL label titrates with an apparent pK_a of approximately 5.5, suggesting that this might be preferentially sensitive to a later stage in the insertion of the toxin. At pH 5.0, corresponding to completion of the titration, the number of motionally restricted lipids per *bound* toxin is approximately 40, whereas at pH 6.2 this stoichiometry is reduced to 24, indicating that the diphtheria toxin molecule is inserted in the lipid bilayer to a greater extent at the lower pH. On the basis of previous hydrophobic photolabeling experiments, it is suggested that only the translocation domain of the B fragment of the toxin is inserted at the higher pH and that the increased number of motionally restricted lipids at the lower pH corresponds to insertion also of the catalytic A domain.

Bacterial protein toxins with intracellular targets reach the cell cytosol via a four-step process consisting of (1) binding to the cell surface, (2) internalization into intracellular vesicles, (3) membrane translocation from the vesicle lumen into the cytosol, and (4) target modification (Montecucco et al., 1994). Of these steps, the translocation across lipid membranes is that least understood in the entire cell intoxication process. Study of the interaction of these toxins with lipid membranes is important not only to understand this stage in the mechanism of action but also because generally the membrane insertion of water-soluble proteins is of basic interest in membrane biosynthesis and secretion. It has been proposed that proteins cross membranes via a molten globular state (Bychkova et al., 1988; Van der Goot et al., 1992). Specifically, the requirement of a partially unfolded, compact state with a native-like secondary structure for the membrane translocation has been indicated for the maltose-binding protein precursor in *Escherichia coli* (Randall & Hardy, 1986), for precursors of the F_1-ATPase b subunit and of cytochrome c_1 in *Neurospora* (Schleyer & Neupert, 1985), and for mouse dihydrofolate reductase fused to a mitochondrial presequence (Eilers & Schatz, 1986). For different proteins, the transition to the molten globule state can be induced by different factors such as temperature, pH, removal of metal ions, and reduction of disulfide bonds (Kuwajima, 1989). A change in pH is the triggering factor most commonly observed to produce this conformational change and also is necessary for the membrane insertion of bacterial toxins *in vivo* (Parker & Pattus, 1993).

Diphtheria toxin is produced by *Corynebacterium diphtheriae* as a single polypeptide chain of 58 kDa, which is cleaved at an exposed loop by the cell-associated proteinase furin, or by trypsin, to generate a disulfide-linked two-chain toxin. Of these two chains, the C-terminal fragment B (342 residues, 38 kDa) is responsible for cell binding to a recently identified receptor (Naglich et al., 1992) and for penetration into the cell of the fragment A (23 kDa). Fragment A blocks cell protein synthesis by catalyzing the selective ADP-ribosylation of elongation factor 2 (Collier, 1990). The crystallographic structure of DT has been solved recently and shows the presence of three domains (Choe et al., 1992). Domain A has a mixed secondary structure and presents a cavity that binds NAD^+. The amino-terminal domain of

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fragment B (termed T) is composed of 9 α-helical segments, and the carboxyl-terminal part (termed R) contains 10 β-strands.

After receptor binding via the R domain, DT[1] enters the endosomal pathway. Acidification of the endosomal lumen produces a conformational change in DT (Blewitt et al., 1985; Cabiaux et al., 1989); the protein becomes hydrophobic and can penetrate lipid membranes (Donovan et al., 1981; Kagan et al., 1981; Hu & Holmes, 1984; Montecucco et al., 1984; Defrise-Quertain et al., 1989; Demel et al., 1991). Membrane insertion is favored by the presence of anionic lipids, independent of the nature of the negatively charged polar head group (Hu & Holmes, 1984; Papini et al., 1987; Chung & London, 1988; Demel et al., 1991). That the low pH-induced conformational change of DT is the trigger for DT membrane insertion and translocation is demonstrated by the fact that DT can be induced to enter and intoxicate cells from the plasma membrane simply by lowering the extracellular pH (Draper & Simon, 1980; Sandvig & Olsnes, 1980, 1981). For a review on the membrane interactions of DT, see London (1992).

