Calorimetric Investigations of the Structural Stability and Interactions of Colicin B Domains in Aqueous Solution and in the Presence of Phospholipid Bilayers*

Alicia Ortega‡§, Stephan Lambotte§, and Burkhard Bechinger§

From the ‡Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, México City 04510, Mexico and §Max-Planck-Institut für Biochemie, Martinried 82152, Germany

Received for publication, August 23, 2000, and in revised form, December 23, 2000
Published, JBC Papers in Press, January 16, 2001, DOI 10.1074/jbc.M007675200

The effects of pH and temperature on the stability of interdomain interactions of colicin B have been studied by differential-scanning calorimetry, circular dichroism, and fluorescence spectroscopy. The calorimetric properties were compared with those of the isolated pore-forming fragment. The unfolding profile of the full-length toxin is consistent with two endothermic transitions. Whereas peak A (T_m = 55 °C) most likely corresponds to the receptor/translocation domain, peak B (T_m = 59 °C) is associated with the pore-forming domain. By lowering the pH from 7 to 3.5, the transition temperature of peaks A and B are reduced by 25 and 18 °C, respectively, due to proton exchange upon denaturation. The isolated pore-forming fragment unfolds at much higher temperatures (T_m = 65 °C) and is stable throughout a wide pH range, indicating that intramolecular interactions between the different colicin B domains result in a less stable protein conformation. In aqueous solution circular dichroism spectra have been used to estimate the content of helical secondary structure of colicin B (~40%) or its pore-forming fragment (~80%). Upon heating, the ellipticities at 222 nm strongly decrease at the transition temperature. In the presence of lipid vesicles the differential-scanning calorimetry profiles of the pore-forming fragment exhibit a low heat of transition multicomponent structure. The heat of transition of membrane-associated colicin B (T_m = 54 °C at pH 3.5) is reduced and its secondary structure is conserved even at intermediate temperatures indicating incomplete unfolding due to strong protein-lipid interactions.

Membrane proteins are characterized by water-exposed extramembranous as well as intramembranous regions, which are embedded in the interior of the lipid bilayer. As a consequence, the energetics of stabilization of membrane-inserted proteins is expected to include contributions from these two regions in different proportions. Whereas the extramembranous region is more likely to be organized by forces similar to those described for water-soluble proteins (1, 2), the membrane-inserted regions are very stable because of considerable strengthening of hydrogen-bonding interactions within the lipidic milieu (3).

The transition of proteins from a soluble to a membrane-associated state offers a new challenge for describing the energetics involved in the process of membrane insertion, which are critical for the proper folding of membrane proteins (3, 4). A key aspect in lipid-protein interactions is the partitioning of the protein from water into the membrane. The conformational changes, however, that are associated with protein insertion into, transport across, and maintenance within membranes remain poorly understood.

Pore-forming colicins are water-soluble proteins that insert into membranes. Merely by offering a different environment and without the need of chemical modifications, these proteins can be investigated as well in solution as in their membrane-inserted state. Therefore, they provide excellent model systems to study the structural changes during membrane insertion. Pore-forming colicins (e.g. colicin A, B, N, E1, and Ia) invade Escherichia coli cells by parasitizing receptors of the outer membrane and use the Tol or Ton protein complexes for translocation. In a final step these proteins cause voltage-dependent pore formation. The three functions correlate with three distinct domains of the polypeptide chain, which are arranged along the primary sequence in the following order: translocation, receptor recognition, and channel formation.

Here we study colicin B (ColB), which is a 54.6-kDa protein secreted by Escherichia coli as a monomeric unit. Primary overall sequence comparison and functional studies indicate that ColB is most closely related to colicins A, N, and Ia (5–7). It has been suggested that, after binding to the target cell but before insertion occurs, the colicin pore-forming domains undergo conformational changes to adopt an insertion-competent state. This transition is induced in vitro by acidic pH. Membrane-active colicins thereby exhibit close similarity to other proteins such as diphtheria, tetanus, and botulinum toxins (8).

Previous calorimetric studies have contributed some understanding of the structural transitions of the colicin A and colicin E1 pore-forming domains (9, 10). However, the conforma-
tional changes of full-length colicin B or its thermolytic fragments, which are necessary for membrane binding and insertion, have so far not been characterized.

