pH-Induced Transitions in Cholera Toxin Conformation: A Fluorescence Study†

Marc J. S. De Wolf,*,‡ Guido A. F. Van Dessel,§ Albert R. Lagrou,‡ Herwig J. J. Hilderson,‡ and Wilfried S. H. Dierick‡,§

RUCA-Laboratory for Human Biochemistry and UIA-Laboratory for Pathological Biochemistry, University of Antwerp, B2020 Antwerp, Belgium

Received July 14, 1986; Revised Manuscript Received January 29, 1987

ABSTRACT: Determination of the ratio of intrinsic fluorescence with dibrominated Bry 96 (F) relative to that with unbrominated Bry 96 (F_0), at neutral pH and in the presence of 0.2 M NaCl, reveals that the A subunit of cholera toxin (CT A) has a somewhat higher affinity for this mild detergent than intact cholera toxin (CT) and its B subunit (CT B). Receptor (GM_1 or oligo-GM_1) binding has no influence on the very low detergent binding of CT and CT B. Activation of CT A by treatment with dithiothreitol (20 mM) also does not affect detergent binding. The weak hydrophobic nature of CT A is also reflected by the negative modulatory action of anionic phospholipids and deoxycholate on its mono-ADP-ribosyltransferase activity and the ability of the former to decrease its intrinsic fluorescence intensity in a salt-resistant way. Detergent binding of CT A is only slightly pH dependent whereas, upon lowering the pH, detergent binding to CT or CT B becomes significant. In the pH range 6.5–4.2 a gradual increase in detergent binding to CT and CT B occurs. In the narrow pH range 4.2–4.0 a sharp and time-dependent enhancement of brominated Bry 96 quenching is observed. The increase in detergent binding upon lowering the pH is fully reversible, salt dependent, and complete within 10 min ($t_{1/2}$ = 2 min at 25 °C). Solute quenching experiments with the neutral polar quencher acrylamide reveal that upon lowering the pH to 5.0 a marked increase in the exposure of the lone Trp-88 residue in each β-polypeptide chain of CT B occurs. This phenomenon is also fully reversible, salt dependent, and complete within 10 min ($t_{1/2}$ = 1.2 min at 25 °C). The acrylamide fluorescence quenching of the A subunit (CT A) is not affected in the pH range (7.0–3.0) investigated. Below pH 4.0 a progressive dissociation of CT B is observed as monitored by following the change in steady-state fluorescence anisotropy (r_s) of dansylated CT B. At 25 °C and pH 3.2, dissociation is complete in 30 min, and a rate constant of 0.150 min^{-1} is calculated. Complex formation of CT B with GM_1 or oligo-GM_1 prevents the dissociation of CT B. This appears to be the result of shielding from protonation of amino acid residues committed to β-polypeptide association.

Cholera toxin (CT,[1] M_r ~84 000) is an oligomeric protein composed of two structural and functional distinct subunits, CT A and CT B (M_r ~29 000 and ~55 000, respectively). CT B contains five identical polypeptide chains (M_r 11 600) arranged in a noncovalently associated ring-like pentameric configuration. CT A consists of two polypeptides, A_1 or α-chain (M_r 23 000) and A_2 or γ-chain (M_r 5500), linked by a single disulfide bridge. CT action is initiated by rapid binding to the outer cell membrane through interaction between CT B and the monosialoganglioside GM_1 followed by entry of polypeptide A_1 into the cell where it is able to stimulate adenylate cyclase by catalyzing the ADP-ribosylation of the $G_{s\alpha}$ subunit of the stimulatory GTP binding regulatory protein [for reviews, see Finkelstein (1973), Holmgren et al. (1973), Finkelstein (1975), Bennett and Cuatrecasas (1977), Gill (1977), Holmgren (1978), Moss and Vaughan (1979), Lai (1980), Johnson (1982), and Gilman (1984)].

The mechanisms of entry of the A_1 polypeptide chain remain poorly defined. Several mechanisms of entry have been proposed such as the creation of a hydrophilic channel following binding of CT B to GM_1. This channel may be formed by the CT B itself (Gill, 1976) or by intrinsic membrane proteins acting as translocators of the A_1 polypeptide chain (Gill, 1978). An alternative explanation is that multivalent binding of the CT B to several receptors would induce a perturbation in both the toxin and membrane structure (Kohn et al., 1980; Fishman, 1982). This would result in the dissociation of CT into CT A and CT B and subsequent penetration of the A component into the cell membrane. The initial highly specific interaction of CT with the oligosaccharide moiety of GM_1 (oligo-GM_1) appears to occur without any substantial conformational change (De Wolf et al., 1981b; Tomasi et al., 1984). It was shown (De Wolf et al., 1981a,b; Sillerud et al., 1981; Tomasi et al., 1984; Ludwig et al., 1985) that the lone Trp-88 residue of each β-polypeptide chain is an important determinant in this interaction. In a previous paper (De Wolf et al., 1985), the pH dependence of the fluorescence characteristics of CT that are related to this lone Trp-88 were examined.

This study is concerned with the effect of environmental factors (pH, ionic strength, reducing agents) on the structure of CT and its subunits with the emphasis on the formation or exposure of hydrophobic domains. The potential implications in the mechanism of translocation into or through the membrane are discussed.

Materials and Methods

Highly purified CT was obtained from List Biological Laboratories Inc. (Campbell, CA). CT A and CT B were prepared according to Finkelstein (1974) except that NaCl (0.2 M) was omitted from the elution buffer during gel filtration over Bio-Gel P-60. This resulted in a better resolution

† This work was supported in part by U.S. Public Health Service Grant 1RO1 AM32136-03 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, U.S. Public Health Service, and by Belgian FGWO Grant 3.0057.81.

‡ RUCA-Laboratory for Human Biochemistry.

§ UIA-Laboratory for Pathological Biochemistry.

[1] Abbreviations: Bry 96, poly(oxyethylene) 10 oleyl ether; CT, cholera toxin; CT A, A subunit of cholera toxin; CT B, B subunit of cholera toxin; Dns, dansyl [5-(dimethylamino)-1-naphthalenesulfonate]; GM_1, galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/87/0426-3799$01.50/0

