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Thermal Stability and Intersubunit Interactions of Cholera Toxin in Solution and in Association with Its Cell-Surface Receptor Ganglioside G_{M_1}†

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ABSTRACT: The thermal stability of cholera toxin free in solution and in association with its cell-surface receptor ganglioside G_{M_1} has been studied by using high-sensitivity differential scanning calorimetry and differential solubility thermal gel analysis. In the absence of ganglioside G_{M_1}, cholera toxin undergoes two distinct thermally induced transitions centered at 51 and 74 °C, respectively. The low-temperature transition has been assigned to the irreversible thermal denaturation of the active A subunit. The second transition has been assigned to the reversible unfolding of the B subunit pentamer. The isolated B subunit pentamer exhibits a single transition also centered at 74 °C, suggesting that the attachment of the A subunit does not contribute to the stability of the pentamer. In the intact toxin, the A subunit dissociates from the B subunit pentamer at a temperature that coincides with the onset of the B subunit thermal unfolding. In aqueous solution, the denatured A subunit precipitates after dissociation from the B subunit pentamer. This phenomenon can be detected calorimetrically by the appearance of an exothermic heat effect. In the presence of ganglioside G_{M_1}, the B subunit is greatly stabilized as indicated by an increase of 20 °C in the transition temperature. In addition, ganglioside G_{M_1} greatly enhances the cooperative interactions between B subunits. In the absence of ganglioside, each monomer within the B pentamer unfolds in an independent fashion whereas the fully ganglioside-bound pentamer behaves as a single cooperative unit. On the contrary, the thermotropic behavior of the A subunit is only slightly affected by the presence of increasing concentrations of ganglioside G_{M_1}. The exothermic process at the onset of the B subunit pentamer unfolding is not present when the toxin molecule is bound to ganglioside G_{M_1}. This effect is observed with both micellar and membrane-bound gangliosides but not with pure phospholipid vesicles. Differential solubility thermal gel analysis indicates that under these conditions the A subunit remains associated with the micellar ganglioside G_{M_1} or ganglioside G_{M_1}-containing membranes.

Cholera toxin is a multisubunit protein consisting of five identical binding (B) subunits (M_r 11 500) forming a pentameric ring which supports the A subunit (Gill, 1976). Treatment of the A subunit with reducing reagents produces two subunits: an ADP-ribosylating protein termed A_1 (M_r 21 000) which activates adenylate cyclase and a small protein, A_2 (M_r 6000), which plays a structural role in holding the A_1 and B subunits together.

The interaction of cholera toxin with the cell surface occurs when the B subunit recognizes and binds, with high affinity and specificity, to ganglioside G_{M_1}. This association is believed to cause a conformational change in the protein that facilitates the exposure and subsequent penetration of the A subunit into the membrane. Evidence for this conformational change has been derived from fluorescence studies using cholera toxin incubated either with ganglioside G_{M_1} (Mullin et al., 1976) or with the oligosaccharide moiety of G_{M_1} (Fishman et al., 1978) and NMR studies (Sillerud et al., 1981). Various techniques including photoaffinity labeling (Wisnieski & Bramhall, 1981; Tomasi & Montecucco, 1981; Tomasi et al., 1982), hydrodynamic studies (Dwyer & Bloomfield, 1982), and differential scanning calorimetry (Goins & Freire, 1985) have provided indirect evidence that upon association of cholera

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toxin with lipid vesicles containing ganglioside G_{M_1}, the A subunit penetrates into the hydrophobic core of the lipid bilayer. More recently, three-dimensional structural studies at 15-Å resolution (Ludwig et al., 1986; Ribi & Kornberg, 1987) have revealed that the A subunit initially does insert into the lipid milieu and that upon reduction of the A subunit there is a further penetration of the enzymatically active A_1 subunit into the membrane. The A_1 subunit is very hydrophobic in nature and can be reconstituted into lipid vesicles even in the absence of ganglioside (Goins & Freire, 1985).

In this paper, the thermal stability of the intact cholera toxin and cholera toxin subunits has been determined by using high-sensitivity differential scanning calorimetry (DSC) and differential solubility thermal gel analysis (Rigell & Freire, 1987). These studies have permitted us to characterize the magnitude of the thermodynamic parameters that determine the stability of this multisubunit protein, to evaluate the cooperative interactions between subunits, and to assess how these parameters depend on the association of the toxin molecule with its cell-surface receptor ganglioside G_{M_1}.

