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Thermodynamic Identification of Stable Folding Intermediates in the B-Subunit of Cholera Toxin†

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Received December 27, 1990; Revised Manuscript Received March 5, 1991

ABSTRACT: The structural stability and domain structure of the pentameric B-subunit of cholera toxin have been measured as a function of different perturbants in order to assess the magnitude of the interactions within the B-subunits. For these studies, temperature, guanidine hydrochloride (GuHCl), and pH were used as perturbants, and the effects were measured by high-sensitivity differential scanning calorimetry, isothermal reaction calorimetry, fluorescence spectroscopy, and partial protease digestion. At pH 7.5 and in the absence of any additional perturbants, the thermal unfolding of the B-subunit pentamer is characterized by a single peak in the heat capacity function centered at 77 °C and characterized by a ΔH_{cal} of 328 kcal/mol of B-subunit pentamer and $\Delta H_{vh}/\Delta H_{cal}$ of 0.3. Lowering the pH down to 4 or adding GuHCl up to 2 M results in a decrease of the calorimetric enthalpy with no significant effect on the van't Hoff enthalpy. The transition enthalpy decreases in a sigmoidal fashion with pH, with an inflection point centered at pH 5.3. Isothermal titration calorimetric studies as a function of pH also report a transition centered at pH 5.3 and characterized by an enthalpy change of 27 kcal/mol of B-subunit pentamer at 27 °C. Below this pH, the enthalpy change for the unfolding transition is reduced to approximately 100 kcal/mol of B-subunit pentamer. Similar behavior is obtained with GuHCl. In this case, a first transition is observed at 0.5 M GuHCl and a second one at 3 M GuHCl. Trypsin digestion studies show that at pH 5.0 the B-subunit is 4 times more susceptible to digestion than at pH 7.0 and that at pH 5.0 limited proteolysis results in two fragments of ~7 and ~5 kDa. These studies provide strong evidence that the B-subunits of cholera toxin are composed of two folding/unfolding domains and that the interactions between the two domains within the same subunit and between subunits are able to account for the cooperative behavior of the entire pentameric ring.

Cholera toxin, the enterotoxin of *Vibrio cholera*, is a globular protein (M_r 85000) composed of two protomeric species, A (M_r 27000) and B (M_r 58000) (Finkelstein, 1973; Sattler et al., 1975; Lai, 1980). The A-promoter contains two nonidentical subunits: an ADP-ribosylating protein, A1 (M_r 21000), which activates adenylate cyclase, and a small subunit, A2 (M_r 6000), which plays a structural role in holding the A- and B-subunits together. The two components of the A-subunit are linked by a single disulfide bond (Gill & King, 1975). The B-promoter contains five identical polypeptide chains (each of M_r 11500) arranged in a noncovalently associated, ringlike pentameric configuration surrounding the dimeric A-subunit (Gill, 1976; Ribi et al., 1988; Spangler & Westbrook, 1989). Cholera toxin binds specifically through the B-subunit to ganglioside GM1 present on the plasma membrane of most eukaryotic cells. This association is believed to cause a conformational change in the protein (Sillerud et al., 1981; Fishman et al., 1978; Goins & Freire, 1985, 1988a; Schon & Freire, 1989; Surewicz et al., 1990) that facilitates the exposure and subsequent penetration of the A-subunit into the membrane.

Previous studies on the thermal stability of intact cholera toxin and cholera toxin subunits from this laboratory (Goins & Freire, 1985, 1988a,b) have shown that cholera toxin undergoes two distinct thermally induced transitions; a low-temperature transition (51 °C) due to the irreversible denaturation of the A-subunit and a high-temperature, reversible transition centered at approximately 75 °C due to the thermal unfolding of the B-subunit. The characteristics of the B-subunit transition are not affected by the presence or absence of the A subunit. In the absence of ganglioside GM1, the B-subunit pentamer exhibits a single transition in aqueous solution and is characterized by little or no intersubunit cooperative interactions. Upon binding to ganglioside GM1, the unfolding process becomes highly cooperative, and the pentameric B-subunit ring effectively behaves as a single cooperative unit (Goins & Freire, 1988; Schon & Freire, 1989).

†Supported by grants from the National Institutes of Health (NS-24520 and RR-04328).