of CT A and CT B (V_e/V_0 for A = 1.13; V_e/V_0 for B = 1.61). Biological activity was determined by the rabbit skin permeability test (Craig, 1971). Concentrations of CT, CT A, and CT B were calculated from published values for extinction coefficients and molecular weights (Finkelstein, 1973). 5-(Dimethylamino)-1-naphthalenesulfonate (dansyl, Dns) derivatives of CT were prepared as previously described (De Wolf et al., 1981b). Dns-CT A and Dns-CT B were derived from Dns-CT according to the same method applied for the preparation of native subunits. The degree of labeling, i.e., the molar ratio of Dns to CT, was determined from absorbance measurements at 282 nm for CT [ϵ = 95 844 L/(mol·cm)] (Finkelstein, 1973) and at 340 nm for the dye [ϵ = 3300 L/(mol·cm)] (Horton & Koshland, 1967). The GM_1 used in these studies was derived from a Supelco (Bellafonte, PA) preparation by preparative thin-layer chromatography (Mullin et al., 1976). The ganglioside concentration was quantitated from its sialic acid content with a micromodification of the resorcinol method of Svennerholm (1957) with *N*-acetylneuraminic acid (Calbiochem) as a standard. The oligosaccharide moiety of GM_1 was obtained from an ozonolysis reaction followed by (i) an alkaline fragmentation procedure as described by Wiegandt and Bücking and (ii) chromatography on Dowex 1-X8 (Wiegandt & Bücking, 1970). Its purity (over 95%) was confirmed by chromatography on thin-layer silica gel G plates with 1-propanol and an aqueous solution of 0.2% $CaCl_2$ (8:2) as the eluent (Fishman et al., 1978; De Wolf et al., 1981a). Dibrominated Bry 96 was prepared by titration of an aqueous solution of Bry 96 with concentrated bromine water (Blewitt et al., 1984). Acrylamide was purchased from Merck and recrystallized from ethyl acetate before use. The stock solution (8 M) of acrylamide was used within a few days of preparation. To evaluate the pH-dependence of Br-Bry 96 and acrylamide quenching of protein fluorescence, the following buffering systems were used: from pH 2.9 to pH 3.5, 0.05 M glycine hydrochloride/0.2 M NaCl; from pH 3.7 to pH 5.0, 0.05 M sodium acetate/0.2 M NaCl; from pH 5.6 to pH 6.6, sodium citrate/0.2 M NaCl; from pH 6.5 to pH 7.5, 0.05 M Tris-HCl/0.2 M NaCl. Protein solutions were obtained from the same stock solution by 10-fold dilution in these buffers. Fluorescence intensities, anisotropies, and excited-state lifetimes were recorded on a SLM 4800 spectrofluorometer equipped with a Hewlett-Packard 85 calculator and a 7225A plotter as previously described (De Wolf et al., 1985). The excitation wavelength was 295 nm; emission wavelengths were 340, 345, and 330 nm for CT, CT B, and CT A, respectively. Attenuation of the excitation light intensity by acrylamide (ϵ_M = 0.23 at 295 nm) and dilution effects were corrected for by applying correction factors as described by Parker (1968). Mono-ADP-ribosyltransferase activity was assayed measuring the formation of [*carbonyl*-^{14}C]nicotinamide from [*carbonyl*-^{14}C]NAD^+ (~50 mCi/mmol, Amersham) in the presence of 60 mM L-arginine methyl ester as an artificial ADP-ribose acceptor (De Wolf et al., 1981c). Phospholipids were purchased from Sigma. Phospholipids dissolved in chloroform–methanol (2:1) were dried under a stream of nitrogen and subsequently ultrasonically dispersed in buffer (1 mg/mL, Braun-Sonic 300, setting 60). Sonication times were 10 min for phosphatidylcholine, 3 min for phosphatidylethanolamine, and 2 min for phosphatidylserine and phosphatidylinositol at 0 °C.

Results

Detergent and Phospholipid Binding of CT, CT A, and CT B. Using the recently described technique of fluorescence quenching detection of detergent binding (Blewitt et al., 1984), we examined the binding of the mild detergent Bry 96 to CT, CT A, and CT B. Determination of the ratio of intrinsic fluorescence with brominated Bry 96 (F) relative to that with unbrominated Bry 96 (F_0), both in the presence of 0.2 M NaCl, indicates that at neutral pH the A subunit has a somewhat higher affinity for this mild detergent than the holotoxin and CT B (Table I).

Table I: Quenching of Intrinsic Fluorescence of CT, CT A, and CT B by Brominated Bry 96

compound	F/F_0[a] pH 7.0	F/F_0[a] pH 4.0	τ/τ_0[a] pH 7.0	τ/τ_0[a] pH 4.0
CT	0.82 (0.81)[b]	0.40	1.02	0.91
CT + GM_1	0.85	0.82		
CT A	0.57	0.56	0.95	0.92
CT A (activated)	0.55	0.56	0.93	0.96
CT B	0.81 (0.80)[b]	0.41	1.03	0.95
CT B + GM_1	0.82	0.81		
CT A + GM_1	0.51	0.47		

[a] F/F_0 and τ/τ_0 are respectively the ratios of intrinsic fluorescence intensities and excited-state lifetimes with brominated Bry 96 (F, τ) relative to that with unbrominated Bry 96 (F_0, τ_0) in the presence of 0.2 M NaCl and at 25 °C. Activated CT A was prepared by treating the native subunit with 20 mM dithiothreitol for 1 h at 30 °C. The protein and GM_1 concentrations were 1 and 20 μM, respectively. [b] F/F_0 obtained with CT or CT B initially incubated for 1 h at pH 4.0 and subsequently pH readjusted to pH 7.0.

The affinity appears to be low since no quenching (detergent binding) is observed upon reducing the Br-Bry 96 concentration down to 0.005%. The decrease in fluorescence intensity by the addition of Br-Bry 96 is not paralleled by a similar drop in average fluorescence excited-state lifetime (Table I). Addition of GM_1 or oligo-GM_1 to CT or its subunits does not alter detergent binding at neutral pH. Also, activation of CT A by addition of dithiothreitol (20 mM), which is essential for expression of catalytic (mono-ADP-ribosyltransferase) activity, has no further effect on its detergent binding (Table I).

The presence of hydrophobic domains on CT and its subunits was also investigated by looking at their interaction with phospholipids. As indicated in Figure 1, various phospholipid dispersions are able to decrease the fluorescence intensity of CT, CT A, and CT B. The efficacy of quenching is to some degree dependent on the nature of the phospholipid added. Acidic phospholipids being somewhat more effective than the zwitterionic phospholipids. Addition of phospholipids does not induce a shift in the fluorescence emission spectra of CT, CT A, and CT B. Whereas increasing the ionic strength partially or almost completely reverses the phospholipid-induced quenching of fluorescence of CT and CT B (Figure 1b,f), addition of NaCl enhances the phospholipid quenching of the fluorescence of CT A (Figure 1d).

Phospholipids are also able to modulate the mono-ADP-ribosyltransferase activity of activated CT A. Anionic phospholipids cause partial inhibition of transferase activity; phosphatidylcholine, on the other hand, significantly enhances activity (Table II). Sodium deoxycholate (0.1%) completely blocks the transferase activity whereas the neutral detergent Bry 96 has no effect.

Effect of pH on Detergent Binding of CT, CT A, and CT B. Detergent binding of CT A is only slightly pH dependent whereas upon lowering the pH to 4 detergent binding to CT and CT B is significantly enhanced (Table I). In the pH range 6.5–4.2, a gradual and time-dependent increase in detergent binding to CT and CT B occurs (Figure 2). In the narrow pH range 4.2–4.0, a sharp enhancement of Br-Bry 96 quenching is observed. As also shown in Figure 2, pH-induced