Macromolecules are stabilized by the cooperative action of numerous weak forces. Because such highly cooperative structures undergo phase transition-like conformational changes upon exposure to heat, relevant thermodynamic, and structural information is obtained from differential-scanning calorimetry (DSC) and circular dichroism (CD) studies. Unlike the many calorimetric studies conducted with water-soluble proteins, the stability of only a few membrane proteins has been measured calorimetrically, and even less is known about the thermal stability of proteins that insert into membranes (9–14). Investigations of full-length colicin B as well as its C-terminal fragment both in solution and the membrane-associated state allows for a direct comparison of the thermal characteristics in these different environments. In addition, by investigating the pH-dependent properties of full-length colicin and its pore-forming fragment, important information on the domain structure and inter-domain interactions is obtained (1). The observed modulations of thermal stability by interactions between domains and with the membrane have important implications also for the insertion and translocation competence of colicin B.

EXPERIMENTAL METHODS

Preparation of ColB and ColB PPF—ColB was expressed in E. coli. The full-length protein as well as its thermolytic fragment were purified following procedures described elsewhere (15, 16). This latter domain of the homologous colicin A protein has been shown to exhibit pore-forming activities (e.g. Ref. 17) and the thermolytic fragment is, therefore, abbreviated PPF. SDS-polyacrylamide gel electrophoresis of ColB and its pore-forming fragment shows one single band in Coomassie Blue-stained gels.

Preparation of Lipid Vesicles—All phospholipids were from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Small unilamellar vesicles of POPC:cardiolipin:POPE (5:1:2, w/w) were prepared from a homogeneous solution of the three lipids in chloroform/methanol (1:1). The solvent was completely removed with a stream of nitrogen and by exposure to high vacuum overnight. Thereafter, buffer was added and the suspension was subjected to several freeze-thaw cycles. A clear suspension of small unilamellar vesicles was obtained after repeated extrusion first through 100-nm (5 times) and thereafter 50 nm polycarbonate filters (Avestin, Milsch, Laudenbach, Germany). The lipid concentration for DSC, CD, and fluorescence experiments was 50 mg/ml.

Differential-scanning Calorimetry—DSC was used to measure the transition temperatures ($T_m$) of full-length ColB and its pore-forming fragment. $T_m$ is defined as the temperature at which the excess heat capacity is maximal. Lyophilized protein was dissolved in buffer (0.1 M KCl, 0.02 M glycine at pH 3.5–4.5; or 0.1 M KCl, 0.02 M tris-malate at pH 6.0–7.0), followed by dialysis overnight against the same buffer. Excess heat (C$_H$) versus temperature scans were obtained from 37 μM protein solutions using a high sensitivity differential scanning calorimeter MicroCal VP-DSC. The sample and reference solutions were carefully degassed under vacuum for 5 min before loading the cells (0.56 mL). After equilibration of the system at 10 °C, the temperature was increased to 100 °C and decreased to 10 °C at scan rates of 1 °C/min. Thereafter another 10–100 °C scan was obtained. This rescan, which in all cases showed no evidence of reversibility, was subtracted from the initial scan, and the resulting baseline was corrected.

Circular Dichroism (CD) Spectropolarimetry—CD spectra (190–250 nm) were recorded on a Jasco J-715 spectropolarimeter using a quartz cuvette of 0.1-mm path length. The protein concentration was 3 μM. For temperature scans at 222 nm, a heating rate of 1 °C/min was used (20–85 °C). CD spectra were recorded every 5–10 °C in the temperature range 20–80 °C as indicated in the figures. The helicity of the peptides was determined quantitatively only for spectra recorded at 25 °C, because limited protein aggregation was observed above the transition temperatures. All measurements were corrected for buffer contributions. The lipid concentration was kept constant.

Intrinsic Emission Fluorescence Measurements—All emission fluorescence experiments were performed using a PerkinElmer LS-50 luminescence spectrometer with a water-thermostat cell holder. Excitation wavelengths of 290 nm were used, and the emission spectra were recorded within the range 300–500 nm. The temperature dependence of the fluorescence intensity was recorded at a constant heating rate of 1 °C/min from 25–85 °C at 320 nm. The protein concentration of ColB or its pore-forming fragment was 3 μM.

RESULTS

To test for the effect of pH on the thermal stability of ColB and its pore-forming fragment in solution as well as in the presence of lipids, the proteins were investigated in a temperature-dependent manner by DSC, fluorescence, and CD spectroscopy.