0006-2960/91/0430-5055$02.50/0

The detailed molecular mechanism of this cooperative enhancement has not been fully characterized yet, even though it is known to involve a conformational rearrangement triggered by the association of the oligosaccharide region of ganglioside GM1 to the B-subunit of the toxin (Schon & Freire, 1989). In order to get more insight into the thermodynamic structure and interactions within the cholera toxin B-subunit pentamer, we have performed a systematic characterization of its conformational and thermal stability as a function of pH and guanidine hydrochloride concentration. This characterization has been performed by using high-sensitivity differential scanning calorimetry, fluorescence spectroscopy, isothermal titration calorimetry, and limited protease digestion. These studies demonstrate that low pH or GuHCl can trigger the presence of partially folded intermediates of the B-subunit and that these intermediates are able to modulate the cooperative behavior of the entire pentameric ring.

Materials and Methods

Protein. Cholera toxin B-subunit was purchased from Sigma (St. Louis, MO) as a lyophilized powder and was reconstituted with water to give the desired concentration of protein in 50 mM Tris-HCl, 0.2 M NaCl, 3 mM NaN_3, and 1 mM Na_2EDTA, pH 7.5. The purity of the protein was checked by SDS–polyacrylamide gel electrophoresis. Different solvent conditions were obtained by dialyzing the protein overnight against 4000 volumes of the desired buffer at 4 °C. The buffers used were 10 mM potassium phosphate, 0.2 M NaCl, and 3 mM NaN_3 for pH 7.5–6.0 and 10 mM acetate, 0.2 M NaCl, and 3 mM NaN_3 for pH 5.5–4.0. For the studies with guanidine hydrochloride, the protein was dialyzed in 10 mM potassium phosphate, 0.2 M NaCl, and 3 mM NaN_3, pH 7.4, overnight at 4 °C. A 4 M GuHCl stock solution was prepared by lyophilizing GuHCl crystals (grade 1, Sigma Chemical Co., St. Louis, MO) overnight and dissolving the dried crystals in potassium phosphate buffer the next day. Samples for the various studies were prepared by mixing the dialyzed cholera toxin B-subunit with the appropriate GuHCl solution to get the desired protein concentration. The pH of the buffer was checked after the addition of GuHCl. In all experiments, the protein concentration was determined by the method of Lowry et al. (1951).

Since a pH of 3 or lower has been reported to dissociate the B-subunit pentamer into its monomeric components (Gill, 1976), the integrity of the B-subunit pentamer was checked under the experimental conditions used in this paper. This was done by time-resolved fluorescence spectroscopy and native gel electrophoresis analysis. Rotational correlation times of 23.8 and 22.1 ns were obtained at pH 7.5 and 4.0, respectively (V. Bhakuni, unpublished data). The similarity of rotational correlation times under both conditions indicates the absence of a significant change in molecular weight, consistent with the absence of pentamer dissociation. Gel electrophoresis experiments under nondenaturing conditions gave single bands with similar mobilities for the B-subunit pentamer at pH 7.5 and 4, also consistent with the absence of pentamer dissociation.

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 about 0.9 mg/mL; 1.3 mL of sample was introduced into the sample cell and a similar amount of buffer into the reference cell. Samples were scanned at a rate of 90 °C/h. The samples were degassed for 15 min at room temperature prior to being scanned in the calorimeter. Data reduction and analysis were performed with software developed in this laboratory.

Fluorescence Measurements. Steady-state fluorescence measurements were made with a Perkin Elmer LS-5 spectrofluorometer equipped with a thermostated cell compartment. The excitation wavelength was 280 nm, and the emission spectrum was recorded between 300 and 420 nm. The emission and excitation slits were 3 and 5 nm, respectively. All the fluorescence measurements were done at 27 °C.

Titration Calorimetry. The calorimetric determination of the heat effects associated with protein transitions triggered by pH was performed with a Microcal Omega titration calorimeter equilibrated at the desired temperature (27 °C). For these experiments, the protein was dissolved in an unbuffered solution of 200 mM KCl, pH 6.5, at a concentration of 2 mg/mL, placed in the calorimeter cell, and then titrated in the same calorimeter cell by the stepwise addition of 10 μL/injection of a 15 mM HCl solution loaded in the injection apparatus of the calorimeter. Control experiments were performed to estimate the heat of HCl injection and dilution and the heat of protein dilution. The heat effects observed upon each injection were measured directly. The heat of reaction after each injection was obtained by subtracting the heats of dilution and injection from the measured total heat. The pH after each HCl injection was experimentally determined by using a separate aliquot of the same protein solution and subjected to the same procedure used in the calorimetric experiments.