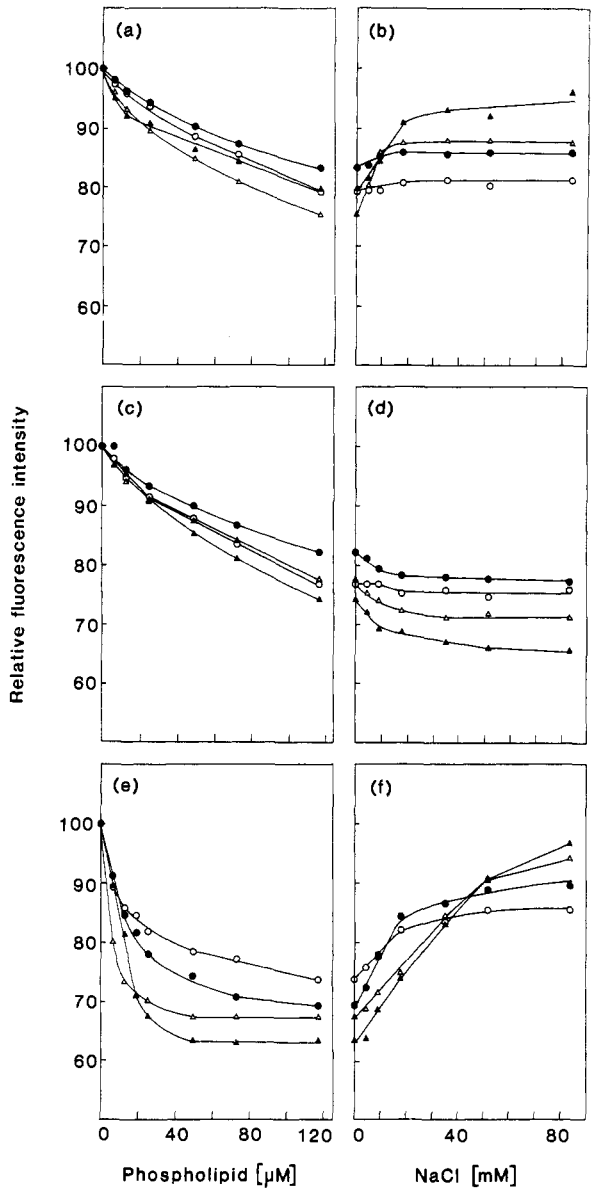

FIGURE 1: Effect of phospholipids and NaCl on the fluorescence intensity of CT (a, b), CT A (c, d), and CT B (e, f). Phospholipids were dissolved in chloroform–methanol (2:1) and dried under a stream of nitrogen. After buffer was added to the dry lipid to obtain a final concentration of 1 mg/mL, the suspension was sonicated to clarity with a Braun sonifier under a stream of nitrogen at 0 °C. Protein concentration was around 1 μM. (○) Phosphatidylcholine; (△) phosphatidylinositol; (●) phosphatidylethanolamine; (▲) phosphatidylserine.

changes in Br-Bry 96 fluorescence quenching of CT or CT B are superimposed on but clearly distinct from strong intrinsic quenching.

In contrast to the pH-induced intrinsic quenching, which occurs without any appreciable time lag, the increase in detergent binding is time dependent. Upon measuring Br-Bry 96 quenching immediately after mixing of CT or CT B with appropriate buffer, a less pronounced increase in Br-Bry 96 binding without a sharp transition with a midpoint at pH 4.1 is observed (Figure 2). At pH 3.75 and 25 °C, detergent binding reaches its maximum in 10 min with a $t_{1/2}$ of 2 min (Figure 3). During this time, the fluorescence intensity is not further affected (Figure 3). The pH-induced conformational change of CT or CT B resulting in an increase of detergent binding is fully reversible as evidenced by the complete reversal of Br-Bry 96 quenching after readjusting the pH from 4.0 to 7.0 with a dilute NaOH solution (Table I). Increased Bry 96 binding to CT and CT B (at pH 4.0) is salt sensitive (Figure 4) but independent of the nature of the electrolyte (Table III).

Table II: Effect of Phospholipids and Detergents on Mono-ADP-ribosyltransferase Activity of CT A[a]

effector	concentration	activity (%)
none		100
phosphatidylcholine	50 μM	108
	250 μM	155
phosphatidylethanolamine	50 μM	100
	250 μM	107
phosphatidylserine	50 μM	66
	250 μM	25
phosphatidylinositol	50 μM	45
	250 μM	21
sodium deoxycholate	0.005%	72
	0.01%	21
	0.1%	<0.5
Triton X-100	0.005%	100
	0.01%	92
	0.1%	84
Bry 96	0.005%	100
	0.01%	100
	0.1%	100

[a] CT A (1.5 μM) was preincubated with the indicated amounts of phospholipid or detergent for 15 min at 37 °C and subsequently incubated with 200 mM sodium phosphate, pH 7.4, 2 mM [*carbonyl*-^{14}C]-NAD$^+$ (53 mCi/mmol), 60 mM L-arginine methyl ester, and 20 mM dithiothreitol for 1 h at 37 °C. Values are the means for three separate experiments.

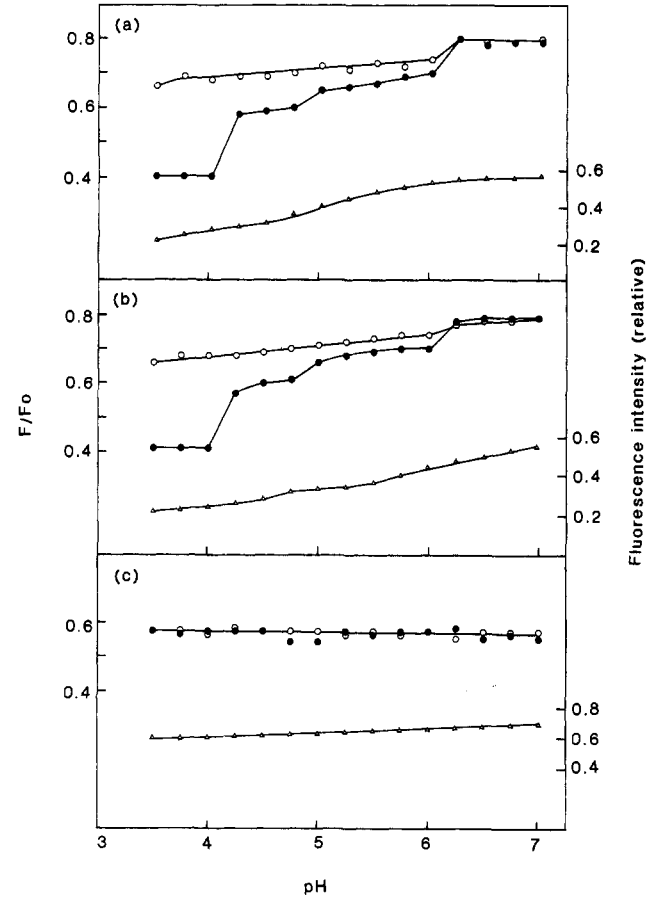

FIGURE 2: pH dependence of the fluorescence intensity and Br-Bry 96 quenching of the fluorescence of (a) CT, (b) CT B, and (c) CT A. Fluorescence intensities in the absence or the presence of 0.1% unbrominated Bry 96 (F_0) and 0.1% Br-Bry 96 (F) were measured on a series of solutions of CT, CT A, and CT B obtained by 10-fold dilution of stock solutions with appropriate buffers. (△) Fluorescence intensities; (○) F/F_0 ratios measured immediately after mixing of protein and buffer; (●) F/F_0 ratios measured 1 h after mixing of protein and buffer.