Materials and Methods

Protein. Intact cholera toxin and cholera toxin B subunit were purchased from Sigma (St. Louis, MO). The proteins were supplied as lyophilized powders and were reconstituted with distilled water to give the appropriate concentration of protein in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 0.2 M NaCl, 3 mM NaN_3, and 1 mM disodium ethylenediaminetetraacetate (Na_2EDTA), pH 7.5. The purity of the protein samples was checked by sodium dodecyl sulfate (SDS) gel electrophoresis using the procedure described by Laemmli (1970). EDTA-free protein samples for calorimetric and differential solubility gel analysis experiments were prepared by dialyzing the toxin stock solution against 4000 volumes of buffer containing no EDTA overnight at 4 °C. All protein stocks and experimental protein samples were assayed for protein concentration by the method of Lowry (1951).

Liposome Preparation. Small unilamellar vesicles were prepared according to Schullery et al. (1980). Egg phosphatidylcholine (egg PC), purchased from Avanti Polar Lipids (Birmingham, AL), was N_2 dried from a chloroform solution and lyophilized overnight. The dried lipid was resuspended in 50 mM Tris-HCl, 0.2 M NaCl, and 3 mM NaN_3, pH 7.5, to give a lipid concentration of 9 mM. The lipid suspension was then sonicated until clear using a bath sonicator (Model G112 SP1G, Laboratory Supplies, Hicksville, NY) and centrifuged at 15000*g* for 1 h to remove residual multilamellar vesicles. The upper two-thirds of the supernatant following centrifugation was used in the differential solubility thermal gel analysis experiments. To avoid fusion of the vesicles prior to use, they were kept in a thermostated block set for 30 °C. Vesicles were prepared fresh for each experiment.

Ganglioside G_{M_1}. The ganglioside G_{M_1} used in these experiments was prepared by enzymatic cleavage of sialic acid residues from di- and trisialogangliosides using a procedure similar to the method of Thompson et al. (1985). The di- and trisialogangliosides used for the isolation procedure were obtained from the Folch extract of bovine brain (Avanti Biochemicals, Birmingham, AL) using the procedure described by Myers et al. (1984). Briefly, 0.5 g of mixed gangliosides and 1 g of egg phosphatidylcholine were dissolved in $CHCl_3/CH_3OH$ (1:2), N_2 dried, and reconstituted with 80 mL of 20 mM sodium acetate buffer, pH 5.6, containing 0.02% NaN_3. A total of 10 units of neuraminidase (*Clostridium perfringens* type VI; Sigma, St Louis, MO) in two aliquots was added to this ganglioside/lipid mixture and then incubated at 37 °C for 24 h. Following enzymatic hydrolysis, the sample was lyophilized, redissolved in a small volume of water, dialyzed extensively to remove all salts, and again lyophilized. The sample was dissolved in $CHCl_3/CH_3OH$ (1:1) and adsorbed onto DEAE-Sephadex (acetate form). The DEAE-ganglioside slurry was packed in a 0.9 × 24 cm column, washed with 3 column volumes of $CHCl_3/CH_3OH/H_2O$ (30:60:8) followed by 1 column volume of methanol, and finally eluted with 75 mM ammonium acetate in methanol. Fractions were evaporated, redissolved in water, and dialyzed extensively against distilled water. An aliquot of these fractions was spotted on an E. Merck silica gel 60 plate (Alltech Associates, Deerfield, IL). The plate was developed in $CHCl_3/CH_3OH/0.02\%$ $CaCl_2$ (55:45:10) (Kundu, 1981). Gangliosides were detected with resorcinol and compared to ganglioside standards obtained from Suppelco (Bellefonte, PA).

All monosialoganglioside-containing fractions were dissolved in $CHCl_3/CH_3OH$ (1:1) and run on an Anasil S silica gel column (2.5 × 40 cm). The gangliosides were eluted from the column with the following series of $CHCl_3/CH_3OH/H_2O$: 65:25:4, 60:30:5, 60:35:8, and 60:40:10, and finally with pure methanol. Aliquots of fractions were rechecked for purity by thin-layer chromatography as described above. Fractions containing ganglioside G_{M_1} were dried, redissolved in water, pooled, and dialyzed extensively against water at 4 °C. Following dialysis, the sample was lyophilized and stored in powder form at −20 °C. The yield from 500 mg of crude gangliosides was between 100 and 150 mg of pure G_{M_1}.

Stock ganglioside solutions were checked by the total sialic acid determination using the resorcinol method described by Spiro (1966). Incorporation of ganglioside into phospholipid vesicles was performed by adding a given amount of G_{M_1} stock solution in Tris buffer and incubating the mixture above the phospholipid phase transition temperature for 90 min. Following incubation, the vesicles were used immediately for differential solubility thermal gel analysis experiments. This allows for an asymmetric distribution of ganglioside in which ganglioside is found only on the outer leaflet of the vesicles (Felgner et al., 1981). Ganglioside concentrations were 10 mol % of the total lipid concentration for the differential solubility gel analysis experiments.