Different models for the membrane translocation of the A fragment into the cytosol have been proposed [for a review, see Montecucco et al. (1994)]. According to the tunnel model proposed by Boquet et al. (1976), the A fragment translocates the membrane through a protein pore formed by the B fragment. The cleft model proposes that the translocation occurs through the interface between the membrane-inserted B domain, which probably is arranged in a multimeric aggregate including the membrane toxin receptor, and the phospholipid acyl chains (Montecucco et al., 1992). It has also been proposed that the release of the A fragment into the cytoplasm is produced directly by lysis of the endocytic organelle (Hudson & Neville, 1985). Some experimental evidence favors the cleft model. It has been shown that the isolated A domain is able to penetrate and interact hydrophobically with the membrane (Montecucco et al., 1985). Hydrophobic photoreactive probes, which reside in the hydrocarbon region of the lipid bilayer, are able to label the A fragment (Hu & Holmes, 1984). It has also been shown by this method that the A domain inserts in the membrane in a pH-dependent manner with an apparent pK that is 0.7 units lower than that of the B domain. This observation is consistent with the possibility that the insertion of the B and A chains occurs sequentially as the medium becomes progressively more acidic (Papini et al., 1987).

In the present work, we have studied the interaction of DT with dioleoylphosphatidylglycerol membranes by using ESR spectroscopy of spin-labeled phospholipids. The changes that are induced by DT in the ESR spectra of phosphatidylglycerols spin-labeled at the 5- and 14-positions of the *sn*-2 chain (5-PGSL and 14-PGSL) incorporated in membranes composed of dioleoylphosphatidylglycerol have been recorded as a function of pH. It is found that insertion of DT in the membranes causes a direct interaction with the lipid chains. This is evidenced by the appearance of a second spectral component that corresponds to spin-labeled lipid chains which are more strongly motionally restricted than are those in the fluid bilayer regions of the membrane. The relative populations of these motionally restricted lipids have been compared at different pH values corresponding to the suggested sequential insertion, and the selectivity for the interaction of DT with spin-labeled phospholipids of different polar head group species was studied.

MATERIALS AND METHODS

Materials. DT was prepared from culture filtrates of *C. diphtheriae* as described by Rappuoli et al. (1983). DOPG and DOPC were from Avanti Polar Lipids (Pelham, AL). Spin-labeled phosphatidylglycerols (n-PGSL) were prepared as described by Marsh and Watts (1982).

Preparation of LUVs. DOPG or DOPC were codissolved with spin-labeled phospholipids in a proportion of 100:1 (w/w). The chloroform solution was dried with a nitrogen gas stream, and the lipids then were kept overnight under vacuum. The dried lipids were suspended to a final concentration of 10 mM in a buffer containing 10 mM HEPES and 0.1 mM EDTA at pH 7.4. The lipid dispersions were frozen and thawed five times and then extruded ten times through polycarbonate filters of 100 nm pore diameter. An extruder from Avestin (Ottawa, Canada) was used.

Sample Preparation. Aliquots of the LUV suspensions containing 0.25−0.5 mg of lipid were mixed with different amounts of DT ranging from 0.05 to 0.5 mg. The final volume was 0.3 mL. A small magnetic stirrer and a microelectrode for measuring the pH were placed in the suspension. The pH, initially at a value of 7.4, was adjusted to the desired value by titrating with 25 mM succinic acid solution. A 10 μL positive-displacement pipette was used, and the succinic acid solution was added slowly, close to the stirring bar. After incubation for 30 min at 20 °C, the samples were spun down at 100000*g* for 30 min. The centrifugation was performed at 20 °C. The pellets were removed with a Teflon spatula and transferred to glass capillaries. After ESR measurements, the samples were dissolved in 0.2 mL of 10% SDS and 0.5 M NaOH. Aliquots of these solutions were taken to quantify phosphate using the method of Bartlett (1959) and protein with a modified method of Lowry (Peterson, 1977).

DT alone self-aggregates and precipitates from solution at pH 5.0. In order to verify that the lipid−protein complexes contain protein effectively bound to the membrane and not unbound self-aggregated protein, representative samples were centrifuged on a continuous sucrose density gradient. Samples containing DOPG LUVs and DT at pH 5.0 were loaded on a 15−40% sucrose density gradient at pH 5.0 and centrifuged in a SW70 rotor at 100000*g* for 9 h. A single band containing both the lipid and the protein was obtained. No lipid or protein was found in the rest of the gradient. The band was collected and the sucrose removed by dialysis at 4 °C against the HEPES buffer at pH 5.0. The material was processed for ESR measurements as described above. The lipid to protein ratio and the fraction of motionally restricted spin label components as measured by ESR spectral subtraction were indistinguishable from those obtained before the

[1] Abbreviations: DT, diphtheria toxin; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; LUV, large unilamellar vesicle; n-PGSL, 1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphoglycerol; 14-SASL, 14-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid; 14-PCSL, 14-PESL, and 14-PASL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphocholine, -phosphoethanolamine, and -phosphoric acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; ESR, electron spin resonance.

density gradient separation. In view of this result, samples were prepared routinely following the procedure described above, omitting the sucrose gradient centrifugation step. At pH 6.0 and above, DT alone does not sediment on centrifugation at 100000*g* for 30 min.