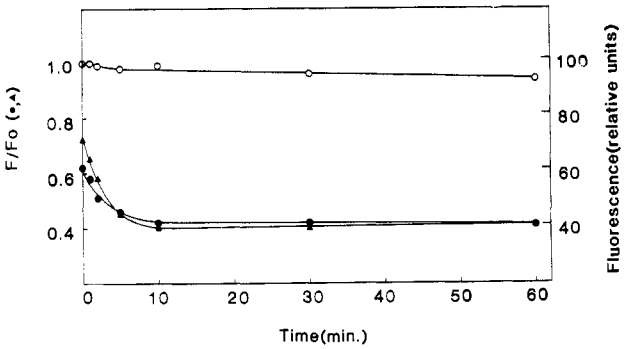

FIGURE 3: Kinetics of acid-induced changes in CT conformation. The fluorescence intensity (○), acrylamide quenching (●), and brominated Bry 96 quenching (▲) of CT (0.5 μM) in 0.05 M sodium acetate buffer, pH 3.75, containing 0.2 M NaCl were recorded at 25 °C as a function of time. Acrylamide and Br-Bry 96 concentrations were 0.4 M and 0.1%, respectively.

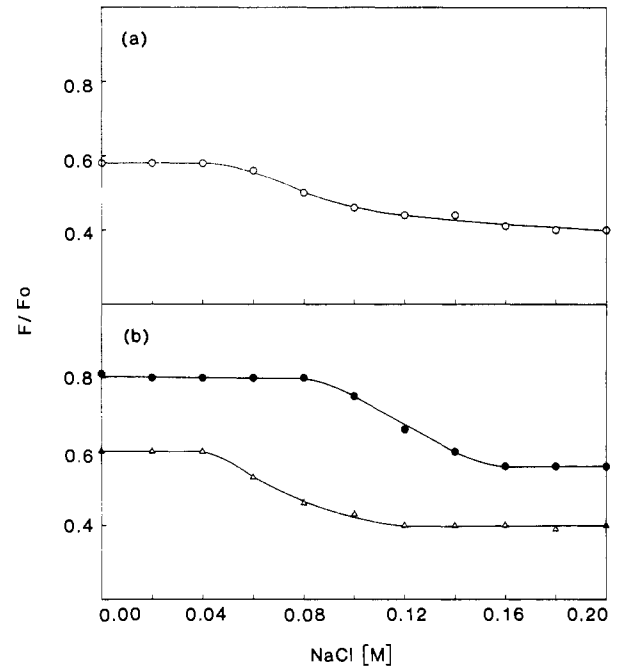

FIGURE 4: Effect of NaCl on Br-Bry 96 quenching of CT (a) and CT B (b). Fluorescence intensities in the presence of 0.1% unbrominated Bry 96 (F_0) and 0.1% Br-Bry 96 (F) were measured as a function of NaCl concentration on CT (○) and CT B (△) in 0.005 M sodium acetate buffer, pH 3.9, and on CT A (●) in 0.005 M Tris-HCl buffer, pH 7.0. Fluorescence intensities were measured after incubation for 1 h at room temperature.

Since the detergent binding itself is salt dependent as indicated by the salt effect on Br-Bry 96 quenching of the fluorescence of CT A (Figure 4), it is difficult to evaluate the exact influence of ionic strength on the pH-induced exposure of hydrophobic domains on CT B. In the presence of GM_1 and oligo-GM_1, no pH-induced enhancement of detergent binding of CT and CT B is observed.

Effect of pH on the Acrylamide Fluorescence Quenching of CT, CT A, and CT B. Further characterization of the pH-induced conformational changes in toxin structure was achieved by performing fluorescence quenching experiments with acrylamide. Acrylamide is a neutral polar quencher that reports not only on the "steady state" but on the "dynamic" exposure of Trp residues in a protein as well (Eftink & Ghiron, 1976, 1977). The quenching is not affected by local electrostatic effects, reducing the ambiguity in the interpretation of the quenching properties.

Figure 5a shows the effect of pH on the acrylamide quenching of the fluorescence of CT. In the pH range 7.0–5.0

Table III: Effect of Salts upon pH Dependence of Fluorescence Quenching of CT B and CT A with Br-Bry 96

salt	F/F_0[a] CT B (pH 3.75)	F/F_0[a] CT A (pH 7.0)
none	0.62	0.83
200 mM NaCl	0.40	0.56
200 mM KCl	0.41	0.61
200 mM LiCl	0.41	0.57
200 mM KOAc	0.40	0.56
150 mM $CaCl_2$	0.42	0.58
150 mM $Mg(OAc)_2$	0.42	0.57
200 mM NaCl + 10 mM EGTA	0.41	0.56

[a] F/F_0 is the ratio of fluorescence intensity of CT B and CT A in the presence of 0.1% Br-Bry 96 (F) relative to that with unbrominated Bry 96 (F_0). Fluorescence intensities were measured at 25 °C with an excitation wavelength of 295 nm and emission wavelengths of 330 and 345 nm for CT A and CT B, respectively. Buffer concentrations were 0.005 M.

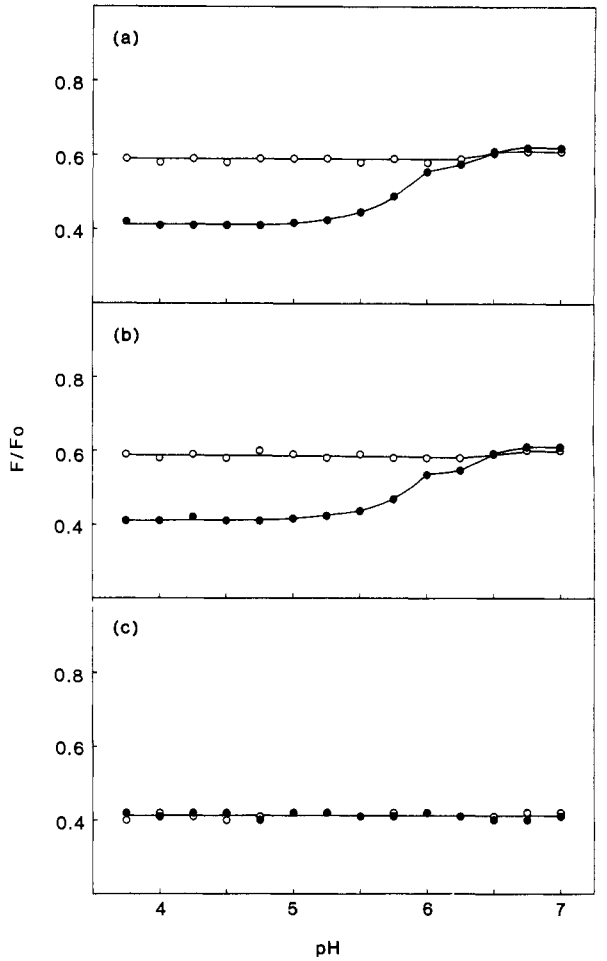

FIGURE 5: pH dependence of acrylamide quenching of the fluorescence intensity of (a) CT, (b) CT B, and (c) CT A. Fluorescence intensities in the absence (F_0) and presence (F) of acrylamide (0.4 M) were measured on a series of solutions of CT, CT A, and CT B obtained by 10-fold dilution of stock solutions with appropriate buffers containing 0.2 M NaCl. The excitation wavelength was 295 nm; emission wavelengths were 340, 345, and 330 nm for CT, CT B, and CT A, respectively. (○) F/F_0 ratios measured immediately after mixing of protein and buffer; (●) F/F_0 ratios measured 1 h after mixing of protein and buffer.

and in the presence of 0.2 M NaCl, a significant increase in fluorescence quenching occurs. This pH dependence is not detected when the quenching is measured immediately after mixing of CT with appropriate buffer but becomes clearly observable after standing of the solution for 1 h. A similar effect of pH on the acrylamide quenching of the fluorescence