Differential Scanning Calorimetry. All calorimetric scans were performed with a Microcal MC-2 differential scanning calorimeter. The calorimetric unit was interfaced to an IBM PC microcomputer using an A/D converter board (Data Translation DT-2801) for automatic data collection and analysis. The protein concentration of each sample used for the calorimetric experiments was ~2 mg/mL; 1.3 mL of sample was injected into the sample cell, and the same amount of buffer was used in the reference cell. Samples were heated at a rate of 60 °C/h. Cholera toxin samples were degassed 15 min at room temperature prior to being scanned. To prepare samples containing cholera toxin and ganglioside G_{M_1}, stock solutions were degassed separately for 15 min after which time aliquots of each were added to the desired volume of buffer and incubated under vacuum an additional 5 min at room temperature prior to being scanned.

Differential Solubility Thermal Gel Analysis. The thermotropic behavior of cholera toxin alone and in the presence of ganglioside G_{M_1} was also examined by using a variation of the technique of differential solubility thermal gel analysis (Rigell & Freire, 1987). This technique allows one to investigate the thermal denaturation of complex multisubunit

proteins by examining solubility changes in individual subunits induced during the heating of the protein. Briefly, a separate aliquot of cholera toxin (100 μL of a 1 mg/mL stock solution dissolved in cholera toxin buffer) was prepared for each temperature point of the thermal denaturation profile. In addition, each sample contained either 0.09 μmol of micellar ganglioside G_{M_1}, 0.9 μmol of egg PC vesicles, 0.9 μmol of egg PC vesicles containing 10 mol % ganglioside G_{M_1}, or buffer. A constant volume of 200 μL was used in all experiments. When present, the concentration of ganglioside G_{M_1} in the samples corresponded to a 76-fold excess in order to ensure complete saturation of the toxin.

For these experiments, a programmable scanning water bath (Neslab RTE-8 and temperature programmer ETP3) was used. The temperature was continuously monitored by a thermistor connected to a Hewlett-Packard logging multimeter. At each designated temperature, the appropriate samples were placed for 1 min in the bath, removed, and placed on ice for 10 min. Immediately after, each sample was centrifuged at 1500 rpm for 90 s and then transferred to a microfuge tube for centrifugation at 15000*g* for 15 min. Iodine and resorcinol detection of thin-layer chromatograms (Kates, 1972) developed in $CHCl_3/CH_3OH/H_2O$ (65:25:4) were utilized to monitor the lipid and ganglioside content, respectively, of the supernatant and pellet fractions. Under these conditions, both the lipid and ganglioside remained in the supernatant fraction of samples heated up to 100 °C, and only free A subunit (see Results) was pelleted, thus allowing one to quantitate the amount of A subunit uptake by ganglioside micelles or phospholipid vesicles.

Since ganglioside G_{M_1} and lipid both interfere with SDS gel electrophoresis (Gill, 1977), they were removed from the supernatant fractions of each sample prior to electrophoresis by incubating with 0.7 mL of cold acetone on ice for 15 min. The samples were centrifuged at 15000*g* for 15 min, and the acetone-containing supernatant was discarded. To remove any residual lipid, the pellet was washed with 0.7 mL of cold chloroform/methanol (2:1). Following a 15-min incubation, the chloroform/methanol mixture was discarded and the pellet dried under vacuum for 30 min. The pellet was then reconstituted with 200 μL of cholera toxin buffer and processed for electrophoresis.

Usually the sample was incubated overnight with 40 μL of a 5× concentrated electrophoresis sample buffer containing 15% 0.4 M Tris-HCl, pH 7.4, 50% sucrose, 4% SDS, and bromophenol blue, as the tracking dye. A 30-μL aliquot of each sample (~12.5 μg of protein) was run on a 15% polyacrylamide gel overlaid with a 5% polyacrylamide stacking gel following the procedure of Laemmli (1970). Electrophoresis was run for 2 h at 60 V (constant) and then for 4 h at 150 V (constant). Gels were stained in methanol/H_2O/acetic acid (5:4:1) containing 0.2% Coomassie brillant blue and subsequently destained in water/methanol/acetic acid (8:1:1).

Densitometry of the destained gels was performed using a LKB laser densitometer (2202 Ultrascan) interfaced to an IBM PC microcomputer using a Data Translation (DT-2805) A/D converter board. The gels were scanned from bottom (low molecular weight species) to top (high molecular weight species). Data collection and subsequent data manipulation to obtain normalized peak intensities and areas were performed with software developed in this laboratory.