Trypsin Digestion of Cholera Toxin B-Subunits. Digestion of cholera toxin B-subunit (1 mg/mL) with trypsin (TPCK-treated; Sigma Chemical Co., St. Louis, MO) was carried out at various protease/protein mole ratios at pH 7.5 and 5.0. The samples were incubated at 30 °C for 10 min. The reaction was terminated by heating the sample at 90 °C for 3 min. Samples were then stored at −20 °C until analysis. The samples were analyzed by SDS–polyacrylamide gel electrophoresis using essentially the method of Giulian et al. (1985), except that 4.8% cross-linking was used in the separating gel. The gels were stained with Coomassie brilliant blue and scanned on an 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). Data collection and subsequent data manipulation to obtain normalized peak intensities and areas were performed with software developed in this laboratory (Goins & Freire, 1988). Results are expressed as the percent area of each peak relative to the total of all peaks.

Results

Effect of pH on the Thermal Stability of Cholera Toxin B-Subunit. The thermal stability of cholera toxin B-subunit was measured at different pHs and GuHCl concentrations by high-sensitivity differential scanning calorimetry. The excess heat capacity (C_p) versus temperature profiles for the B-subunit pentamer at decreasing pH values are shown in Figure 1A. At pH 7.5, the thermal unfolding of cholera toxin B-subunit is characterized by a single peak in the heat capacity function centered at 76.9° in agreement with previous studies (Goins & Freire, 1988). This transition is characterized by a calorimetric enthalpy (ΔH_{cal}) of 328 kcal/mol of B-subunit pentamer and a van't Hoff to calorimetric enthalpy ratio ($\Delta H_{vh}/\Delta H_{cal}$) of 0.29. For each experiment, the van't Hoff enthalpy was calculated by using the standard formula ΔH_{vh}

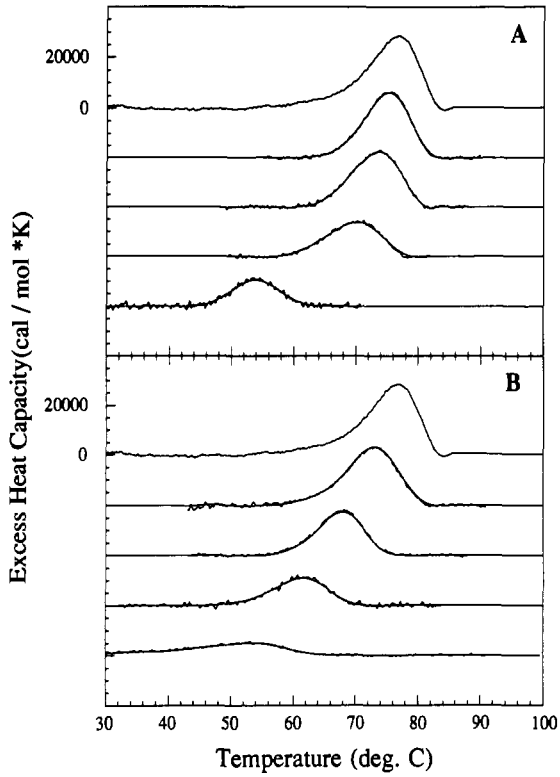

FIGURE 1: Excess heat capacity versus temperature for the B-subunit pentamer of cholera toxin as a function of pH (panel A) and guanidine hydrochloride concentration (panel B). From top to bottom, the experimental pHs in panel A are 7.5, 6.0, 5.5, 5.0, and 4.0. Similarly, the GuHCl concentrations in panel B are from top to bottom 0, 0.5, 1.0, 2.0, and 3.0 M. The buffers used were 10 mM potassium phosphate, 200 mM NaCl, and 3 mM NaN_3 for pH 7.5–6.0 and 10 mM acetate, 200 mM NaCl, and 3 mM NaN_3 for pH 5.5–4.0. For the experiments in the presence of GuHCl, the buffer was the same as for pH 7.5. The curves have been shifted in the y axis for display purposes. The solid lines were obtained by Fourier-transform smoothing of the data.

$= 4RT_m^2C_{p,max}/\Delta H_{cal}$ [see Privalov (1979)]. The low $\Delta H_{vh}/\Delta H_{cal}$ ratio has been interpreted as being indicative of a lack of cooperative interactions within the pentameric B-subunit ring, resulting in the independent unfolding of each subunit within the pentamer. It must be noted that the pentamer does not dissociate upon thermal unfolding and that the transition is reversible provided that the protein is cooled down immediately after the unfolding transition has occurred (Goins & Freire, 1988).