To perform the experiments on pH titration (see Figure 2, later), the pH of a suspension containing LUVs and DT, initially at pH 7.4, was reduced to the desired value as described above, and 5 μL aliquots of the whole suspension at the different values of the pH were taken for ESR measurements. To study the selectivity of the interaction of DT with different lipids, DOPG LUVs free of spin-labeled lipids were mixed with DT and the pH was decreased to 5.0. After incubation at 20 °C for 30 min, the sample was divided into several aliquots, and the different spin-labeled lipids in concentrated ethanolic solution were added to the separate aliquots. After an incubation for 4 h at 20 °C, unbound spin label was removed by dilution and centrifugation, and the pellets were processed for ESR measurements as described above. This procedure ensures that all the samples were of exactly the same lipid to protein ratio. Chemical analysis after ESR measurement confirmed that these samples had effectively the same lipid to protein ratio.

ESR Spectroscopy. ESR spectra were recorded on a Varian Century Line 9 GHz spectrometer equipped with nitrogen gas flow temperature regulation. The capillaries (1 mm i.d.) containing the sample were placed in a 4-mm quartz tube that contained light silicone oil for thermal stability. The sample temperature was measured with a thermocouple placed inside the quartz tube just above the top of the ESR cavity. Spectral subtraction and other data analysis were performed as described in Marsh (1982).

RESULTS

Perturbation of Lipid Mobility. The ESR spectra from the 5-PGSL and 14-PGSL phosphatidylglycerol spin-label positional isomers in LUVs composed of DOPG, and of 14-PGSL in LUVs composed of DOPC/10% DOPG, in the presence and absence of DT, are shown as a function of pH in Figure 1. At pH 7.0, the ESR spectra of both lipid spin labels are unchanged for samples in the presence of DT compared with those for the DOPG LUVs alone. As the pH is decreased, the outer hyperfine splitting ($2A_{max}$) in the spectra of the 5-PGSL spin label increases for the samples containing DT, whereas that for DOPG LUVs alone does not change (Figure 1A). This increase in A_{max} corresponds to a decrease in the lipid chain mobility on interaction of DT with DOPG LUVs at acidic pH. The spectra of the 5-PGSL at intermediate pH values in the titration region appear as a single component. If two components coexist in these spectra, they either are not resolved or are averaged by exchange. Concomitantly with the increase in outer hyperfine splitting of the 5-PGSL spin label, a second component of larger outer hyperfine splitting appears in the outer wings of the ESR spectra of the 14-PGSL spin label, as the pH is decreased for samples containing DT (Figure 1B). This additional spectral component, which is not seen in the spectra of DOPG LUVs alone, corresponds to spin-labeled lipids whose chain motion is restricted by direct interaction with the toxin (cf. Görrissen et al., 1986). Its appearance gives direct evidence for insertion of the toxin in the lipid membrane at acidic pH. For DOPC LUVs