Results

Thermal Stability of Cholera Toxin. The excess heat capacity versus temperature for intact cholera toxin (Figure 1A–C) and for isolated pentameric B subunits (Figure 1D) is shown in Figure 1. The calorimetric scan of intact cholera toxin (Figure 1A) is characterized first by the appearance of a peak centered at 51 °C with an enthalpy change of 90 kcal/mol. This peak is followed by an exothermic process at ~70 °C associated with the appearance of a white precipitate. If intact cholera toxin is scanned only up to the temperature at which the precipitate begins to appear (Figure 1B) and then cooled and rescanned, the calorimetric profile shown in Figure 1C is observed. This scan is characterized by a single peak centered at 74 °C and an enthalpy change of 520 kcal/mol. This calorimetric scan is similar to the one obtained with isolated B subunit pentamers (Figure 1D) and indicates that the peak at 74 °C corresponds to the denaturation of the binding subunits in the intact toxin. The magnitude of the enthalpy changes for these transitions is comparable to those obtained for the denaturation of other proteins [see Privalov (1982) for a review].

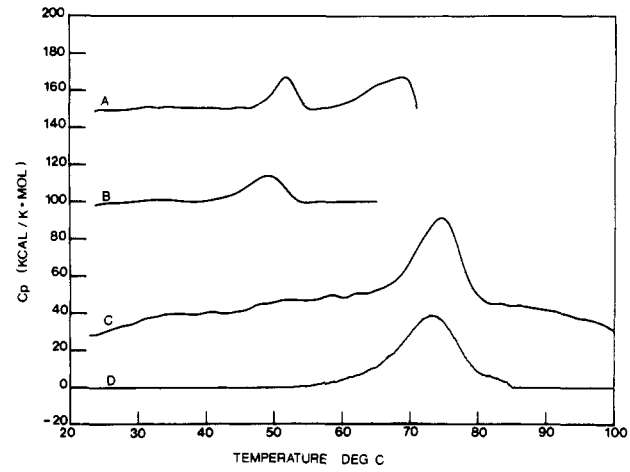

FIGURE 1: Excess heat capacity function versus temperature for cholera toxin free in aqueous solution (curve A) showing the initial portion of the exothermic effect at 70 °C (see text for details). Curve B depicts a scan of a different sample of toxin stopped immediately prior to the exotherm. Curve C shows the rescan of the sample in curve B. Curve D shows the calorimetric heat capacity profile for the isolated B subunit pentamer. All the calorimetric scans in this paper were performed with a toxin concentration of 2 mg/mL in a total volume of 1.3 mL.

Gel electrophoresis analysis of the precipitate and the supernatant obtained after centrifugation of a sample scanned up to 70 °C revealed that the A subunit was present exclusively in the precipitate and that the supernatant contained only the B subunit. These experiments indicate that the A subunit denatures at 51 °C but it is only released from the toxin molecule at the onset of the B subunit pentamer unfolding.

Thermotropic Behavior of Cholera Toxin in the Presence of Ganglioside G_{M_1}. As shown in Figure 2, the unfolding transition of the B subunit pentamer of intact cholera toxin centered at 74 °C (trace A) is shifted to higher temperatures and broadened after the addition of ganglioside G_{M_1} micelles at low toxin:ganglioside molar ratios (see also Figure 4). It should be noted that the concentration of ganglioside present in all calorimetric samples was above the critical micellar concentration of 1×10^{-6} M (Masserini & Freire, 1987). The broad multiple peaks evident at low protein:ganglioside mole ratios give rise to a single and progressively sharper peak centered at 95 °C at increasing ganglioside concentrations. The upward shift of 20 °C in the transition temperature denotes a stabilization of the B subunit native conformation in the presence of ganglioside G_{M_1}.

At saturating concentrations of ganglioside, the heat capacity profile is noticeably sharper than in the absence of

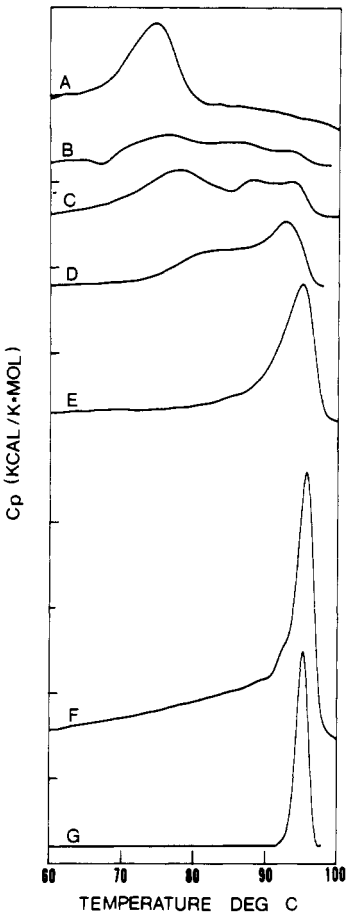

FIGURE 2: Enlargement of the B subunit transition region from heat capacity profiles of intact cholera toxin incubated with increasing amounts of ganglioside G_{M_1}. The mole ratios of ganglioside G_{M_1} to intact toxin are 0 (A), 0.46:1 (B), 1.5:1 (C), 3.7:1 (D), 7:1 (E), 23:1 (F), and 77:1 (G). Scans are offset for presentation purposes. Each interval on the y axis represents 50 kcal/(K·mol).

