pH-dependence of the phospholipid interaction of diphtheria-toxin fragments

Cesare MONTECUCCO,* Giampietro SCHIAVO* and Maurizio TOMASI†

*Centro C.N.R. per la Fisiologia dei Mitochondri e Laboratorio di Biologia e Patologia Molecolare, Istituto di Patologia Generale, Via Loredan 16, 35131 Padova, and †Laboratorio di Biologia Cellulare, Istituto Superiore di Sanità, Viale Regina Elena, 00164 Roma, Italy

Photoreactive phospholipids have been used to probe the lipid interaction of diphtheria toxin. Low pH values induce the membrane insertion of both the binding and enzymic fragments of the toxin. The efficiency of this process is much higher with asolectin than with egg lecithin (phosphatidylcholine)/cholesterol liposomes. The low-pH-induced interaction of the toxin fragments with the membrane hydrocarbon phase is more evident for the enzymic A-chain than for the binding B-chain, and it is fully reversed by returning the pH to neutrality.

INTRODUCTION

Diphtheria toxin (DT) is secreted by *Corynebacterium diphtheriae* as a single polypeptide chain (M_r 58 342) of known sequence (Ratti *et al.*, 1983; Kaczorek *et al.*, 1983; Greenfield *et al.*, 1983). Proteinases activate this non-toxic form by splitting the molecule into two domains held together by a single disulphide bridge. The C-terminal part, fragment B (M_r 37 194), is able to bind to a specific DT receptor present on most eukaryotic cells. After reduction with thiol-reducing agents, the N-terminal part, fragment A (M_r 21 164), ADP-ribosylates elongation factor 2 specifically, thereby blocking protein synthesis. It has been estimated that a single A-fragment is able to kill a cell (Pappenheimer, 1977; Uchida, 1982).

The chain of events leading the hydrophilic polypeptide A across the hydrophobic membrane barrier to reach its cytoplasmic target is not yet clear (Olsnes & Sandvig, 1983).

There is evidence that, after binding to the cell, DT is endocytosed and that a low pH, such as that found in endosomes, is sufficient to cause its entry into the cell (Sandvig & Olsnes, 1980; Draper & Simon, 1980). The role of the DT receptor in this process, if any, is unknown. It is generally assumed that lipid bilayers provide a biologically relevant model system to study the membrane penetration of DT (Alving *et al.*, 1980; Boquet, 1980; Kagan *et al.*, 1981; Donovan *et al.*, 1981, 1982; Deleers *et al.*, 1983; Cabiaux *et al.*, 1984; Misler, 1984). Studies with black lipid membranes (BLM) have suggested that, at low pH, the B-fragment forms a hydrophilic channel permitting the passage of the A-chain across the membrane; the hydrophobic central portion of the B-chain has been implicated in this process (Kagan *et al.*, 1981; Donovan *et al.*, 1981; Deleers *et al.*, 1983). In this model, fragment A is protected from the contact with lipids by the larger B fragments (Olsnes & Sandvig, 1983).

To probe the membrane interaction of DT we have used here a labelling method able to demonstrate the penetration of proteins into the membrane (Brunner,

1981; Bayley, 1983; Bisson & Montecucco, 1985*a,b*). It is based on the use of trace amounts of photoactivatable radioactive phospholipids interdispersed among other lipids in liposomes. On illumination the dark-stable reagents generate a highly reactive intermediate able to cross-link, and hence specifically label, the lipid-exposed protein regions.

MATERIALS AND METHODS

Chemicals

DT was obtained from Connaught Laboratories, Willowdale, Ont., Canada, and further purified by chromatography on DEAE-cellulose DE-52 as described by Collier & Kandel (1971). Fragment A was purified and carboxymethylated as described by Chung & Collier (1977). DMP, DCP, EL and AL were from Sigma. AL was purified as described by Kagawa & Racker (1971). Chol (Merck) was recrystallized from ethanol. PC I (sp. radioactivity 2.8 Ci/mmol) and PC II (sp. radioactivity 174 Ci/mol) were prepared as previously described (Bisson & Montecucco, 1981). DCCD (sp. radioactivity 54 Ci/mol) was purchased from Amersham International.