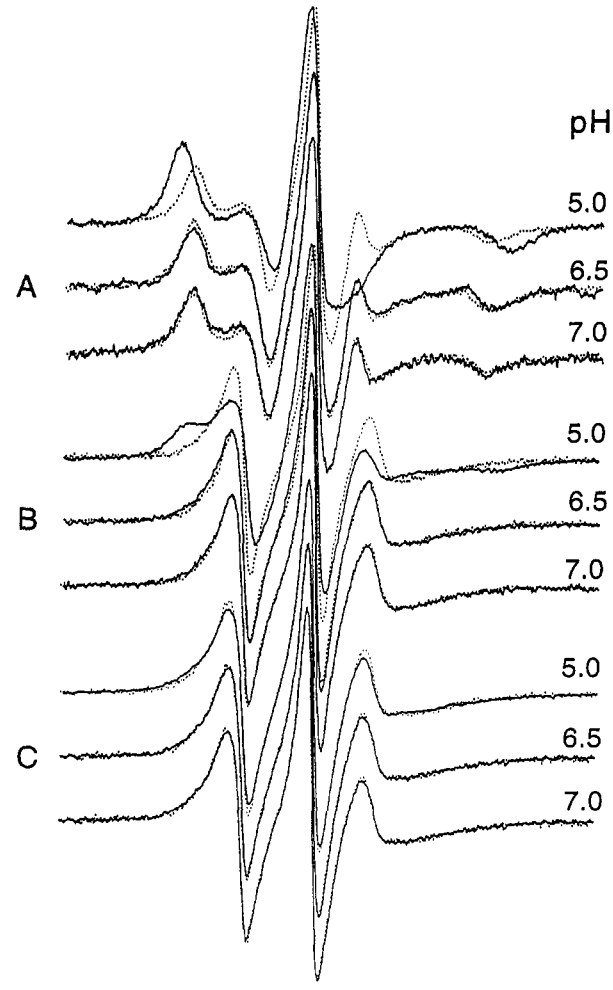

FIGURE 1: ESR spectra of (A) 5-PGSL and (B) 14-PGSL spin labels in LUVs of DOPG in the presence (continuous line) and in the absence (dotted line) of DT at pH (from upper to lower) 5.0, 6.5, and 7.0. The sample temperature was 10 °C. (C) ESR spectra of 14-PGSL in DOPC/10% DOPG LUVs in the presence (continuous line) or in the absence (dotted line) of DT at pH (from upper to lower) 5.0, 6.5, and 7.0 and a temperature of 5 °C. The initial lipid to protein ratio was 1:1 w/w. Total scan width: 100 G.

containing only 10% of DOPG (Figure 1C), the proportion of the second component in the spectrum of 14-PGSL for samples containing DT at low pH is less than that obtained with DOPG LUVs, indicating that a smaller amount of toxin is inserted in lipid vesicles containing a high proportion of zwitterionic lipid. At pH 4.4 (data not shown), it is more evident than in the spectrum at pH 5.0 that is shown in Figure 1C. (For these less negatively charged bilayers, the surface enhancement of the H^+ ion concentration is lower.)

Two processes compete at low pH, namely, binding to the membrane and toxin self-aggregation. When LUVs of DOPG alone are present, the strong membrane binding overcomes the self-aggregation and lipid–protein complexes with relatively low lipid to protein ratio are obtained. For toxin added at a ratio of lipid to protein of 2:1 mg/mg (145 mol/mol), precipitated lipid–protein complexes with lipid to protein ratio in the region of 100 mol/mol were obtained with DOPG LUVs at pH 5.0. When DOPC-containing vesicles are used, the weaker interaction with zwitterionic lipids and the relatively high protein concentration used in these experiments favor the protein self-aggregation; low binding to the zwitterionic lipid-containing membrane is then

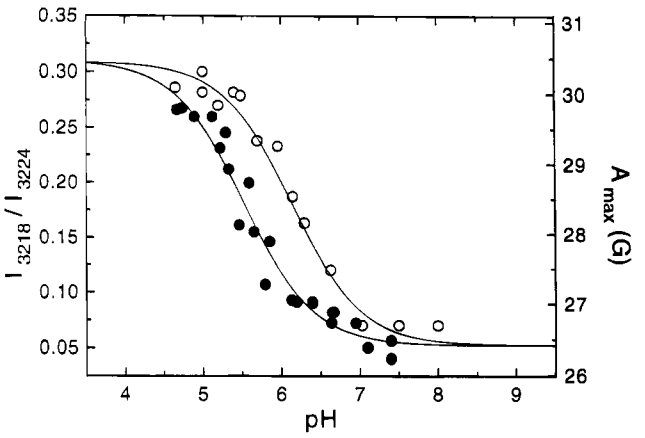

FIGURE 2: Dependence on pH of the maximum hyperfine splitting, A_{max}, in the spectra from 5-PGSL (●, right-hand ordinate) and of the ratio of the intensity at 3218 G to the intensity at 3224 G in the spectra from 14-PGSL (○, left-hand ordinate) in DOPG LUVs, in the presence of DT. The total lipid to protein ratio was 1:1 w/w. The sample temperature was 20 °C. The full lines represent fits to a single titration with pK_a = 5.5 and 6.1, respectively.