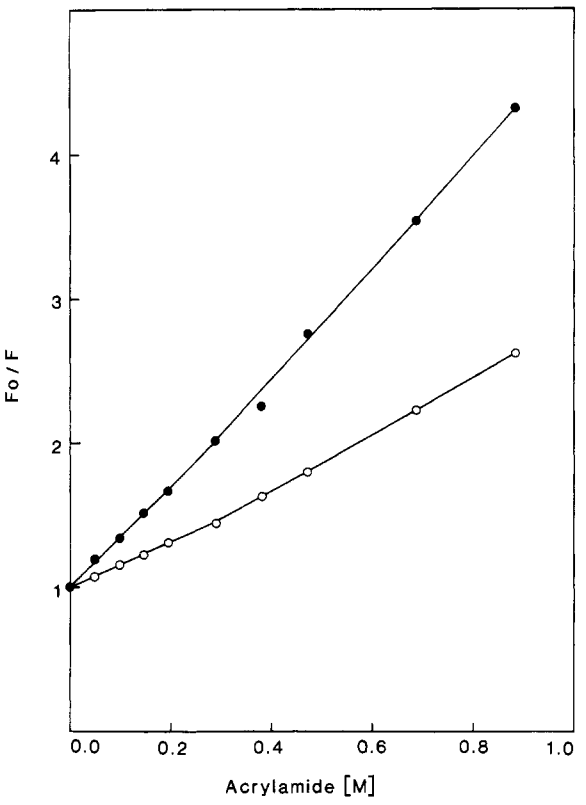

FIGURE 6: Stern–Volmer plots of fluorescence quenching of the CT B by acrylamide at pH 7.0 (○) and pH 4.75 (●). The excitation wavelength was 295 nm, and the emission wavelength was 345 nm.

of CT B is noticed (Figure 5b) whereas in the pH range 7–3.0 the quenching of CT A is not affected (Figure 5c).

The variations of fluorescence intensity of CT B with respect to acrylamide concentration at neutral and acidic pH (pH 4.75) show upward curving Stern–Volmer plots (Figure 6). From the initial slopes of these plots, apparent K_{sv} values of 1.5 and 3.4 M^{-1} at pH 7.0 and pH 4.75, respectively, were calculated. Since the fluorescence of CT and CT B is related to the indole fluorescence of the lone Trp-88 residue of each β-polypeptide chain (De Wolf et al., 1981a,b), changes in acrylamide quenching are indicative of changes in exposure of Trp-88.

Increased acrylamide quenching of the fluorescence of CT and CT B at low pH is not the result of an increase in the average fluorescence excited-state lifetime of Trp-88. On the contrary, upon lowering the pH to 5.0 of a solution of CT B, a significant drop (from 3.6 to 2.8 ns) in the average fluorescence excited-state lifetime has been observed (De Wolf et al., 1985).

Although lowering the pH results in an immediate and strong depression of the fluorescence intensities of CT and CT B (Figure 2), the time-dependent enhanced exposure of Trp-88 is not associated with further changes in fluorescence intensity (Figure 3), and no shift in fluorescence emission spectrum can be observed. The kinetics of exposure of Trp-88 are shown in Figure 3. Increased exposure of Trp-88 is complete in 10 min with at $t_{1/2}$ = 1.2 min at 25 °C.

The pH-induced increase in exposure of Trp-88 is salt dependent (Table IV). In the absence of 0.2 M NaCl, almost no pH-induced increase in fluorescence quenching of CT (not shown) and CT B is observed. The effect of salt appears to be independent of the type of ion present, indicating ionic strength is important.

The conformational change that enhances the exposure of Trp-88 is completely reversible. Reversing the pH from 5.0 to 7.0 restores the accessibility of Trp-88 for acrylamide to its original value. In the presence of GM_1 or oligo-GM_1, no pH-induced conformational change in the CT or CT B structure can be detected as monitored by acrylamide quenching of fluorescence. This is not surprising in the light of the localization of Trp-88 in the binding site of CT. We have previously shown that complex formation of CT or CT B with GM_1 or oligo-GM_1 results in an almost complete shielding of Trp-88 from interaction with charged (I^-) and neutral (acrylamide) solute quenchers (De Wolf et al., 1981b, 1983).

Table IV: Effect of Salts upon pH Dependence of Fluorescence Quenching of CT with Acrylamide

salt	F/F_0[a] pH 4.75	pH 7.0
none	0.56	0.61
200 mM NaCl	0.40	0.61
200 mM KCl	0.42	0.62
200 mM LiCl	0.41	0.61
200 mM KOAc	0.40	0.61
150 mM $CaCl_2$	0.41	0.62
150 mM $Mg(OAc)_2$	0.40	0.61
200 mM NaCl + 10 mM EGTA	0.41	0.60

[a] F/F_0 is the ratio of fluorescence intensity of CT in the presence of 0.4 M acrylamide (F) relative to that in the absence of acrylamide (F_0). Fluorescence intensities were measured at 25 °C with an excitation wavelength of 295 nm and emission wavelength of 340 nm. Buffer concentrations were 0.005 M.

Effect of pH on Pentameric Structure of CT B. The effect of pH on the degree of dissociation of CT B can be easily followed by measuring the "steady-state" fluorescence anistropy (r_s) of Dns-CT B. This is evidenced by (i) the coincident change in elution volume after gel filtration on a Bio-Gel P-60 column and (ii) the almost identical r_s values obtained with Dns-CT B after standing for 30 min at pH 3.2 and with nitrophenylsulfenylated Dns-CT B. In the latter case, it has been shown that, after this specific modification of the single Trp-88 residue of each β-polypeptide chain, CT B is present (even at neutral pH) in its monomeric form (De Wolf et al., 1981a).

Upon monitoring for 1 h r_s of Dns-CT B in the pH range 7–4, no significant dissociation of CT B is apparent. Therefore, changes in Br-Bry 96 and acrylamide quenching of the fluorescence of CT B in this pH range cannot be attributed to a loss of pentameric structure. Below pH 4.0, however, a loss of pentameric structure becomes apparent. As shown in Figure 7, dissociation is complete within 30 min at 25 °C and pH 3.2. Under these conditions the dissociation is first order with time, displaying a rate constant of 0.150 min^{-1}. In the presence of GM_1 or oligo–GM_1, dissociation of CT B at low pH is prevented (Figure 7). This is also demonstrated by the elution of the acidified (pH 3.2) CT B–GM_1 complex at the void volume of a Bio–Gel P–60 column.