The transition temperature (defined as the temperature location of the maximum in the excess heat capacity function) depends strongly on pH. As shown in Figure 2A, the T_m decreases approximately 3 °C upon lowering the pH from 7.5 to 5.5, but it decreases about 20 °C after lowering the pH from 5.5 to 4.0. The measured enthalpy change for the transition decreases in a sigmoidal fashion with pH and exhibits an inflection point around pH 5.3 as shown in Figure 2B. At pH 7.5, the observed enthalpy change is 328 kcal/mol of B-subunit pentamer and decreases to about 134 kcal/mol at pH 5.0. Further decrease of the pH to 4.0 does not induce any major additional decrease in the enthalpy change for the transition. It is interesting to note that whereas the calorimetric enthalpy (ΔH_{cal}) decreases substantially with pH, the van't Hoff enthalpy (ΔH_{vh}) remains essentially constant throughout the entire pH range studied. As a result, the ratio of the van't Hoff to calorimetric enthalpy also exhibits a sigmoidal relationship with pH and is maximal at the lowest pH studied. As shown in Figure 2C, the pH dependence of the $\Delta H_{vh}/\Delta H_{cal}$ ratio has an inflection point near pH 5.3. Table I summarizes all the experimentally determined thermodynamic results.

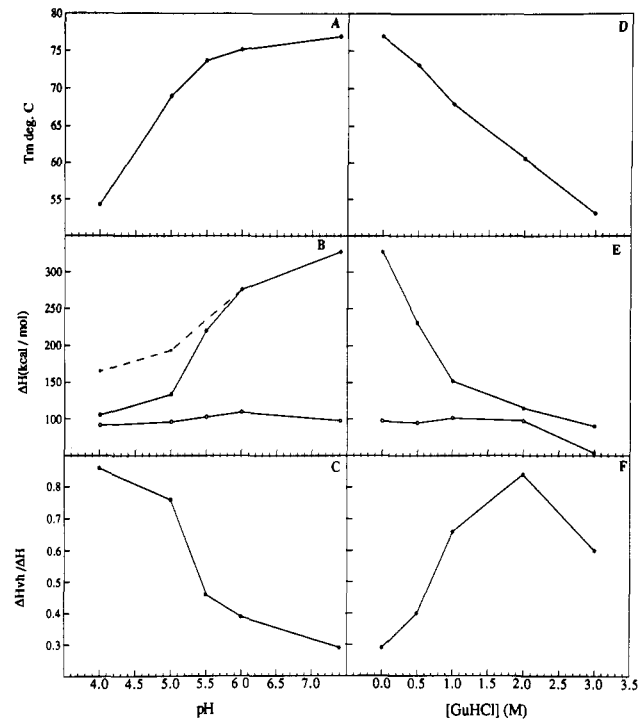

FIGURE 2: pH (left panels) and GuHCl concentration (right panels) dependence of the transition temperature, calorimetric and van't Hoff enthalpy changes, and van't Hoff to calorimetric enthalpy ratio associated with the thermal unfolding of the B-subunit of cholera toxin. In panel B and E, the closed circles correspond to the calorimetric enthalpy and the open circles to the van't Hoff enthalpy. The dashed line in panel B is the intrinsic enthalpy change for the structural change associated with the unfolding of the B-subunit. It was obtained from the total enthalpy change (solid line) after correction for the heat of histidine protonation as discussed in the text.

Table I: Thermodynamic Parameters for the Thermal Unfolding of Cholera Toxin B-Subunit[a]

pH	T_m (°C)	ΔH_{cal} (kcal/mol)	ΔH_{vh} (kcal/mol)	$\Delta H_{vh}/\Delta H_{cal}$
7.5	76.5 ± 0.3	328 ± 7.5	95 ± 2.8	0.29
6.0	74.9 ± 0.2	274 ± 8	107 ± 2	0.39
5.5	73.4	220	101	0.46
5.0	69.0 ± 0.1	134 ± 10	96 ± 2.5	0.72
4.0	53.2 ± 0.1	105 ± 4	91 ± 3	0.86

[GuHCl](M) at pH 7.5	T_m (°C)	ΔH_{cal} (kcal/mol)	ΔH_{vh} (kcal/mol)	$\Delta H_{vh}/\Delta H_{cal}$
0.0	76.5 ± 0.3	328 ± 7.5	95 ± 2.8	0.29
0.5	72.2 ± 0.9	224 ± 6.5	97 ± 2.2	0.42
1.0	67.9 ± 0.3	152 ± 7.5	101 ± 3	0.66
2.0	60.7	115	98	0.84
3.0	53.1	90	53	0.60

[a] The quoted errors represent the experimental variation over two independent protein preparations.