Liposome preparation

All operations were performed under a red safety light. Liposomes were prepared by mixing, in chloroform/methanol (2:1, v/v), DMP or EL or AL with cholesterol (2.5:1 molar ratio, taking 750 as the M_r of a AL phospholipid), PC I (0.02/0.12 mol%) and PC II (0.03/0.06 mol%). After drying under an N_2 flux, resuspension in diethyl ether, drying and vacuum-pumping, the lipids were sonicated in 50 mM-Tris/acetate, pH 8.0, in a stoppered vial under N_2 until optical clarity was achieved. In some experiments DMP/Chol/DCP liposomes were prepared as described above with the molar ratio 5:3.75:1.

Abbreviations used: DT, diphtheria toxin; DMP, dimyristoyl phosphatidylcholine; DCP, dicetyl phosphate; EL, egg lecithin (phosphatidylcholine); Chol, cholesterol; PC I, 1-palmitoyl-2-(2-azido-4-nitro)benzoyl-*sn*-glycero-3-phospho[3H]choline; PC II, 1-myristoyl-2-[12-amino-(4*N*-3-nitro-1-azidophenyl)]dodecanoyl-*sn*-glycero-3-phospho[^{14}C]choline; AL, soya-bean asolectin; DCCD, *N,N'*-dicyclohexyl[^{14}C]carbodi-imide; BLM, black lipid membranes; SDS, sodium dodecyl sulphate.

Photolabelling and radioactivity determinations

The liposomal preparation was subdivided in 0.5 ml fractions and the 'nicked' toxin or the A-fragment was added at a lipid/protein molar ratio varying from 4200 to 12600 in different experiments.

After 10 min incubation at 37 °C the mixture was adjusted to the desired pH by adding 10% (v/v) acetic acid. pH was measured with a Beckman 71 pH-meter standardized between 4.00 and 7.00. The ionic strength of the different samples was adjusted with NaCl. After 5 min at 37 °C the samples were illuminated with a long-wave u.v. lamp (Mineralight, San Gabriel, CA, U.S.A.) with the protection of a glass/water filter for 10 min. When required, 2 M-Tris was used to return the pH from 5.00 to 8.00. The protein was recovered by cold-acetone precipitation and diethyl ether washing as described previously (Tomasi & Montecucco, 1981) and subjected to SDS/polyacrylamide-gel electrophoresis on 15% (w/v) polyacrylamide gels as described by Kadenbach *et al.* (1983). After Coomassie Blue staining, destaining and densitometric recording, the gels were cut in 1.8 mm-thick slices and incubated overnight with a tissue solubilizer. After a 24 h incubation with a scintillation cocktail the samples were counted for radioactivity in a Packard Tri-Carb 300C liquid-scintillation counter. Radioactivity associated with the protein bands was determined as d.p.m. and corrected for differences in protein content (determined by integration of the Coomassie Blue peaks).

Labelling with [¹⁴C]DCCD

A 0.5 ml portion of AL/Chol liposomes, prepared as described above in the same buffer and containing in addition 1 mM-lysine, was added to a vial in which 1 μ Ci of [¹⁴C]DCCD was previously dried under a N₂ stream from a toluene stock solution. After 30 min incubation, with stirring, at room temperature, 30 μ g of DT or 10 μ g of fragment A was added and the incubation prolonged for a further 5 min. The pH was adjusted to 4.5 or 5.5 and the mixture incubated for 1, 2 or 4 h either at 20 °C or at 37 °C. The protein was recovered by addition of cold 5% acetic acid in acetone and the radioactivity associated with the protein was determined as described above.

RESULTS

Hydrophobic photolabelling of DT

To monitor the lipid interaction of DT we have used both a shallow probe, PC I (the structural formula appears as an inset to Fig. 1), whose photoreactive group is strictly localized at the membrane surface, and a deep probe, PC II, whose reporter group is bound at the fatty acid methyl terminus (see inset to Fig. 1).