observed. For DOPC/10% DOPG lipids dispersed in buffer containing DT at an initial lipid/protein ratio of 2:1 mg/mg (145 mol/mol) and then titrated to pH 5.0, the lipid to protein ratio of the lipid-containing bands obtained on a sucrose density gradient (used to separate aggregated protein) was 300 mol/mol.

pH Titration. The pH dependences of the ESR spectral characteristics for the 14-PGSL and 5-PGSL spin labels in LUVs of DOPG in the presence of DT are given in Figure 2. The ratio of the intensity at 3218 G to the intensity at 3224 G in the spectra from 14-PGSL, which correspond to the positions of the low-field peaks of the motionally restricted and the fluid components, respectively, was taken as a measure of the relative intensity of these components. Both this parameter for the 14-PGSL spin label and the outer hyperfine splitting constant, A_{max}, of the 5-PGSL spin label display a pronounced titration that reflects the decrease in the lipid chain mobility on insertion of the toxin at low pH. An apparent pK_a of 6.1 from 14-PGSL and of 5.5 from 5-PGSL is obtained for this overall process. The discrepancy in these two values may arise in part from the fact that empirical parameters are used to characterize overlapping spectra, but the size of the difference does suggest that the two spin labels may be reflecting preferentially different processes with distinct pK_as. The pH dependences of the 5-PGSL and 14-PGSL spectra would be expected to be similar if they are reflecting a single process that is perturbing the lipid chain mobility. This, for instance, is what is found for the pH-dependent insertion of α-lactalbumin in lipid membranes (Montich and Marsh, unpublished results). For a two-stage process—as is found here because the number of motionally restricted lipids per bound toxin changes with pH (see below)—the pH dependences will be different if the relative strength of the response to the two processes differs at the two label positions, e.g., if the two processes are associated with different depths of toxin penetration. Papini et al. (1987) have found that the pH dependences for membrane insertion of the A and B domains of DT differ and titrate with apparent pK_as of 5.2 and 5.9, respectively. Separate titration curves predicted for pK_as of 5.5 and 6.1 are given in Figure 2, where the shift in pK_a between the 14-PGSL and 5-PGSL spin labels is found to be equal to

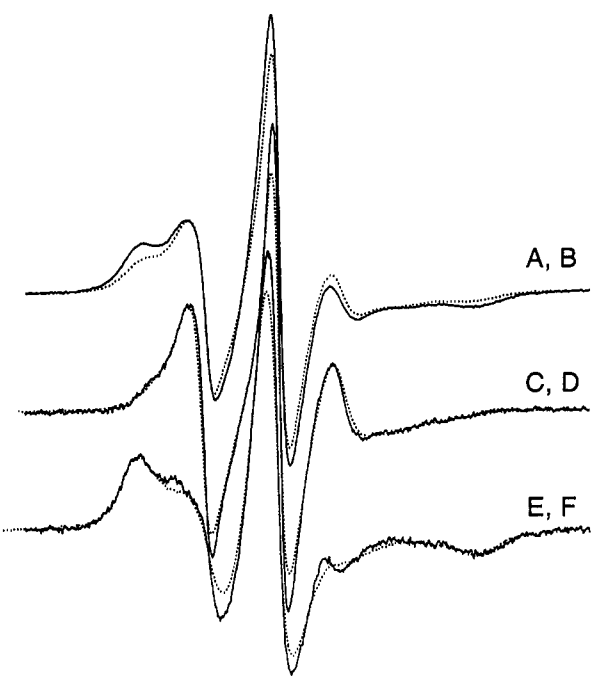

FIGURE 3: (A and B) ESR spectra from 14-PGSL in DOPG/DT complexes at pH 5.0 and 10 °C. The lipid to protein ratio of the complexes was (A) 55 mol/mol (full line) and (B) 64 mol/mol (dotted line). (C, full line) Fluid component difference spectrum resulting from subtraction of 76% of the integrated intensity from spectrum B, by using spectrum A. (D, dotted line) Spectrum of 14-PGSL in DOPG membranes at 5 °C. (E, full line) Motionally restricted component difference spectrum resulting from subtraction of 63% of the integrated intensity from spectrum A by using spectrum B. (F, dotted line) Matching single-component spectrum from gel-phase lipid vesicles. Total scan width: 100 G.

that for insertion of the B and A domains of the toxin, respectively. The difference between the absolute values of these pK_as and those found by Papini et al. (1987) for insertion of the A and B domains might be attributed to differences in electrostatic enhancement of the surface H^+ ion concentration that arises from the different negatively charged lipid content of the membranes used. Assuming an intrinsic pK_a for binding to zwitterionic membranes of 5.1, i.e., equal to that for the conformational change induced in DT in solution (Chung & London, 1988), an effective surface potential of $\psi = -(kT/e) \ln 10 \times \Delta pK_a^{el} \approx -60$ mV is deduced from the apparent pK_a of 6.1 observed here for DT/DOPG with the 14-PGSL spin label [cf., e.g., Cevc and Marsh (1987)].