The stabilizing effect is probably due to shielding from protonation of amino acid residues involved in the association of β-polypeptide chains. This lack of protonation of amino acid residues is suggested by the following experiment. As shown in Figure 8 and previously described in more detail (De Wolf et al., 1985), a strong depression of fluorescence intensity of CT B is observed upon lowering the pH to 4.5. Complex formation of B with GM_1 or oligo–GM_1 (not shown) prior to lowering the pH prevents this quenching. Addition of GM_1 or oligo-GM_1 after lowering the pH however does not reverse this quenching. This indicates that also in a CT B–GM_1 complex protonated amino acid residues are still quenching. At pH 4.5 the binding of GM_1 or oligo–GM_1 to CT B appears

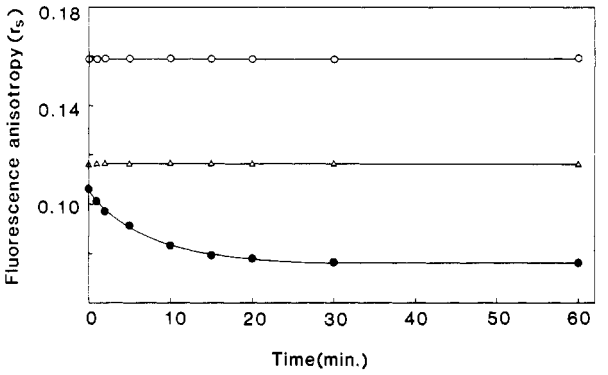

FIGURE 7: Low pH induced changes in steady-state fluorescence anisotropies (r_s) of Dns-CT B in the absence (●) and presence of GM_1 (4 μM) (○) or oligo-GM_1 (4 μM) (△) as a function of time. The Dns-CT B (2.6 dansyl residues/CT B molecule) concentration was 0.2 μM. Measurements were in glycine hydrochloride buffer (0.05 M) (pH 3.2) containing 0.2 M NaCl at 25 °C. The excitation and emission wavelengths were 340 and 525 nm, respectively.

normal as evidenced by the characteristic "blue shift" in the fluorescence emission maximum (Figure 8). However, the quenching persists after reversing the pH of the protonated CT B–GM_1 complex to pH 7.0 or even pH 9.0.

Discussion

Using fluorescence quenching detection of detergent binding, we examined the binding of the mild detergent Bry 96 to CT and its subunits. Our data suggest that CT A displays a somewhat higher affinity for this mild detergent than CT and CT B. Fluorescence quenching of CT A by brominated Bry 96 is probably not the result of a collisional interaction between the detergent and indole groups (the α-polypeptide chain of CT A contains three Trp residues whereas the γ-polypeptide chain is devoid of Trp residues) since the average fluorescence excited-state lifetime of these equally accessible (De Wolf et al., 1981a) indole side chains is not affected.

The partial hydrophobic nature of CT A is also reflected by its ability to interact with phospholipids as evidenced by the salt-enhanced fluorescence quenching and the ability of phospholipids and deoxycholate to modulate the mono-ADP-ribosyltransferase activity of CT A. Since in the latter case the substrates of the enzymatic reaction (NAD^+, L-arginine methyl ester) are water soluble, an interaction of the phospholipids with the enzyme is inferred. Because of its salt resistance, it is unlikely that this interaction is electrostatic in nature. The differential effects of anionic and neutral phospholipids and detergents in the mono-ADP-ribosyltransferase action are probably resulting from the net negative charge of the ADP-ribose donor NAD^+. Previous studies using charge-shift electrophoresis (Ward et al., 1981) were unable to reveal any detergent binding to CT and to its subunits. The lower ionic strength in the electrophoretic separations and the low affinity probably account for this discrepancy. Our data also suggest that in the holotoxin the hydrophobic domain on CT A is shielded from interaction with Br-Bry 96 and that receptor binding does not induce the exposure of this hydrophobic domain on the CT structure. One should, however, also consider the possibility that in the intact toxin detergent binding (as measured by fluorescence quenching) to CT A escapes from detection because of strong internal quenching of the fluorescence of the Trp residues residing in CT A. The occurrence of a strong intrinsic quenching of the fluorescence of Trp residues present in CT A of the holotoxin is suggested by the similar average fluorescence excited-state lifetimes, solute quenching characteristics of CT and CT B, and the low

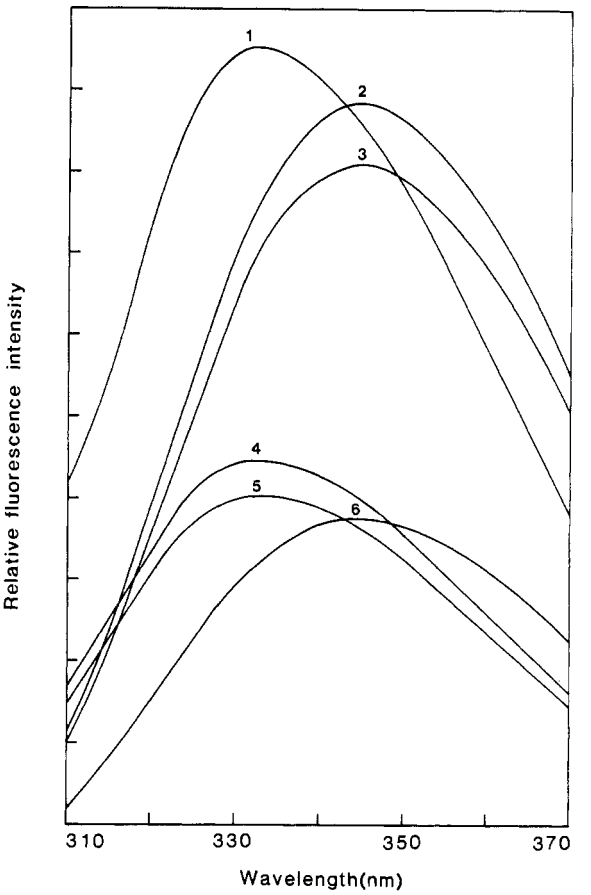

FIGURE 8: Fluorescence emission spectra of CT B under different experimental conditions. CT B solutions were of constant ionic strength (0.2 M NaCl), and pH adjustments were performed by adding small aliquots of 0.05 M HCl or 0.075 M NaOH. The excitation wavelength was 282 nm, and spectra were recorded 30 min after addition of ligand or adjustment of pH. CT B and GM_1 concentrations were 0.5 and 10 μM, respectively. (1) CT B complexed with GM_1 at pH 7.0 and subsequently acidified to pH 4.5; (2) CT B at pH 7.0; (3) CT B initially acidified to pH 4.5 and subsequently readjusted to pH 7.0; (4) CT B acidified to pH 4.5 and after complexation with GM_1 readjusted to pH 7.0; (5) CT B acidified to pH 4.5 and subsequently complexed with GM_1; (6) CT B acidified to pH 4.5.

quantum yield of CT as compared to its constituent subunits (De Wolf et al., 1981b, 1985). However, using Triton X-100 micelles containing a photoreactive azidoglycolipid, Tomasi et al. (1984) also found a negligible interaction between CT and this neutral detergent, supporting the view of a lack of binding of detergent to CT A in intact CT. Reduction of the disulfide bridge, which leads to a slow dissociation of the α-chain from $\gamma\beta_5$, resulted in a significant photolabeling of the α-polypeptide chain. This would suggest that the unmasked hydrophobic region is located at the intersubunit interface of the α- and γ-polypeptide chains. Since activation of CT A by treatment with dithiothreitol does not affect detergent binding, our data suggest that the hydrophobic domain on CT A is rather located at the intersubunit interface between the α- and β-polypeptide chains whereas dissociation of α from γ does not contribute to the formation of hydrophobic regions but may be important for the exposure of the catalytic domain.