There are several lines of evidence showing that the labelling with these photoactivatable phosphatidylcholines is exclusively restricted to the lipid bilayer (Bisson *et al.*, 1979; Montecucco *et al.*, 1980, 1983; Tomasi & Montecucco, 1981; Hoppe *et al.*, 1983). This holds also in the case of DT. In fact, as shown in Fig. 1, when the 'nicked' active form of DT was incubated with liposomes of different lipid composition at pH 8.0 and illuminated, no labelling of the toxin was found. In contrast, when pH was lowered to 4.5, both fragments A and B were labelled with the two probes. This indicates that both polypeptide chains of the nicked toxin are

inserted into the lipid bilayer, but only at low pH. The insertion and consequent labelling of the partially hydrophobic B-fragment is in agreement with the results of previous studies, which have demonstrated its ability to increase the conductance of BLMs. The present data show that the hydrophilic fragment A is also able to penetrate the hydrophobic membrane phase. This was also observed in recent studies with a reagent carrying the photoreactive group near the fatty acid methyl end (Hu & Holmes, 1984; Zalman & Wisniewsky, 1984). The toxin fragments expose their protein surface at the different membrane regions probed by PC I and PC II. However, the reactivity of the amino acid residues in contact with the photoreactive group of the two probes must be different in order to explain the finding that PC II is about 20 and 15 times more efficient than PC I in labelling fragments A and B respectively. The lower labelling ability of PC I with respect to PC II cannot be accounted for by the higher quenching of its photoreactive group by water (Bisson & Montecucco, 1981).

It has been shown that DT binds much more efficiently to liposomes containing negatively charged lipids and that the AL/lipid mixture displays the maximum DT-binding capacity (Alving *et al.*, 1980; Donovan *et al.*, 1982). In agreement with these findings, a 10–12 times higher toxin labelling with AL with respect to EL liposomes was consistently found at pH 4.5 (see Fig. 1).

Hydrophobic photolabelling is at the present time unable to provide an estimate of the fraction of toxin actually inserted in the membrane, because the yield of protein photo-cross-linking is low and highly dependent on the reactivity of the various protein residues towards the photogenerated intermediate (Richards & Brunner, 1980; Bayley, 1983; Bisson & Montecucco, 1985a,b). However, the yield of toxin labelling with AL vesicles at low pH is similar, for PC II, to that found with several integral membrane proteins (Bisson & Montecucco, 1981), indicating that a major portion of the toxin becomes inserted in the membrane.

The same profile of labelling was also obtained with a carbene-generating phosphatidylcholine (Brunner *et al.*, 1983; C. Montecucco, J. Brunner, G. Schiavo & M. Tomasi, unpublished work), indicating that the distribution of radioactivity between the two DT fragments is not the result of the reactivity properties of the nitroaryl nitrene generated by PC I and PC II.

pH-dependence of DT labelling

Owing to the greater extent of labelling observed with AL liposomes, these were used to study the pH-dependence of DT insertion into the lipid bilayer. Fig. 2 shows that between pH 6 and 5 there is a very large increase in the extent of DT labelling with both PC I and PC II. This is not due to different reactivities of the reagent at the different pH values, because it was not observed with other proteinaceous toxins such as abrin and ricin (C. Montecucco & M. Tomasi, unpublished work). The latter result also tends to exclude an effect mediated via a liposome structural transition. This conclusion is further supported by the lack of difference found with changing pH in negatively stained asolectin liposomes (results not shown) and by their impermeability to K⁺ at pH 4.0 (Boquet & Duflo, 1982).

Fig. 1. Hydrophobic photolabelling of DT liposomes at different pH values

(a) Coomassie Blue profile of photolabelled DT on a 15% polyacrylamide gel run as described by Kadenbach *et al.* (1983). The gel was overrun in order to remove the unbound photoreacted lipids, which run near the tracking dye. Patterns of DT labelling in the presence of AL at pH 8.0 ($\times$) or at pH 4.5 ($\bullet$) or in the presence of EL at pH 4.5 (stippled areas) with PC I (b) and PC II (c) are also shown. The structural formulae of PC I and PC II are given as insets.