The DSC experiments described above indicate the existence of a pH-induced transition centered around pH 5–5.5. This transition is characterized by an enthalpy change of ~120 kcal/mol at 70 °C. This value was obtained by using acetate buffer which is characterized by a very small ΔH of ionization (0.12 kcal/mol). In order to dissect the purely conformational change from any histidine protonation effects upon unfolding at pH 5, additional experiments were also performed in MES buffer, which is characterized by a high protonation enthalpy (3.7 kcal/mol). The calorimetric enthalpy in MES buffer was

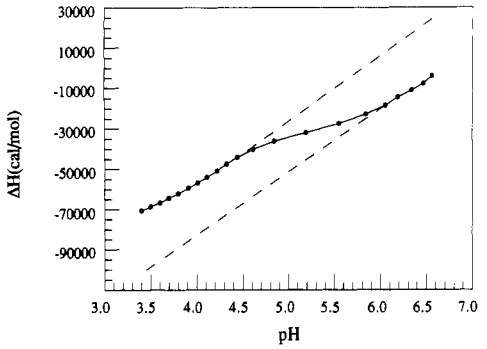

FIGURE 3: Results of isothermal calorimetric determination of the pH titration of the B-subunit of cholera toxin. Shown in the figure is the change in enthalpy as a function of pH measured in 27 °C. For these experiments, the B-subunit (2 mg/mL) was dissolved in an unbuffered solution of 200 mM KCl, pH 6.5, placed in the calorimeter cell (1.3 mL), and then titrated by the stepwise addition of 10 μL/injection of 15 mM HCl.

31.5 ± 7 kcal/mol higher than in acetate buffer at the same temperature. This value can be used to calculate the number of protein groups (n_p) that become protonated upon unfolding by means of the formula:

$$n_p = \frac{\Delta H_{cal,B} - \Delta H_{cal,A}}{\Delta H_{i,B} - \Delta H_{i,A}}$$

where $\Delta H_{cal,A}$ and $\Delta H_{cal,B}$ are the transition enthalpies measured in buffers A and B at the same temperature and pH and $\Delta H_{i,A}$ and $\Delta H_{i,B}$ are the enthalpies of ionization of buffers A and B, respectively. The above formula indicates that the unfolding of the B-subunit pentamer is accompanied by the protonation of 9 ± 2 groups (i.e., approximately 2 groups per B-subunit monomer). Since the enthalpy of protonation of histidine is −6.9 kcal/mol (Privalov et al., 1986), it follows that below pH 6 histidine protonation will lower the overall transition enthalpy by 62 kcal/mol. Since the observed decrease is ~120 kcal/mol, it can be concluded that the enthalpy change associated with the conformational change is also on the order of 60 kcal/mol at 70 °C. This pH-induced conformational change does not represent the unfolding of the entire molecule as evidenced by the fact that even after this change has occurred the calorimetric transition is still observed, albeit with a reduced enthalpy.

The occurrence of the pH-induced transition can also be illustrated by considering the apparent ΔC_p for the transition as a function of pH. In general, the enthalpy change associated with protein unfolding is a linear function of temperature, the slope being equal to the heat capacity difference between the denatured and native states of the protein. Since the transition temperature is dependent on pH, it is expected for the calorimetric enthalpy change to vary with pH; however, the apparent ΔC_p should be independent of pH unless there is a pH-dependent protein transition. In the case of the B-subunit pentamer, the apparent ΔC_p has a maximum of ~0.6 cal/(K·g) around pH 5.5. For globular proteins, ΔC_p is on the order of 0.08–0.16 cal/(K·g) and independent of pH (Privalov & Khechinashvilli, 1974; Privalov, 1979). The anomalous ΔC_p behavior observed for the B-subunit pentamer is also indicative of a pH-induced transition. If the observed value of 0.6 cal/(K·g) were due to the intrinsic ΔC_p between the denatured and native states, the protein will exhibit cold denaturation near room temperature, which is not the case (Goins & Freire, 1988).

Isothermal Titration Calorimetry. The enthalpy change associated with the pH-induced conformational change of the B-subunit pentamer was also studied at constant temperature.