The increased quenching of CT with Br-Bry 96 upon lowering the pH suggests that there is a pH-induced conformational change unmasking or forming hydrophobic domains on the CT surface. These pH-induced hydrophobic protein regions are formed on CT B because of the similar results obtained for both CT and CT B. The increased quenching with Br-Bry 96 might be the result of the enhanced accessibility

(exposure) of Trp-88 of each β-polypeptide chain and/or enhanced binding of detergent. Because of the significant enhancement in acrylamide quenching of CT and CT B upon lowering the pH to 5.0 and taking into consideration that the fluorescence of CT B is related to the lone Trp-88 residue of each β-polypeptide chain, it can be assumed that the hydrophobic indole side chain of Trp-88 indeed becomes more exposed. Furthermore, both the pH-induced exposure of Trp-88 and increased detergent binding are reversible and display similar kinetics, further pointing to a causal relationship. The Br-Bry 96 quenching as a function of pH, however, displays a different profile. Maximal quenching is observed below pH 5.0, indicating that the hydrophobicity is increased independently of further exposure of Trp-88. The quenching in the pH range 7–4.0 is not the result of dissociation of CT B into its constituent monomers since (i) the quenching is reversible, (ii) binding to GM_1 or oligo-GM_1, which is dependent on the multimeric nature of CT B, is not affected (similar "blue shift" in fluorescence emission spectra and shielding of the fluorescence of CT B-GM_1 from quenching with acrylamide), and (iii) the steady-state anisotropy of Dns-CT B in the pH range 7–4 is not significantly altered within 1 h. The sharp increase in Br-Bry 96 quenching at pH 4.1 suggests that the conformational change exposing hydrophobic domains on CT B is triggered by a cooperative process involving the breaking of salt bridges, creation of charge repulsions, and changes in hydrogen bonding. Since the effect is time dependent, it is not merely the result of a deionization of carboxyl groups in CT B.

Upon decreasing the pH below 4, dissociation of CT B is observed, which seems not to be associated with further formation or exposure of hydrophobic domains. Complex formation of CT B with GM_1 or oligo-GM_1 however prevents the dissociation of CT B into its constituent monomers. This could be the result of shielding from protonation of amino acid residues committed to subunit association and/or cross-linking of the β-polypeptide chains by oligo-GM_1.

A lack of protonation of certain amino acid side chains in the CT B–GM_1 complex, at low pH, was also suggested by the previous observation that complex formation of CT B with GM_1 or oligo-GM_1 prevents the pH-dependent intrinsic quenching of the Trp-88 fluorescence (De Wolf et al., 1985). In the same report it was also proposed that the amino acid side chains involved in the pH-induced intrinsic fluorescence quenching of Trp-88 are respectively Glu-11 and His-13 in the acidic pH range and Tyr-12 in the alkaline pH range (De Wolf et al., 1985). These amino acid side chains are believed to be in close proximity of Trp-88 because of the single intrachain disulfide bridge between positions 9 and 86 joining the C- and N-terminal regions of the β-polypeptide chain of CT B.

In this study we further show that, after protonation of these amino acid side chains, addition of GM_1 or oligo-GM_1 is not able to reverse the fluorescence quenching. Under these conditions binding appears to be normal as evidenced by (i) the characteristic blue shift in the fluorescence emission spectrum and (ii) the strong inhibition of acrylamide quenching. This demonstrates that in the CT B–GM_1 complex protonated amino acid side chains are still able to quench Trp-88 fluorescence. Moreover, neutralization or even alkalinization of the protonated CT B–GM_1 complex does not reduce the quenching whereas reversing the pH of acidified CT B to neutral pH almost completely restores the fluorescence intensity. These experiments suggest that some protons are trapped in the CT B–GM_1 (CT B–oligo-GM_1) complex and further illustrate the lack of exchange of these protons with the medium.

The foregoing also supports our previous contention that the binding sites of CT are located in clefts formed by adjacent β-polypeptide chains of CT B (De Wolf et al., 1981b). Since oligo-GM_1 exerts a similar stabilizing effect upon the pentameric structure of CT as GM_1, one could imagine that the carbohydrate moiety of GM_1 actually causes cross-linking of the β-polypeptide chains of CT B. The terminal galactose residue could for instance interact with Trp-88 (via hydrogen bonding) on one β-polypeptide chain and the sialic acid residue interacting with a positively charged amino acid residue (Arg-35 or Lys-?) on the adjacent β-polypeptide chain.

Our data also imply that Glu-11, Tyr-12, and His-13 located in or near this cleft forming the binding site for GM_1. In this respect it is interesting to note that this particular sequence (-Cys-Ala-Glu^{11}-Tyr^{12}-His^{13}-) comprises the region (-Cys-Ala-Glu-Tyr-), which has been shown (Ledley et al., 1976; Kurosky et al., 1977) to be significantly homologous to the highly preserved region (-Cys-Ala-Glu-Tyr-Cys-) of the β-subunit of the glycoprotein hormones (TSH, LH, hCG, and FSH) and serine proteases.

In conclusion, our experiments show that, in contrast to CT and CT B, CT A is slightly hydrophobic and that there are pH-induced (reversible) conformational changes in the CT B of the holotoxin that appear to form or unmask hydrophobic domains. The results are also in agreement with previous studies (De Wolf et al., 1981b; Tomasi et al., 1984) showing that the mere binding of CT to its receptor is not sufficient to induce a conformational rearrangement in the toxin structure allowing the α-subunit to interact with the lipid bilayer. Presumably some processing of the toxin is a prerequisite for penetration of the active part into or through the lipid bilayer of the cell membrane. Cleavage of the interchain disulfide bridge joining the α- and γ-subunits by a thiol:protein disulfide oxidoreductase might provide such a function (Moss et al., 1980). Binding of CT B to GM_1 would serve to bring the α-subunit in close proximity of the lipid bilayer. Following dissociation of the α-subunit from the stable $\beta_5\gamma$ $(GM_1)_5$ complex, its weak hydrophobic nature might be sufficient to allow initial interaction with the lipid bilayer.

The role of the acid-induced exposure of hydrophobic domains of CT B in the penetration process is still obscure. In contrast with diphtheria toxin (Olsnes & Sandvig, 1983; Blewitt et al., 1985), the importance of low pH in membrane penetration of CT is not evident. Although ultrastructural studies (Joseph et al., 1978) show internalization of CT, biochemical studies using lysosomotropic amines provide no conclusive evidence that receptor-mediated endocytosis and exposure to acidic pH is an essential step in CT action (Houslay & Elliott, 1981; Hagmann & Fishman, 1981; Gill et al., 1981). Furthermore, studies using photoreactive glycolipid and phospholipid compounds incorporated into model membranes demonstrated that only CT A was labeled when the photoactivated label was buried in the phospholipid bilayer (Tomasi & Montecucco, 1981; Wisnieski & Bramhall, 1981; Tomasi et al., 1982). These data were therefore not compatible with the formation of a hydrophilic channel by the β-polypeptide chains of CT B. However, these experiments do not unequivocally show that CT A is actually penetrating the lipid bilayer to which the CT B is bound. In the latter case, the photoaffinity labeling of CT A should show saturation kinetics (optimal labeling when all the toxin is bound), which is not evident from their data (Tomasi & Montecucco, 1981; Wisnieski & Bramhall, 1981; Tomasi et al., 1982). Therefore,

these experiments do not exclude the possibility that CT A of the holotoxin is interacting directly in a nonspecific manner with the different model membranes.

Finally, binding of the CT B to GM_1 stabilizes its quaternary structure. This might offer an explanation for the observation that although a rapid internalization of CT occurs (Joseph et al., 1978), ^{125}I-CT remains persistently (several days) bound to human fibroblasts and is degraded slowly (Chang et al., 1983). Since during this time the toxin is probably recycled back to the cell surface, the stabilizing effect of GM_1 might protect against dissociation and loss of binding at the acidic pH of intracellular compartments such as endosomes and lysosomes. It is therefore not excluded that this persistent binding of CT enables the CT A to penetrate into the cell at both the level of the cell surface and the level of the membranes of the vacuolar apparatus, which is in accordance with the partial protective effects of lysosomotropic amines (Houslay & Elliott, 1981; Hagmann & Fishman, 1981; Gill et al., 1981).