ganglioside, indicating an increase in the cooperative interactions between individual monomers within the B subunit pentamer. This is reflected in the ratio of the van't Hoff enthalpy to calorimetric enthalpy ($\Delta H_{vH}/\Delta H_{cal}$). When the calorimetric enthalpies are normalized to the concentration of intact toxin, a value of 0.169 is calculated for the experiments in the absence of ganglioside G_{M_1}, whereas at saturation (trace G), this value becomes 0.932. These results indicate that in the absence of ganglioside the individual monomers of the B subunit pentamer unfold independently of one another but that at saturation the B subunit pentamer unfolds as a single cooperative unit. Similar thermotropic behavior is exhibited by the B subunit pentamer alone when scanned in the presence of ganglioside G_{M_1} (unpublished observation; Dalziel et al., 1984).

Also, it should be noted that the exothermic effect, observed in the absence of ganglioside, is absent in the calorimetric scans obtained in the presence of ganglioside. These results together with gel electrophoresis analysis (see below) indicate that the A subunit does not precipitate if the toxin is bound to ganglioside G_{M_1}, in agreement with previous observations that the A subunit or at least part of the A subunit partitions into the hydrophobic interior of the ganglioside micelle.

Figure 3 shows the excess heat capacity function associated with the thermal denaturation of the A subunit. These curves were obtained from calorimetric scans of intact cholera toxin in the presence of increasing concentrations of ganglioside G_{M_1}. As shown in the figure, saturating concentrations of ganglioside

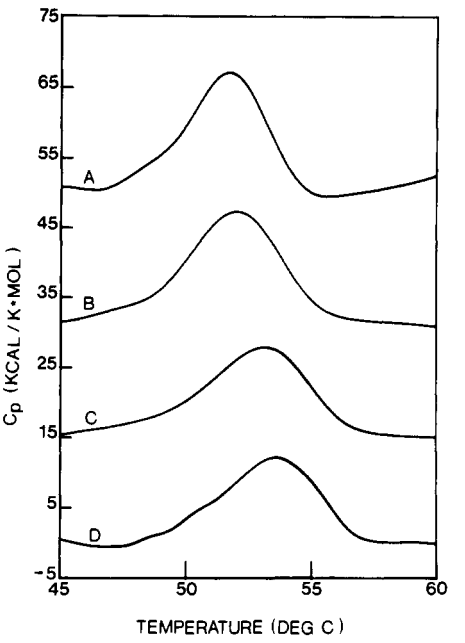

FIGURE 3: Enlargement of the A subunit transition region from calorimetric scans of intact cholera toxin scanned with increasing concentrations of ganglioside G_{M_1}. The mole ratios of ganglioside G_{M_1} to intact toxin are 0 (A), 0.46:1 (B), 9:1 (C), and 23:1 (D). Scans are offset for presentation purposes.

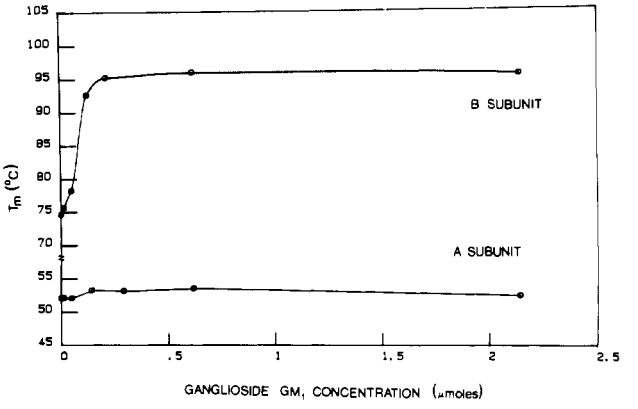

FIGURE 4: Dependence of the transition temperature on the concentration of ganglioside G_{M_1} for the A and B subunit transitions of cholera toxin.

G_{M_1} induce a shift of only 1 °C on the A subunit transition even though the transition of the B subunit has been shifted 20 °C to higher temperatures. These results indicate that the interactions between the A and B subunits are probably not affected by the association of ganglioside G_{M_1} with the B subunit, suggesting that these interactions are only of a very local nature. This conclusion is also consistent with the observation that the unfolding profile of the B subunit is not affected by the presence or absence of the A subunit in the toxin molecule. This should be contrasted with the dramatic enhancement of the inter-B-subunit cooperative interactions induced by ganglioside G_{M_1}.