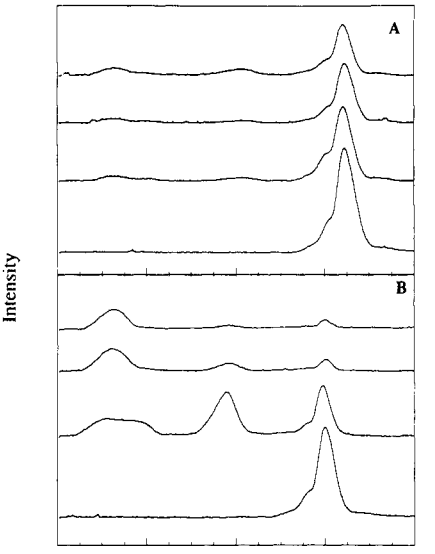

FIGURE 4: Densitometer traces of SDS gels of the B-subunit of cholera toxin after trypsin digestion at pH 7.5 (panel A) and pH 5.0 (panel B). From bottom to top, the densitometric traces correspond to 0, 0.001, 0.006, and 0.008 trypsin/B-subunit molar ratios. The B-subunit samples (1 mg/mL) were incubated with the protease at 30 °C for 10 min as detailed in the text. The reaction was terminated by heating the samples to 90 °C for 3 min and then analyzed by SDS–polyacrylamide gel electrophoresis.

In these experiments, a fixed amount (10 μL) of 15 mM HCl was injected in a stepwise fashion into the calorimeter cell containing B-subunit pentamer in 200 mM KCl. The heat released after each injection was measured and corrected for dilution heats by subtracting the values obtained in experiments performed under identical conditions except for the presence of the protein. The pH after each HCl injection was experimentally determined by using a separate aliquot of the same protein solution. Figure 3 shows the change in enthalpy as a function of pH for this calorimetric titration. A smooth transition centered around pH 5.3 is observed. The transition is characterized by a ΔH of 27 kcal/mol of B-subunit at 27 °C. These results clearly indicate that the pH-dependent transition in the B-subunit can be induced isothermally. As concluded from the differential scanning calorimetric experiments, the enthalpy of the pH-induced transition was estimated to be ~60 kcal/mol at 70 °C. The reduced enthalpy observed at 27 °C is consistent with a ΔC_p of 0.8 kcal/(K·mol) for the pH-induced conformational transition. As demonstrated by the DSC experiments, the B-subunit is still capable of undergoing a thermally induced transition even after the pH transition has occurred. This observation further supports that the pH 5.3 transition induces only a partial unfolding of the molecule.

Protease Cleavage of the B-Subunit. In order to further characterize and identify the structural regions involved in the pH-induced transition, the B-subunit pentamer was subjected to tryptic digestion at pH 7.5 and 5.0, i.e., under conditions before and after the pH-induced transition. The digestion experiments were performed in the same buffer systems used for the calorimetric measurements. Briefly, the experiments were performed by incubating a fixed concentration of B-subunit with increasing concentrations of trypsin at 30 °C for 10 min. The reaction was terminated by heating the mixture at 90 °C for 3 min, and the reaction products were analyzed by SDS gel electrophoresis. Figure 4 shows typical laser densitometer traces of SDS–polyacrylamide gels obtained for B-subunit pentamer solutions after treatment with increasing

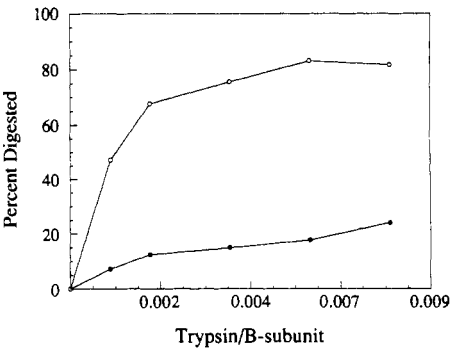

FIGURE 5: Percent of B-subunit digested after exposure to different trypsin/B-subunit molar ratios. The same amount of B-subunit was exposed to different amounts of trypsin for equal lengths of time (10 min) at 30 °C. The solid circles represent data obtained at pH 7.5 and the open circles the data obtained at pH 5.0.