Differential-scanning Calorimetry—The temperature dependencies of the excess molar heat capacity of the full-length ColB between 10 and 100 °C are shown in Fig. 1 as a function of pH. The scans were obtained at heating rates of 1 °C/min. Denaturation was irreversible, because the transitions are absent during cooling or reheating of the samples. In this study the “heat of transition,” $\Delta H$, therefore, relates to the heat absorbed during the process of denaturation and cannot be fully interpreted as the enthalpy of a reversible process. Two superimposed endothermic transitions give the best theoretical fit of the scans obtained at pH 7.0: peak A with $T_m = 54.9 ± 0.5 °C$, and peak B with $T_m = 58.7 ± 0.8 °C (n = 3)$ ($\Delta H_{m(A)} = -3.9 °C$). At pH 3.5 both transitions occur at lower temperatures and with better resolution. The transition temperature of peak A is 29.8 °C and that of peak B 40.9 °C ($\Delta H_{m(B)} = 11.1 °C$). The highest $T_m$ was observed at pH values of >4.5. When the pH is decreased in a stepwise manner from 7.0 to 3.5, peaks A and B decrease by 25.0 °C and 17.8 °C, respectively. Therefore, although both components are affected by pH, peak B is less sensitive to changes in proton activity and more resistant to thermal denaturation than peak A. The transition temperatures obtained at pH 2.5 and pH 9.0 are very similar to those of pH 3.5 and pH 7.5, respectively (not shown).

The heat absorbed during unfolding is obtained by integrating the area under the fitted curves of each transition. Table I lists the unfolding parameters determined using linear and quadratic baselines in a multiple-curve fitting routine of the DSC data. The calculated $\Delta H$ for peaks A and B at different pH are included in Table I. The pH dependence of $\Delta H$ for peak A

---

2 In equilibrium processes the area under the transition enthalps (C$_H$ versus temperature) represents the change of enthalpy of systems at thermodynamic equilibrium throughout the temperature-induced unfolding process (48). Therefore, in the literature reference is often made to “transition enthalpies” also in cases of irreversible protein denaturation during the heating scan (e.g. Refs. 9, 14). One of the reviewers pointed out that this approach might only be valid under some circumstances. Although the excellent line fit obtained during the deconvolution of our traces seems to suggest that application of a reversible model bears some justification (Figs. 1D, 3, 4) we prefer to make reference to “heat of transition” ($\Delta H$) throughout the text.

3 Compare Tables 1–3 for standard deviations of transition temperatures.
protein was dissolved in 100 mM KCl and 20 mM glycine (T_kcal/mol and dotted lines the experimental data; The scan rate was 1 °C/min. The temperature dependence of the excess molar heat capacity of the protein is depicted at different pH values: A, 7.0; B, 6.0; C, 4.5; and D, 3.5. The protein was dissolved in 100 mM KCl and 20 mM tris-malate (C and D) or 100 mM KCl and 20 mM tris-malate (A and B). Solid lines correspond to the experimental data; dotted lines correspond to the best-fit analysis. The scan rate was 1 °C/min.

and B is depicted in Fig. 2. Although ΔH_A increases, ΔH_B decreases at the same rate. The observation of two well-distinguishable transitions could be due to different protein conformations or due to separate conformational unfolding steps of discrete domains within the same protein. To further discriminate between these possibilities, ColB was heated to 35 °C, slightly above the T_m of peak A at pH 3.5, and was kept at this temperature for 10 min. A DSC experiment with partially unfolded ColB was then performed at a heating rate of 1 °C/min between 25 and 85 °C (Fig. 3). A single transition with ΔH = 98 kcal/mol and T_m = 62.1 ± 0.1 °C (S.D., n = 3) was observed, which is 21 °C increased from T_m of the continuous scan. This result indicates that the transition of peak B is subject to interactions with components contributing to peak A.