The transition temperatures calculated from the maximum in the heat capacity function for the thermal transition of the A and B components of cholera toxin upon addition of ganglioside G_{M_1} are shown in Figure 4. As discussed before, the association of ganglioside G_{M_1} with cholera toxin has a profound effect on the B subunit transition temperature but only a slight effect on the A subunit transition temperature. The transition temperature of the B subunit increases monotonically upon the addition of ganglioside G_{M_1} up to the

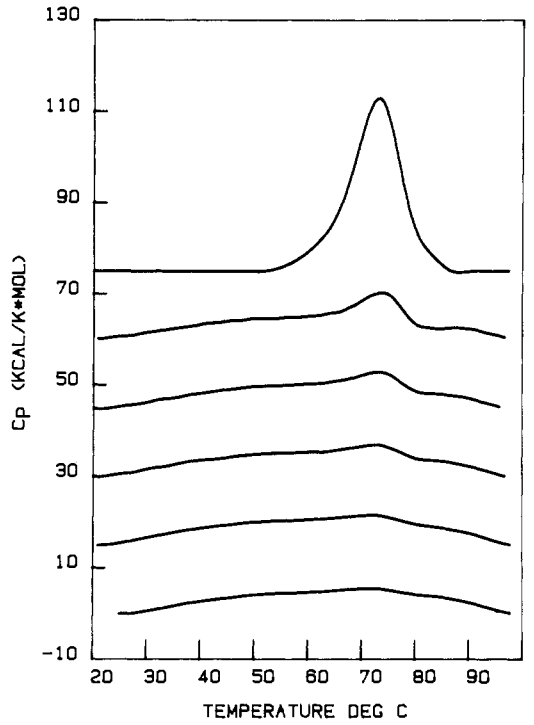

FIGURE 5: Series of consecutive DSC scans of isolated B subunit pentamer. Each successive scan was performed after immediately cooling the sample in the calorimeter and then rescanning under the same conditions. Scans are offset in the *y* axis for presentation purposes.

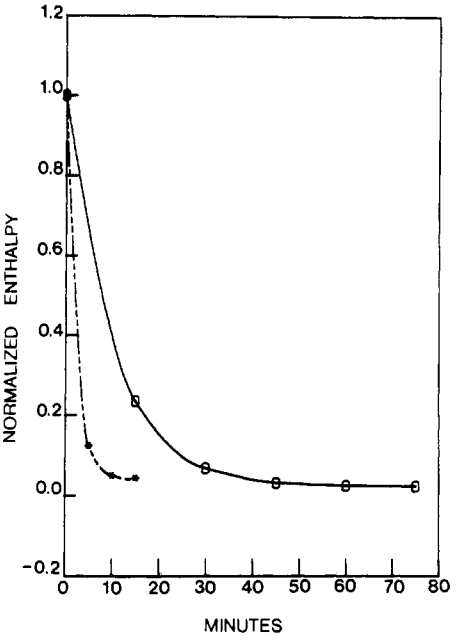

FIGURE 6: Dependence of the degree of reversibility of the B subunit unfolding transition on the time spent by the sample above the transition temperature in the absence (solid line) or presence (dashed line) of excess ganglioside G_{M_1}. Enthalpies were normalized to the enthalpy of the first scan in the series. Raw data points were fitted by using a single-exponential decay curve.

concentration at which saturation is reached at a G_{M_1}:protein ratio of 5:1.

Reversibility of Thermal Transitions. The thermal denaturation of the cholera toxin A subunit is an irreversible process. On the contrary, the thermal denaturation of the B subunit pentamer is a reversible process, provided that the protein is not kept at postdenaturational temperatures for long periods of time. This behavior is illustrated in Figure 5 for successive calorimetric scans of the same sample of isolated B subunit pentamers. As depicted in this figure, the area under the heat capacity curve decreases progressively for each successive scan. In fact, if the areas are plotted as a function of the time that the sample has been kept above the transition region, an exponential decay is obtained, as shown in Figure 6. This exponential decay is characterized by a relaxation time of 10 min.

The reversibility of the isolated B subunit pentamer transition in the presence of excess ganglioside G_{M_1} (B subunit:G_{M_1} ratio of 1:50) was also studied, and the results are shown in Figure 6 (dashed line). An exponential decay characterized by a relaxation time of 2 min was obtained under these conditions. The B subunit pentamer transition of intact cholera toxin in the absence and presence of ganglioside G_{M_1} also shows a similar reversible behavior (data not shown).