Motionally Restricted Lipids. The second motionally restricted component that is induced by DT at low pH in the spectra of 14-PGSL can be quantitated in more detail by spectral subtraction. This is illustrated in Figure 3 for intersubtractions between the spectra of DOPG LUV samples at pH 5.0 that contain different amounts of DT. The spectrum from the sample with lipid/toxin ratio of 55 mol/mol (Figure 3A) clearly contains a larger proportion of the motionally restricted spin-label component than does that of the sample with 64 mol/mol lipid/toxin ratio (Figure 3B). Subtraction of 76% of the normalised double-integrated intensity of spectrum (A) from spectrum (B) yields a difference spectrum consisting of the single fluid component (Figure 3C). This difference spectrum resembles very closely that of 14-PGSL in DOPG alone at 5 °C (Figure 3D) rather than at 10 °C, the temperature for Figure 3C. This

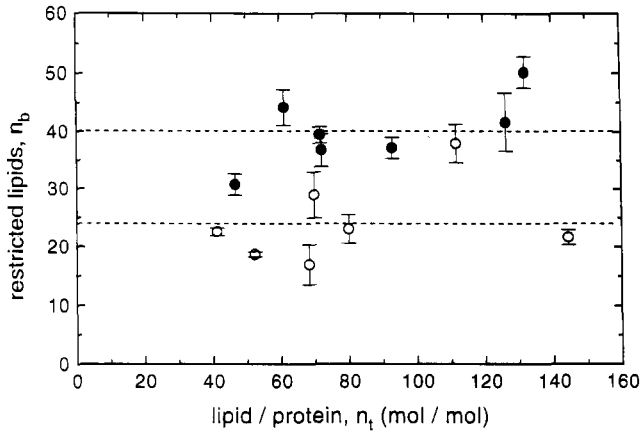

FIGURE 4: Effective number, n_b, of motionally restricted lipids per DT monomer for DOPG/DT complexes of different total lipid to protein ratios, n_t, at pH 5.0 (●) and pH 6.2 (○). Values are deduced from the fraction, f, of motionally restricted spin-labeled lipids and the lipid/protein ratio, n_t. Dashed lines represent mean values.

indicates that binding of the toxin generally decreases the mobility of the membrane lipids somewhat, in addition to the direct motional restriction of the lipid chains that is indicated by the appearance of the second spectral component with larger outer hyperfine splitting. Subtraction of 63% of the normalized double-integrated intensity of spectrum B from spectrum A yields a difference spectrum consisting of the single motionally restricted spin-label component (Figure 3D). The outer hyperfine splitting in this spectrum is $2A_{max}$ = 61 G, which is approaching the limits of motional sensitivity of conventional nitroxide ESR spectroscopy and indicates a very considerable restriction in chain mobility relative to that of the fluid lipids. This value is considerably greater than that found, for instance, for the insertion of apocytochrome *c* into negatively charged lipid bilayers (Görrissen et al., 1986) and is more comparable to the values found with integral transmembrane proteins [see, e.g., Marsh (1985)].

From the results of the two intersubtractions, it is possible to determine the relative amounts of the motionally restricted (fraction, f) and fluid (fraction, $1 - f$) components for the spectra corresponding to both lipid/toxin ratios in Figure 3, panels A and B (cf. Knowles et al., 1981). The dependence of the number of motionally restricted lipids per toxin, n_b, on the lipid/toxin ratio, n_t, is given for complexes of DT with DOPG, both at pH 5.0 and at pH 6.2, in Figure 4. In this it is assumed that $n_b = n_t f$, i.e., that spin-labeled phosphatidylglycerol shows no preferential selectivity for interaction with the toxin as compared with the parent host dioleoylphosphatidylglycerol lipid. Generally, this is found to be the case for the interaction of spin-labeled lipids with integral proteins [see, e.g., Marsh (1985)]. The number of motionally restricted lipids per toxin remains approximately constant with lipid/toxin ratio, to within the experimental scatter. The mean number of motionally restricted lipids per toxin is approximately 40 at pH 5.0 and 24 at pH 6.2, respectively. This result provides further evidence for different extents of membrane insertion of the toxin molecule at pH 6.2 and pH 5.0.