To study the origin of the two transitions of ColB, a 203-amino acid C-terminal fragment of ColB that contains the pore-forming domain was also investigated. Fig. 4 shows the temperature dependence of the excess molar heat capacity of the pore-forming fragment at different pH values at a heating rate of 1 °C/min. Irreversible denaturation was observed after heating to 100 °C. The denaturation profile of the pore-forming fragment in solution shows one main transition corresponding to 90% of the total ΔH. The transition temperature for the main transition is 65.7 ± 1.5 °C at pH 7.0. A minor transition with T_m = 55.5 ± 1.5 °C is consistently present and corresponds to the remaining 10 ± 4% of the total heat of transition. The temperature of the pore-forming fragment is pH-independent between pH 3.5 and 9.0 (Fig. 4 and Table II). The pH-dependent changes in ΔH of the PFF and peak B of ColB exhibit comparable characteristics (Tables I and II). By changing the pH from 3.5 to 7.0 the relative decrements in ΔH are 63 and 59% for ColB peak B and the pore-forming fragment, respectively. A minor increase in heat of transition when the pH was lowered from 3 to 5 was observed for the related ColA PFF (Fig. 2 in Ref. 9). The effect of pH on ColB reveals more than one protonation state, suggesting the existence of several conformational states (Fig. 5A). In contrast, between pH 3.5 and 7.0 both transitions of the PFF occur in a pH-independent manner (Fig. 5B).

The denaturation profile of ColB in the presence of small unilamellar lipid vesicles is shown in Fig. 6A. At pH 7.0 ColB exhibits the same two transitions observed during its unfolding in aqueous solution in agreement with weak membrane-association of the protein at neutral pH. The T_m values of peaks A and B are 55.4 ± 0.4 °C and 57.9 ± 0.4 °C, respectively. At pH 3.5, however, peak A exhibits a T_m of 53.3 ± 0.9 °C and peak B is absent (Fig. 6B). In the presence of lipids the total ΔH of ColB at pH 7.0 is 172 kcal/mol. At pH 3.5 ΔH equals 140 kcal/mol in the presence of lipids, a value similar to the ΔH observed for peak A at pH 3.5 in aqueous buffer alone (Fig. 1D and Table I). However, in the presence of lipids this latter transition has shifted to higher temperatures by at least 20 °C (Fig. 6B).

Fig. 6C shows the DSC profile of the pore-forming fragment in the presence of lipids. The transition occurs over a wide temperature range and ΔH is significantly decreased (Table III). After rescan subtraction and baseline correction line-fitting analysis reveals a pattern of multiple transitions at pH 7.0 or pH 3.5 (Figs. 6, C and D). The calculated values of T_m and ΔH_total are listed in Table III.

CD Spectropolarimetry—Far UV-CD spectra of ColB and the pore-forming fragment in solution and in the presence of lipids at pH 7.0 display a maximum at 191 nm as well as minima at 208 and 222 nm (Figs. 7C, 7D, 8B, and 8C). The CD line shapes are, therefore, characteristic of a large proportion of α-helical secondary structures. When the mean residue ellipticity at 222 nm is taken as an indicator, helix contents of 39 and 82% are obtained for ColB and its PFF, respectively, in aqueous solution before starting the heating scan (Figs. 7C and 8B). In the presence of liposomes optical distortions by absorption flattening and light scattering would result in an underestimation of the helical content (18). These effects are minimized when small unilamellar vesicles prepared by extrusion through 50-nm filters are used (18). Nevertheless, no attempt was made to calculate the helix content quantitatively from the CD spectral line shape in the presence of lipids or after thermal denaturation.

The relative intensity changes of the ellipticity at 222 nm, however, were recorded as a function of temperature and compared with the DSC thermal transitions (Fig. 7, A and B). Fig. 7A presents the temperature dependence of the ColB ellipticity at 222 nm. At temperature scan rates of 1 °C/min (25–85 °C) a single transition is observed for ColB at pH 7.0 with T_m values

![Graph showing temperature dependence of ColB ellipticity](https://example.com/graph.png)
Calorimetric Stability of Colicin B and Its Pore Domain

TABLE I
Calorimetric data of ColB in aqueous solution at 1 °C/min

| pH | $T_m^A$ | $\Delta H_A$ | $T_m^B$ | $\Delta H_B$ | Total $\Delta H$ |
|----|---------|--------------|---------|--------------|-----------------|
| 7.0 | 54.9 ± 0.5 | 162 | 55.7 ± 0.8 | 28 | 190 |
| 6.5 | 55.5 ± NA$^a$ | 163 | 58.5 ± NA | 27 | 190 |
| 6.0 | 52.4 ± 0.4 | 157 | 57.3 ± 0.4 | 31 | 188 |
| 4.5 | 51.3 ± 0.3 | 145 | 57.0 ± 0.5 | 40 | 185 |
| 4.0 | 43.2 ± 0.5 | 132 | 52.5 ± 0.4 | 47 | 179 |
| 3.5 | 29.8 ± 2.0 | 121 | 40.9 ± 1.0 | 75 | 199 |

$^a$ NA, not available.