Distribution of labelling between DT fragments

The large increase in labelling with decreasing pH is not distributed evenly between the two toxin fragments. Instead, as shown in Fig. 3, low pH values influence the phospholipid interaction of the enzymic A-fragment more than that of the binding fragment, B. These data suggest that the A-chain does not simply penetrate into the membrane as a consequence of the pH-driven membrane binding and insertion of the B-chain. It

appears that a low pH-induced conformational transition of the A-fragment is superimposed on the increased binding and labelling of the B-domain. The lipid interaction of the A-chain is fully reversible, since no significant labelling (less than 5%) was found when the liposome/toxin mixture was returned from pH 5 to pH 8 before photoactivation. In the case of the B-fragment, its insertion in the membrane is only partially reversible (60–70%).

Fig. 2. DT-associated radioactivity as function of pH

The amounts of labelling of DT incubated with AL liposomes at different pH values are reported as percentages of that bound to the toxin at pH 4.00. The mean results for three different experiments are shown. *, PC I; □, PC II.

Fig. 3. Distribution of labelling between DT fragments at different pH values

The amount of total radioactivity associated with fragment A with respect to that bound to the entire DT in the same experiment is plotted against pH. The mean results for three different experiments are shown.

Lipid interaction of fragment A

These results suggested the possibility that, at low pH, the A-chain could itself interact with lipids. Table 1 shows that fragment A alone is able to adopt, at low pH, a lipid-interacting conformation and that its insertion into AL liposomes is much more efficient than into phosphatidylcholine vesicles. Negatively charged lipids appear to be involved, and even a molecule as simple as myristic acid appears to be effective. Again the phenomenon is fully reversible.

Fig. 4 shows the pH-dependence of the labelling of fragment A in AL vesicles. With both PC I and PC II the transition occurs between pH 4.5 and 5.5, suggesting an

Table 1. DT and fragment A labelling at pH 4.0 in liposomes of different compositions

Further abbreviations used: MA, myristic acid; A, fragment A of DT.

Liposome composition	Fragment...	Labelling (% of that found with DT and AL/Chol)	
		B	A
DT + AL/Chol		100	100
DT + EL/Chol		8-10	6-8
DT + DML/Chol/DCP		100	55-60
A + AL/Chol		—	130-155
A + DMP/Chol		—	10-12
A + DMP/Chol/MA		—	38-45
A - DMP/Chol/DCP		—	45-55

Fig. 4. pH-dependence of fragment A photolabelling

The amount of radioactivity associated with fragment A after photolabelling in the presence of AL/Chol liposomes is plotted in function of the pH of the incubation medium. Data (means for three experiments) are expressed as percentage of the radioactivity (d.p.m.) of either PC I (○) or PC II (●) bound to the protein at the pH of maximum labelling.

involvement of carboxylic groups. Even in their protonated form, carboxylic groups are polar and hence unlikely to interact with lipids. However, one glutamic acid residue is exposed to lipids in cytochrome *c* oxidase (Casey *et al.*, 1980), ATP synthase (Sebald *et al.*, 1980) and glycophorin (Ross *et al.*, 1982) as inferred from their high labelling with the hydrophobic carboxylate-specific reagent DCCD. Under conditions of high labelling of cytochrome *c* oxidase with [¹⁴C]DCCD and high labelling of DT with the phospholipid probes, there was a negligible binding of [¹⁴C]DCCD to any of the DT fragments or to the A-fragment alone (results not shown). This result suggests that, at low pH, fragment A rearranges or interacts with other A-chains in such a way as to shield the polar carboxylic groups from contact with lipids.

DISCUSSION

The action of DT is thought to involve the following steps: (i) binding to its receptor, (ii) internalization in endocytotic vesicles, (iii) membrane crossing and (iv) modification of elongation factor 2.