These calorimetric results suggest the following transition mechanism for the cholera toxin B subunit:

$$F \rightleftharpoons U \rightarrow P$$

According to the above mechanism, the B subunit pentamer exists in rapid equilibrium between the folded and unfolded states. This equilibrium is followed by an irreversible process, P, which is triggered when the unfolded state becomes populated. The rate of formation of the irreversible denatured species, P, is temperature dependent and characterized by an activation enthalpy of 25 kcal/mol. This mechanism appears to be of a general nature and obeyed by small as well as large proteins including those lacking disulfide bridges. For example, *Staphylococcus* nuclease also exhibits a postdenaturational irreversible step characterized by a relaxation time of ~60 min at pH 6.3 in which the unfolding transition is centered at 52 °C. Similarly, the same phenomenon occurs with ribonuclease A, even though in this case the relaxation time is approximately 4 h (unpublished data from this laboratory).

Differential Solubility Thermal Gel Analysis. Figure 7 shows typical laser densitometer tracings of SDS–polyacrylamide gels of the supernatant of cholera toxin samples heated up to the indicated temperature and then centrifuged as indicated under Materials and Methods. At low temperatures, the densitometer traces are characterized by the presence of two bands corresponding to the B subunit monomer and A subunit, respectively. The A subunit peak is present in the free cholera toxin samples heated up to 65 °C and absent in samples heated to higher temperatures (panel I). The disappearance of the A subunit peak coincides with the presence of a white pellet in the samples heated above 65 °C. Furthermore, this disappearance coincides with the exothermic effect seen in the calorimetric scans of cholera toxin in solution. These results also indicate that the denatured A subunit dissociates from the intact toxin molecule at the onset of B subunit pentamer denaturation. By contrast, the B subunit remains soluble in aqueous solution even at temperatures above denaturation.

The dissociation and precipitation of the denatured A subunit are also evident in the densitometer tracing of cholera toxin samples incubated with lipid vesicles lacking ganglioside G_{M_1} (panel II). On the contrary, the denatured A subunit remains in the supernatant in those samples incubated with ganglioside G_{M_1} either in micellar form (panel III) or when incorporated into lipid bilayers (panel IV). Since the A subunit precipitates when free in aqueous solution, these results indicate that the denatured A subunit stays associated with the ganglioside micelles or the phospholipid vesicles containing ganglioside G_{M_1} but not with the phospholipid vesicles lacking ganglioside. The calorimetric results agree with this obser-

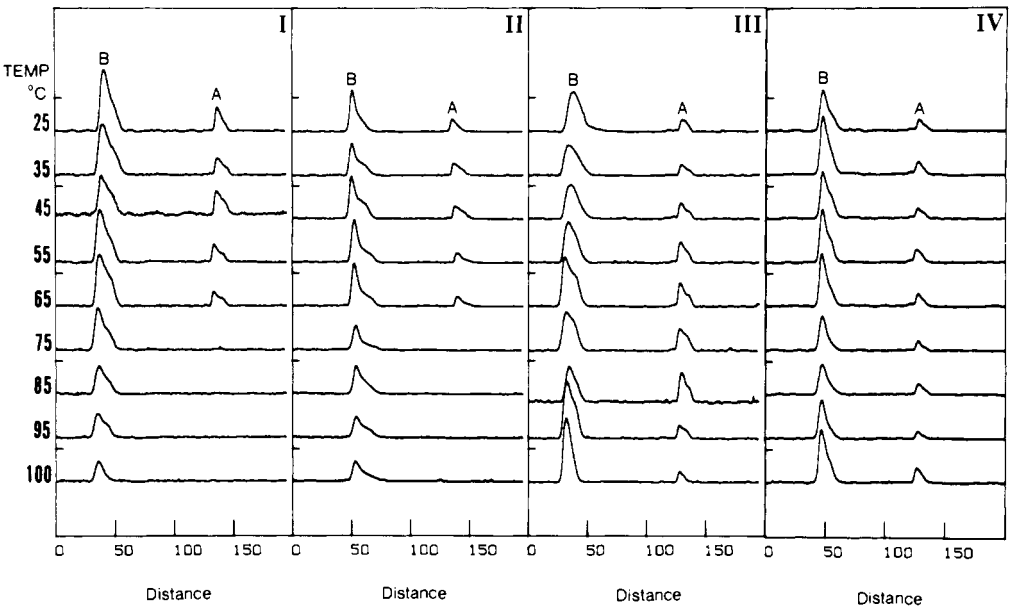

FIGURE 7: Laser densitometer tracing of differential solubility gel analysis experiment of cholera toxin in solution (panel I), in the presence of egg PC small sonicated vesicles (panel II), in the presence of ganglioside G_{M_1} micelles (panel III), or in the presence of egg PC small sonicated vesicles containing 10 mol % of ganglioside G_{M_1} (panel IV). The actual experimental conditions for each panel are described in the text. SDS gels were run with approximately 12.5 μg of protein in each lane. In each instance, the gel was scanned from bottom to top.

vation since no exotherm is seen in the scans when ganglioside G_{M_1} is present. This association of the A subunit is mediated by the B subunit since the A subunit alone is unable to bind lipid vesicles with or without ganglioside G_{M_1}.