DISCUSSION

Structural and Functional Characteristics of Colicin B and Related Colicins—The high resolution X-ray structures of several channel-forming domains of colicins (A, N, E1, Ia) exhibit a 10-helix arrangement with 2 hydrophobic helices being surrounded by two layers of hydrophilic and amphipathic helices (19–22). These conformations, therefore, represent water-soluble forms of the proteins. The primary sequences of the C-terminal parts of colicins A and N exhibit ≥54% identity and 71 or 62% homology with ColB PFF, respectively. In addition, both the high resolution X-ray structure of ColA PFF (19) and the ColB PFF CD spectra (Fig. 8B) exhibit ≥75% $\alpha$-helix contributions. It is, therefore, reasonable to assume that the colicin A and B pore-forming domains adopt closely related structures.
In contrast, the length and amino acid composition of the pore-forming regions of colicins A, B, and N exhibit considerable differences when compared with those of colicins Ia, Ib, and E1 (e.g. Ref. 16). Therefore, these C-terminal fragments are assigned to different families.

However, when the receptor and translocation mechanisms of colicins are compared with each other, the molecules group in a different manner. Although group A colicins (including colicin A and ColN) use the TolA system for translocation, group B colicins (including ColB and ColIa) parasitize the Ton B machinery (6). Despite this functional similarity between ColB and ColIa, the primary sequence homology is modest and the helix content of ColB (Fig. 7B) is considerably lower than that of colicin Ia, in particular when considering the R/T domains alone (22).

Structural details, such as the location of the Ton recognition sequence in ColIa (22), or the distribution of hydrophilic and hydrophobic residues within the pore-forming domains of colicins indicate that upon receptor-binding, translocation and membrane-insertion conformational changes occur. For example, a multitude of investigations show that the helical conformation of ColA PFF is preserved during pH-dependent membrane insertion when at the same time refolding of the tertiary structure occurs (23).

**Thermal Transitions of Colicin B**—The thermal melting characteristics of colicin B and its PFF provide valuable information about the conformational stability and the interactions between the domains also when high resolution structures have not been obtained. The heats of transition of colicin B and its thermolytic fragments in solution are \( \pm 3.6 \) and \( \pm 1.3 \) cal/g, respectively, and, therefore, depending on the experimental conditions, are smaller or within the range previously observed for globular proteins in solution at comparable transition temperatures (11, 24).

The DSC traces of colicin B exhibit two well-separated transitions (Fig. 1) suggesting denaturation of two discrete cooperative units. The R/T domain is known to account for 61% of the total length of colicin B, which corresponds closely to the 68% value obtained for the heat of transition A when averaged between pH 7 and 4. Comparison with the DSC traces obtained from the isolated PFF provides further support for this assignment. Although the transition temperatures of the isolated PFF are considerably higher when compared with peak B of full-length ColB, this difference is largely annihilated after transition A has been uncoupled from transition B by incubation at intermediate temperatures (Fig. 3). In addition, the size and pH dependence of the heat of transition (\( \Delta H \)) of peak B of ColB parallel those of the main transition observed for the isolated pore-forming fragment (Tables I and II). The large reduction in heat of transition of both peak B and the ColB pore-forming fragment during membrane association of the corresponding proteins further demonstrate that transition B is closely associated with the pore-forming domain of full-length ColB (Fig. 6, B–D).

The CD spectra of colicin B and its pore-forming fragments indicate a high content of helical secondary structures of the C-terminal domain at room temperature (Figs. 7 and 8). This is in excellent agreement with previous structural findings for the colicin A pore-forming domain (19, 25) as well as the high degree of primary sequence homology between the pore-forming fragments of CoIA and ColB (16). The calorimetric, fluorescence, and circular dichroism studies presented in this paper demonstrate that the two reported transitions of ColB are due

---

**Table II**

| pH  | \( T_m \)A | \( \Delta H \)A | \( T_m \)B | \( \Delta H \)B |
|-----|------------|----------------|------------|----------------|
| 7.0 | 55.5 ± 1.5 | 2              | 65.7 ± 1.5 | 27             |
| 6.0 | 53.7 ± 0.8 | 8              | 64.3 ± 1.0 | 37             |
| 4.5 | 55.2 ± 0.2 | 8              | 65.0 ± 0.2 | 62             |
| 3.5 | 55.1 ± 1.3 | 5              | 65.1 ± 0.9 | 66             |