To gather more information on the poorly understood aspect of the penetration across the membrane, model systems such as liposomes and BLMs have been widely used. As a result of these studies it has been proposed that, at low pH, the B-domain forms a transmembrane protein channel through which the enzymically active A-chain traverses the membrane without entering into contact with lipids (Olsnes & Sandvig, 1983). This proposal is in agreement with the available primary structure of DT, which shows the presence, in the B-fragment, of hydrophobic stretches about 20 residues long that could be involved in the interaction with the hydrocarbon chains of phospholipids. In contrast, the A-chain shows many charged residues evenly distributed, thus conferring a hydrophilic character.

The present findings do not support this model and provide evidence that the A-fragment at low pH also enters the lipid bilayer both at a superficial and at a deeper level. The membrane penetration of DT is dependent on lipid composition and occurs over the range of pH values that are encountered in endosomal compartments (Steinman *et al.*, 1983).

Both DT fragments are increasingly labelled as the pH is lowered, but this effect is much more pronounced for the A- than for the B-fragment. The low-pH-induced lipid interaction of the A-chain is fully reversible. It is noteworthy that the $^3\text{H}/^{14}\text{C}$ (PC I/PC II) labelling ratio of both the A- and B-fragments ($6.2 \pm 1.5\%$ for A and $7.3 \pm 1.1\%$ for B with respect to the starting ratio) remains constant between pH 4 and 7, suggesting that the hydrophobic lipid-interacting form of DT is present in a very similar conformation, although in a different proportion, over a wide range of pH. The much higher yield of labelling of PC II with respect to PC I indicates that the photoreactive group of PC II does not appreciably fold back to the membrane surface.

The capacity of the highly hydrophilic fragment A to interact with the hydrophobic fatty acid portion of phospholipids at low pH was unexpected on the basis of current ideas on lipid-protein interactions. Fairly recently the membrane insertion of another very hydrophilic protein, apo-(cytochrome *c*), has been well documented (Rietveld & de Kruijff, 1984; Dumont & Richards, 1984). The finding that hydrophilic proteins may adopt conformations that bring their hydrophobic residues together on the protein surface may also be related to the process of cell penetration by several different viruses that occurs through protein domains exposed to the water milieu in the isolated virus (White *et al.*, 1973). The ability of fragment A to penetrate into a lipid bilayer would explain the toxicity of its conjugates, which lack fragment B, with asialofetuin (Cawley *et al.*, 1981), lectins (Uchida *et al.*, 1978) and antibodies (Masuho *et al.*, 1979; Collier & Kaplan, 1984). According to the results obtained with BLMs and K^+ -loaded AL liposomes, its membrane insertion should be electrically silent (Boquet & Duflot, 1982; Misler, 1984).

The pH-dependence of the lipid interaction of fragment A suggests an involvement of carboxy groups. These hydrophilic residues appear not to enter in contact

with lipids, most likely because they are shielded inside the area of protein-protein contact among different A units. If the role of negatively charged lipids is to neutralize the positive charges of the protonated A-fragment, then a complex formed by negative lipids plus a dimer or a multimer of A-chains could be the form able to be inserted into the membrane. Protonation-deprotonation and charge-neutralization processes are also compatible with the fully reversible lipid interaction of fragments A and could contribute to its exit from the membrane when it faces the neutral pH of the cytoplasm.

From studies of hydrophobic photolabelling a rather different picture emerges for the membrane interaction of cholera toxin and of DT (Tomasi & Montecucco, 1981; Tomasi *et al.*, 1982). Both toxins are composed of binding and enzymic subunits held together via a single disulphide bridge, and the active subunit must enter the membrane to perform its action. However, different events appear to be able to trigger the membrane penetration of the active subunit. In fact, chemical reduction is effective with cholera toxin, whereas low pH is needed for DT. Moreover, the binding domain of cholera toxin confines itself to the membrane surface, whereas that of DT penetrates into the hydrophobic core of the lipid bilayer.

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