Discussion

Cholera toxin is a multisubunit protein consisting of two structurally distinct subunits, A and B. Protomer B is a pentameric ring of identical subunits which bind ganglioside G_{M_1} with high affinity. Protomer A consists of two different subunits, A_1 and A_2, linked by a disulfide bond. The subunits are arranged such that the A subunit occupies the central channel of the B subunit pentamer extending well above the plane of the pentameric ring (Dwyer & Bloomfield, 1982; Ribi & Kornberg, 1987). The hydrophobic A subunit is the toxic component capable of activating adenylate cyclase. Since the A subunit does not bind cells, it must rely on the B component for association with ganglioside G_{M_1} molecules on the cell membrane surface.

The A and B components of intact toxin have different thermal stabilities as evidenced by the existence of two well-separated thermal transitions in the heat capacity profile. The A subunit unfolds at 51 °C with an enthalpy change of 90 kcal/mol whereas the B subunit pentamer unfolds at 74 °C with an enthalpy change of 520 kcal/mol of pentamer. The presence of the A subunit does not significantly affect the thermal unfolding of the B subunit pentamer, since within experimental error identical thermotropic behavior is found for the B subunit whether in isolated form or as a part of the holotoxin. These results indicate that the A and B subunits behave independently of each other and that their thermodynamic stability is determined by intrasubunit rather than heterologous intersubunit interactions.

The calorimetric studies in conjunction with differential solubility thermal gel analysis provide the following picture for the mechanism of thermal denaturation of intact cholera toxin. In the absence of ganglioside G_{M_1}, this event consists of (1) unfolding of the A subunit while attached to the holotoxin, (2) dissociation of the A subunit at the onset of B subunit pentamer unfolding, and (3) precipitation of the dissociated A subunit. It should be noted that the denaturation of the A subunit per se does not trigger its dissociation from the B subunit pentamer.

The thermotropic behavior of cholera toxin is greatly influenced by ganglioside G_{M_1} as shown by a dramatic increase in the transition temperature of the B subunit pentamer. This same behavior has been reported (Dalziel et al., 1984) for the isolated B subunit pentamer, suggesting that the behavior of the B subunit is not affected by the presence of the A subunit. By contrast, incubation of intact toxin with ganglioside G_{M_1} results in only a slight increase in the transition temperature for the A subunit transition. The results from several spectroscopic investigations (Fishman et al., 1978; Sillerud et al., 1981; Tomasi et al., 1984) have suggested the existence of a conformational change in the B subunit upon binding to ganglioside G_{M_1}. The calorimetric results presented in this paper revealed that the major effect of ganglioside is on the cooperative interactions between the B subunits and demonstrate further that there is little, if any, direct interaction between the A subunit and ganglioside G_{M_1}. This conformational difference probably does not represent a gross structural rearrangement (Dwyer & Bloomfield, 1982) but rather local amino acid side chain reorientation due to ganglioside G_{M_1} association (Tomasi et al., 1984). The binding site for the ganglioside G_{M_1} has been postulated as being at the interface between B subunit monomers (DeWolf et al., 1981), thus providing a structural basis for the observed enhancement in intersubunit interactions. This additional intersubunit communication in the presence of ganglioside G_{M_1} may play a role in the insertion of the A subunit into the membrane.

The denatured A subunit does not precipitate at the onset of the B subunit unfolding in the presence of ganglioside G_{M_1}. This observation is consistent with the idea that, under these conditions, the A subunit of cholera toxin is located within the lipid milieu as a result of the association of the B subunits of the toxin with the oligosaccharide portion of the ganglioside molecule. The postulated orientation of the toxin molecule upon association is with the A_1 portion facing toward the membrane (Dwyer & Bloomfield, 1982). Previously, we have demonstrated (Goins & Freire, 1985) that the hydrophobic A_1 subunit behaves as an integral membrane protein when incorporated into liposomes using standard membrane re-

constitution techniques. Evidence for direct penetration has accumulated in the last few years using several techniques including hydrophobic photoaffinity labeling (Wisnieski & Bramhall, 1981; Tomasi & Montecucco, 1981; Tomasi et al., 1982), laser light scattering (Dwyer & Bloomfield, 1982), and lipid layer crystallization (Ludwig et al., 1986; Ribi & Kornberg, 1987). Recent isothermal titration calorimetry results (B. Goins and E. Freire, unpublished results) indicate that the insertion of the active A subunit into the interior of the membrane is an energetically favorable process. Within this context, the main role of the B subunit pentamer is to provide a water-soluble carrier to the A subunit and a mechanism to place it in close contact with the membrane. The mechanism involves ganglioside G_{M_1} as a high-affinity membrane surface receptor and as a modulator of the intersubunit interactions within the cholera toxin molecule.

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