Different Spin-Label Head Groups. The ESR spectra of different phospholipid species spin-labeled at the C-14 atom of the *sn*-2 chain are given in Figure 5 for complexes of DT with DOPG at pH 5.1. All spectra are compared with those

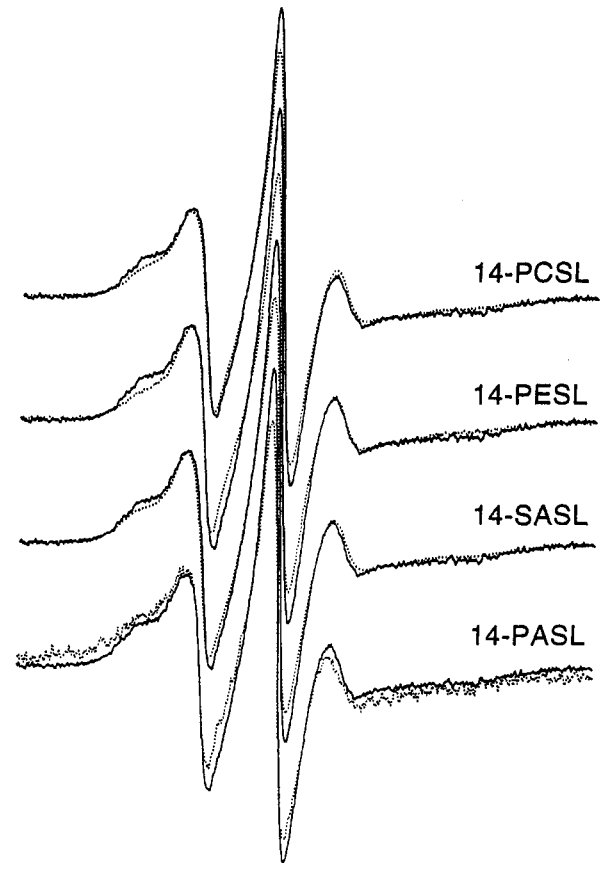

FIGURE 5: ESR spectra of different phospholipid species spin-labeled at position 14 of the *sn*-2 acyl chain in DOPG/DT complexes at pH 5.1. The spectra from 14-PCSL, 14-PESL, 14-SASL, and 14-PASL (dotted lines) are compared with the spectrum from 14-PGSL (continuous line). Lipid/protein ratio = 79 ± 3 mol/mol. Temperature: 10 °C. Total scan width: 100 G.

from spin-labeled phosphatidylglycerol, 14-PGSL. It can be seen that the spectra from the zwitterionic spin labels, 14-PCSL and particularly 14-PESL, contain a smaller proportion of the component from motionally restricted lipids than does that from 14-PGSL. For the negatively charged spin labels, 14-SASL and 14-PASL, the proportion of motionally restricted component is comparable to that for 14-PGSL. The bound DT therefore exhibits a limited selectivity for interaction with negatively charged phospholipids in general.

DISCUSSION

The present work is concerned with the detection of the pH-dependent insertion of DT into lipid membranes, as reflected by the perturbation of the lipid chain mobility. The appearance of a second motionally restricted spin-labeled lipid component, as evidenced by the ESR spectra of 14-PGSL, on interaction of DT with lipid vesicles provides direct evidence for the insertion of the toxin into the hydrophobic core of the lipid membrane at low pH. This is in agreement with previous work using hydrophobic photolabeling (Montecucco et al., 1985; Papini et al., 1987) and fluorescence quenching (Chung & London, 1988). The present experiments with spin-labeled lipids, particularly with 14-PGSL, demonstrate that the membrane insertion of DT is accompanied by a pronounced reduction in the mobility of the lipid chains that is comparable to that induced by integral proteins. The latter result suggests that the inserted portion of the toxin extends beyond the 14-position of the

lipid chains and transverses the membrane, as would be required for a translocation process.

The motionally restricted lipid component is also present in the ESR spectra of 14-PGSL in complexes of DT with a 10:90 mol/mol mixture of DOPG/DOPC (clearly at pH <5.0), although in a smaller proportion than that for complexes with DOPG alone. The motionally restricted component exhibits a limited selectivity for interaction of the toxin with negatively charged lipids that does not depend on the particular anionic lipid species. A specificity between different negatively charged lipids was also not found in the requirement for binding of the toxin to lipid vesicles (Chung & London, 1988).