ACKNOWLEDGMENTS

The skilled technical assistance of R. Goossens is gratefully acknowledged.

Registry No. Mono-ADP-ribosyltransferase, 58319-92-9; sodium deoxycholate, 302-95-4; Triton X-100, 9002-93-1.

REFERENCES

Bennett, V., & Cuatrecasas, P. (1977) in *The Specificity and Action of Animal, Bacterial and Plant Toxins* (Cuatrecasas, P., & Greaves, N. F., Eds.) pp 3–66, Chapman & Hall, London.

Blewitt, M. G., Zhao, J.-M., McKeever, B., Sarma, R., & London, E. (1984) *Biochem. Biophys. Res. Commun. 120*, 286–290.

Blewitt, M. G., Chung, L. A., & London, E. (1985) *Biochemistry 24*, 5458–5464.

Chang, P. P., Fishman, P. H., Ohtomo, N., & Moss, J. (1983) *J. Biol. Chem. 258*, 426–430.

Craig, J. P. (1971) in *Microbial Toxins* (Montie, T. C., & Ajl, S. J., Eds.) Vol. 2A, pp 189–254, Academic, New York.

De Wolf, M. J. S., Fridkin, M., Epstein, M., & Kohn, L. D. (1981a) *J. Biol. Chem. 256*, 5481–5488.

De Wolf, M. J. S., Fridkin, M., & Kohn, L. D. (1981b) *J. Biol. Chem. 256*, 5489–5496.

De Wolf, M. J. S., Vitti, P., Ambesi-Impiombato, F. S., & Kohn, L. D. (1981c) *J. Biol. Chem. 256*, 12287–12296.

De Wolf, M., Van Dessel, G., Hilderson, H. J., Lagrou, A., & Dierick, W. (1983) *Arch. Int. Physiol. Biochim. 91*, B11–B13.

De Wolf, M., Van Dessel, G., Lagrou, A., Hilderson, H. J., & Dierick, W. (1985) *Biochim. Biophys. Acta 832*, 165–174.

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

Eftink, M. R., & Ghiron, C. A. (1977) *Biochemistry 16*, 5546–5551.

Finkelstein, R. A. (1973) *CRC Crit. Rev. Microbiol. 2*, 553–623.

Finkelstein, R. A. (1975) in *Microbiology* (Schessinger, D., Ed.) pp 236–241, American Society of Microbiology, Washington, DC.

Finkelstein, R. A., Boesman, M., Neoh, S. H., LaRue, M. K., & Delaney, R. (1974) *J. Immunol. 113*, 145–150.

Fishman, P. H. (1982) *J. Membr. Biol. 69*, 85–97.

Fishman, P. H., Moss, J., & Osborne, J. C., Jr. (1978) *Biochemistry 17*, 711–716.

Gill, D. M. (1976) *Biochemistry 15*, 1242–1248.

Gill, D. M. (1977) *Adv. Cyclic Nucleotide Res. 8*, 85–118.

Gill, D. M. (1978) in *Bacterial Toxins and Cell Membranes* (Jeljaszewicz, J., & Wadström, T., Eds.) pp 291–332, Academic, London.

Gill, D. M., Hope, J. A., Meren, R., & Jacobs, D. S. (1981) in *Receptor-Mediated Binding and Internalization of Toxins and Hormones* (Middlebrook, J. L., & Kohn, L. D., Eds.) pp 113–121, Academic, New York.

Gilman, A. G. (1984) *J. Cell Biol 36*, 577–579.

Hagman, J., & Fishman, P. H. (1981) *Biochem. Biophys. Res. Commun. 98*, 677–684.

Holmgren, J. (1978) in *Bacterial Toxins and Cell Membranes* (Jeljaszewicz, J., & Wadström, T., Eds.) pp 333–366, Academic, New York.

Holmgren, J., Lönnroth, I., & Svennerholm, L. (1973) *Infect. Immunol. 8*, 208–214.

Horton, H. R., & Koshland, D. E., Jr. (1967) *Methods Enzymol. 11*, 556–565.

Houslay, M. D., & Elliott, K. R. F. (1981) *FEBS Lett. 128*, 289–292.

Johnson, G. L. (1982) in *Molecular Actions of Toxins and Viruses* (Cohen, P., & Van Heyningen, S., Eds.) pp 33–49, Elsevier, Amsterdam.

Joseph, K. C., Kim, S. U., Stieber, A., & Gonatas, N. K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2815–2819.

Kohn, L. D., Consiglio, E., De Wolf, M. J. S., Grollman, E. G., Ledley, F. D., Lee, G., & Morris, N. P. (1980) *Adv. Exp. Med. Biol. 125*, 487–503.

Kurosky, A., Markel, D. E., Peterson, J. W., & Fitch, W. M. (1977) *Science (Washington, D.C.) 195*, 299–301.

Lai, C.-Y. (1980) *CRC Crit. Rev. Biochem. 9*, 171–206

Ledley, F. D., Mullin, B. R., Lee, G., Aloj, S. M., Fishman, P. H., Hunt, L. T., Dayhoff, M. O., & Kohn, L. D. (1976) *Biochem. Biophys. Res. Commun. 69*, 852–859.

Ludwig, D. S., Holmes, R. K., & Schoolnik, G. K. (1985) *J. Biol. Chem. 260*, 12528–12534.

Moss, J., & Vaughan, M. (1979) *Annu. Rev. Biochem. 48*, 581–600.

Moss, J., Stanley, S. J., Morin, J. E., & Dixon, J. E. (1980) *J. Biol. Chem. 255*, 11085–11087.

Mullin, B. R., Fishman, P. H., Lee, G., Aloj, S. M., Ledley, F. D., Winand, R. J., Kohn, L. D., & Brady, R. O. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 842–846.

Olsnes, S., & Sandvig, K. (1983) in *Receptor-Mediated Endocytosis* (Cuatrecasas, P., & Roth, T., Eds.) Series B, Vol. 15, pp 187–236, Chapman & Hall, London.

Parker, C. A. (1968) in *Photoluminescence of Solutions*, pp 220–222, Elsevier, New York.

Sillerud, L. O., Prestegard, J. H., Yu, R. K., Konigsberg, W. H., & Schafer, D. E. (1981) *J. Biol. Chem. 256*, 1094–1097.

Svennerholm, L. (1957) *Biochim. Biophys. Acta 24*, 604–611.

Tomasi, M., & Montecucco, C. (1981) *J. Biol. Chem. 256*, 11177–11181.

Tomasi, M., D'Agnolo, G., & Montecucco, C. (1982) *Biochim. Biophys. Acta 692*, 339–334.

Tomasi, M., Battistini, A., Cardelli, M., Sonnino, S., & D'-Agnolo, G. (1984) *Biochemistry 23*, 2520–2526.

Ward, W. H. J., Britton, P., & Van Heyningen, S. (1981) *Biochem. J. 199*, 457–460.

Wiegandt, H., & Bücking, W. (1970) *Eur. J. Biochem. 15*, 287–292.

Wisnieski, B. J., & Bramhall, J. S. (1981) *Nature (London) 289*, 319–321.