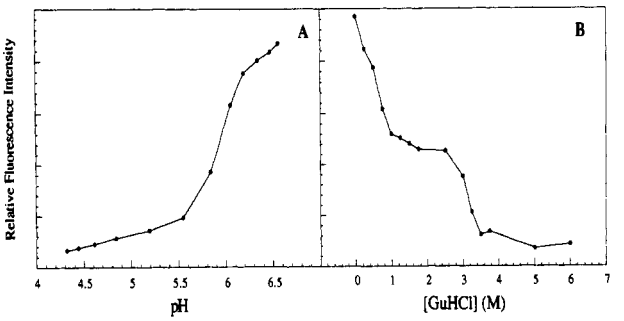

FIGURE 6: Normalized change in fluorescence emission intensity of B-subunit as a function of pH (panel A) and Gu HCl concentration (panel B). The experiments were performed at 27 °C using an excitation wavelength of 280 nm. The buffers are the same as those described in Figure 1. The experiments were done with a B-subunit concentration of 0.5 mg/mL.

concentrations of trypsin. As shown in the figure, three distinctive bands were obtained, one of which corresponds to the intact B-subunit monomer (M_r 11 500) and the other two to fragments derived from the tryptic digestion of the B-subunit. It is interesting to note that only 2 fragments are produced even though there are 11 potential cleavage sites in the molecule. These two fragments of M_r ~7K and ~5K account for the entire B-subunit.

The relative intensities of the bands corresponding to the 7- and 5-kDa fragments changed drastically with increasing trypsin concentration at pH 5.0, but were not much affected at pH 7.5. In fact, at pH 7.5, the 7- and 5-kDa bands never became significantly populated. It must be noted that at pH 5.0 the intrinsic activity of trypsin is ~20% lower than at pH 7.5 [see Lehninger (1982)], indicating that the enhanced digestion observed at pH 5.0 cannot be due to an enhanced intrinsic activity of the enzyme but to a more labile substrate. These results also suggest that each B-subunit monomer is composed of two structural regions or domains. At pH 7.5, the two domains are probably in close contact with each other such that they cannot be separated by protease digestion. At pH <5.5, the close contact between the domains is apparently lost, resulting in the accessibility of the linker or hinge region between the two domains to the protease molecule. The entire 7-kDa domain is also more susceptible to further digestion as demonstrated by its fast rate of degradation. In fact, after prolonged protease incubation, only the 5-kDa fragment remains intact. This result is consistent with the calorimetric data indicating that most of the overall transition enthalpy is lost with the pH transition, and further suggests that the pH-induced transition involves the unfolding of the 7-kDa domain, making it readily accessible to protease digestion. Figure 5 shows the percent degradation of B-subunit pentamer with respect to the trypsin/B-subunit molar ratio. Under the experimental conditions, the maximum cleavage of about 80% was achieved for pH 5.0 at a protease/protein mole ratio of 0.008, whereas at the same ratio only about 20% cleavage was obtained at pH 7.5.

Fluorescence Measurements. The presence of only one tryptophan residue in each of the polypeptide chains constituting the B-pentamer (Kurosky et al., 1977; De Wolf et al., 1981) makes possible the use of fluorescence spectroscopy to study this transition. The pH-induced transition of the B-subunit pentamer is accompanied by a decrease in the emission intensity and a blue shift of about 8 nm (from 350 to 342 nm) in the fluorescence emission maxima. Figure 6 shows the pH dependence of the fluorescence intensity of the B-subunit pentamer. Similar results were obtained by using the shift in the emission maximum. As shown in the figure, a sigmoidal decrease of the fluorescence intensity upon lowering the pH is observed. This dependence is consistent with the existence of a pH-induced transition. Under the experimental conditions of these experiments, an inflection point near pH 5.8 was observed. This inflection point occurs about 0.5 pH unit higher than the one observed calorimetrically. This observation suggests that the pH transition might not be a two-state process and that the protein region defining the environment of the tryptophan might be affected first.

Effect of GuHCl on the Thermal Stability of Cholera Toxin B-Subunit. In order to provide additional experimental verification to the conclusion that the B-subunit of cholera toxin is composed of two interacting domains, GuHCl was used as a perturbant. GuHCl has been used for many years as a protein denaturant [see Pace (1986) for a review] and more recently as an agent to dissect interdomain interactions (Ramsay & Freire, 1990). Figure 6B shows the GuHCl concentration dependence of the fluorescence intensity of the single tryptophan residue in the B-subunit. As shown in the figure, the change in fluorescence intensity is clearly biphasic, showing a first inflection at 0.5 M GuHCl and a second inflection at 3 M GuHCl. These results are also consistent with the existence of two unfolding domains with different structural stabilities.