The pH dependence of the perturbation of the lipid mobility of DT (Figure 2) reveals several interesting features. The chain motion is unperturbed at neutral pH but becomes progressively restricted on titrating to acidic pH. The apparent pK_a for the perturbation of the rotational motion that is recorded by 5-PGSL is 0.6 units lower than that recorded with 14-PGSL. Comparison with the pH dependence in hydrophobic photolabeling experiments (Papini et al., 1987) suggests that 5-PGSL preferentially reflects the insertion of the A domain of DT which occurs at a pH approximately 0.7 units lower than that required for insertion of the B domain. This suggestion is supported by the finding that the number of motionally restricted lipids per inserted toxin is dependent on the pH (Figure 4), rather than maintaining a fixed stoichiometry throughout the titration. It should be noted, however, that the ESR spectra of the lipid spin labels *per se* are not capable of distinguishing between insertion of the A and B domains of the toxin. This assignment is made solely from the comparison with the results on the differential pH dependence of hydrophobic photolabeling of the two domains in the intact toxin [Papini et al. (1987) and see Results].

The number of motionally restricted lipids is approximately 24 per toxin monomer at pH 6.2. From calculations based on molecular packing (Marsh, 1993), this corresponds reasonably well with the number of phospholipids contacting two helical hairpins that traverse the membrane independently, which is estimated to be approximately 28. Such a number therefore would be consistent with the proposal based on the crystal structure of DT that insertion of the translocation domain of the B fragment involves the hairpins composed of hydrophobic or apolar helices 5 and 6, and helices 8 and 9 traversing the membrane (Choe et al., 1992). At pH 5.0, the number of motionally restricted lipids per toxin monomer is increased to approximately 40. The difference from the number of motionally restricted lipids obtained at pH 6.2 is suggested to represent the additional bulk of toxin that is accommodated in the lipid bilayer on insertion also of the A domain at lower pH (cf. above). This suggestion is consistent also with the finding that the A fragment alone can penetrate lipid membranes at low pH, with a lower pK_a $\approx$ 4.9 than that for the whole toxin (Montecucco et al., 1985). The effective area occupied by the whole toxin inserted in lipid monolayers at pH 5.0 corresponds to an intramembranous radius of 26 Å (Demel et al., 1991). On simple geometric grounds (cf. Marsh, 1993), it can be calculated that minimally approximately 37 phospholipids could be accommodated around the perimeter of a transbilayer segment of this size. This value is in accord with the number of motionally restricted lipids found at pH 5.0 from ESR spectroscopy.

The above geometric estimates suggest that the toxin is inserted in a monomeric form in DOPG vesicles. This is in agreement with the suggestion (see Results) that the binding to vesicles composed totally of negatively charged lipids is so strong that it competes effectively with the aggregation reaction of the toxin that occurs at low pH. Alternatively, if the toxin is inserted in a partially aggregated form, the intramembranous sections of the inserted toxin must be sufficiently widely separated that lipids can be accommodated fully around their perimeter, even when the whole toxin (including A fragment) is inserted. It should be noted that the increase in number of motionally restricted lipids per inserted toxin with decreasing pH is not likely to be caused either by a subsequent aggregation of the inserted hydrophobic segments or by an oligomerization of the B domain within the bilayer on titration to lower pH, both of which would be expected to decrease the intramembranous surface of the toxin that is exposed to lipid (cf. Marsh, 1993). For vesicles containing a high proportion of zwitterionic lipid, the binding is less strong than to vesicles composed solely of anionic lipids, and it is likely then that the toxin may be inserted in an aggregated form (see Results).

In conclusion, the present results demonstrate that insertion of DT in lipid membranes at acidic pH is accompanied by a large perturbation of the lipid chain mobility. The extent of this perturbation is consistent with the inserted toxin traversing the entire bilayer. The number of lipid chains that are in direct contact with the toxin molecule increases with decreasing pH, most probably corresponding first to insertion of the translocation domain of the B fragment followed by that of the A fragment. Insertion of the latter then gives rise to a larger perturbation of chain segments closer to the lipid polar head groups. This, together with the correlation with structural and dimensional information, gives further insight into the mode of membrane translocation of DT at low pH in endosomes.

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