Figure 1B shows the excess heat capacity (C_p) versus temperature profiles for the B-subunit pentamer at pH 7.5 and increasing GuHCl concentrations. Upon increasing the GuHCl concentration, the transition temperature shows a linear decrease with GuHCl concentration as shown in Figure 2D. The enthalpy change observed for the transition exhibits an almost linear decrease from 328 kcal/mol at zero GuHCl to 152 kcal/mol at 1 M GuHCl and then decreases gradually to 90 kcal/mol at 3 M GuHCl. The enthalpy decrease observed up to 1 M GuHCl parallels the decrease in fluorescence intensity observed in this GuHCl concentration range. In this case also, the ΔH_{vh} (95 kcal/mol) remained almost constant up to 2 M GuHCl and then dropped down to 53.3 kcal/mol at 3 M GuHCl concentration. As shown in Figure 1B, the calorimetric scan at 3 M GuHCl becomes very broad, indicating a loss in the magnitude of the cooperative interactions within the B-subunit. The dependence of the measured thermodynamic parameters on GuHCl concentration is summarized in Figure 2D–F. The ratio of the van't Hoff to calorimetric enthalpy increases with GuHCl concentration from 0.29 at zero GuHCl to 0.84 at 2 M GuHCl and then decreases to 0.6 at 3 M GuHCl. It is interesting to note that the initial effects of pH and [GuHCl] result in a decrease in the calorimetric enthalpy for the transition with no significant effect in the van't Hoff enthalpy.

Discussion

The results presented in this paper provide evidence for the existence of two folding domains within the B-subunits of cholera toxin. These domains are apparently of roughly equal size (5 and 7 kDa) judging by their electrophoretic mobilities and transition enthalpies. According to the pH and GuHCl stability studies, one of the domains (probably the 5-kDa domain as indicated by the limited proteolysis experiments) is more stable and is able to undergo a cooperative thermal unfolding transition even after the other domain is in the unfolded state.

Even though low molecular weight proteins are usually composed of a single domain, this is not the first time that a protein of this size has been found to have two domains. Two examples are given by T4 lysozyme and also by ribonuclease (Weaver & Matthews, 1987; Wlodawer et al., 1982). In the case of ribonuclease, it has also been shown that under certain solvent conditions it is possible to uncouple the behavior of the two structural domains (Brandts et al., 1989).

It is important to note that the decrease in calorimetric enthalpy elicited by pH or GuHCl is not accompanied by a similar decrease in the van't Hoff enthalpy. ΔH_{vh} decreases only slightly, giving rise to an apparent increase in the magnitude of the cooperative interactions. In fact, once the pH-induced transition has occurred and the less stable domain is in the unfolded state, the rest of the pentameric ring undergoes thermal unfolding as a single cooperative unit. This type of behavior is consistent with a model in which the pentameric ring consists of a highly cooperative core formed by the most stable domains of the B-subunits. This core behaves as a single cooperative unit provided that the stable domains are intact. The overall cooperativity of the transition is, to a large extent, dictated by the cooperativity of the pentameric core. So, at pH 7.5 the calorimetric enthalpy includes the contribution from the less stable domains whereas the van't Hoff enthalpy is primarily dictated by the inner core. Under these conditions, the $\Delta H_{vh}/\Delta H_{cal}$ ratio is low ($\sim$0.29). At pH <5, the calorimetric enthalpy contains contributions from the most stable domains alone (since the less stable domains are already unfolded), and the cooperativity ($\Delta H_{vh}/\Delta H_{cal}$) approaches unity. Previously, we have shown that the association of ganglioside GM1 increases the van't Hoff enthalpy to values close to the overall calorimetric enthalpy (Goins & Freire, 1988). According to the model presented here, the association of ganglioside GM1 will have the effect of coupling the behavior of the less stable B-subunit domains to that of the inner core, thus increasing the cooperativity of the transition to include the entire pentameric ring.

The binding of the B-subunit pentamer of cholera toxin to cell-surface ganglioside GM1 is the first step in the intoxication reaction of cholera toxin (Finkelstein, 1973). The association of the B-subunit pentamer to GM1 results in a series of events that lead to the insertion of the toxic A-subunit into the cell membrane. This process involves changes in the conformation of the B-subunits and changes in their cooperative interactions. The studies presented in this paper suggest that this process might be mediated by domain–domain interactions.

Acknowledgments

We thank Dr. Ludwig Brand and his laboratory for performing the time-resolved fluorescence experiments.

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