**Fig. 4.** Effect of pH on the DSC profile of the colicin pore-forming fragment. The temperature dependence of the excess molar heat capacity of the pore-forming fragment is depicted at different pH values: A, 7.0; B, 6.0; C, 4.5; and D, 3.5. The pore-forming fragment was dissolved in 100 mM KCl and 20 mM glycine (C and D) or 100 mM KCl and 20 mM tris-malate (A and B). The solid lines correspond to the experimental data; the dotted (individual components) and hatched lines (sum) correspond to the best theoretical fit.
to denaturation of two discrete domains of the protein. Far UV-CD spectra indicate that during this process the ColB-pore-forming fragment in solution loses most of its secondary structure (Fig. 8B). This is in contrast to the colicin A pore-forming domain, which at 90 °C and at pH 5 retains a considerably higher proportion of secondary structure in CD spectra (9).

Three out of eight tryptophans of colicin B are located within the pore-forming fragment of colicin B at positions analogous to those in colicin A. Structural comparison, therefore, strongly suggests that at ambient temperature the tryptophan side chains at positions 395, 439, and 449 are buried within the hydrophobic interior of the protein (19, 25). A continuous decrease in fluorescence intensity is observed upon heating, which partly persists when the sample is equilibrated back to room temperature (not shown). This indicates a pronounced change in environment. The remaining five Trp residues in ColB are located within the pore-forming fragment of colicin B at positions analogous to those in colicin A. Structural comparison, therefore, strongly suggests that at ambient temperature the tryptophan side chains at positions 395, 439, and 449 are buried within the hydrophobic interior of the protein (19, 25). A continuous decrease in fluorescence intensity is observed upon heating, which partly persist when the sample is equilibrated back to room temperature (not shown). This indicates a pronounced change in environment. The remaining five Trp residues in ColB are located within the pore-forming fragment of colicin B at positions analogous to those in colicin A. Structural comparison, therefore, strongly suggests that at ambient temperature the tryptophan side chains at positions 395, 439, and 449 are buried within the hydrophobic interior of the protein (19, 25).

**FIG. 5.** pH dependence of $T_m$ of colicin B and its pore-forming fragment. A, dependence of the $T_m$ of ColB on pH. The $T_m$ used in this analysis corresponds to the temperature at the peak of the transition. The solid symbol (●) illustrates the pH dependence of transition A; the open symbol (○) illustrates the pH dependence of transition B. B, dependence of the $T_m$ of ColB pore-forming fragment on pH. The solid symbol (●) corresponds to the pH dependence of transition A and (○) corresponds to the pH dependence of transition B.

**FIG. 6.** Temperature dependence of the excess molar heat capacity of ColB and the pore-forming fragment in the presence of small unilamellar lipid vesicles. ColB at (A) pH 7.0 and (B) 3.5; pore-forming fragment at pH 7.0 (C) and 3.5 (D). The solid line corresponds to the experimental data; the dotted line corresponds to the best-fit analysis. Note that panels A and B, and C and D, are shown at two different scales. The vesicles were prepared by extrusion as described under “Experimental Methods.”
increasing amount of solvent-exposed hydrophilic areas and/or augmentation of protonation reactions at low pH when compared with neutral conditions (11). The observed proton exchange during the unfolding of ColB below its pI, indeed, indicates that residues buried in the native structure are protonated during the ColB thermal transition (27). Similarly, pH-dependent changes in the thermodynamic parameters have been observed with other soluble proteins (1, 28, 29).

The decreased thermal stability of ColB at lowered pH correlates with the previously observed requirement of acidic conditions for optimal channel and molten globule formation of ColB (30), increased membrane association of ColA (31, 32), augmented membrane-insertion rate of ColA (33, 34), and pH-sensitive conformational changes of ColE1 in the presence of membranes (35). The unfolding profile of diphtheria toxin has been published previously (36). This protein shares similarities with membrane-active colicin B such as acidic pH-triggered membrane insertion or the secondary and tertiary structure of the membrane-inserting domain (37). Diphtheria toxin also exhibits two transitions, which have been assigned to the A and B fragment (at pH 8, \( T_m = 54.5 \) and 58.4 °C, respectively) (36). In a similar manner the transition temperatures and transition enthalpies exhibit a strong pH dependence, and the isolated A fragment exhibits a 6.5–10 °C reduction in \( T_m \) when compared with the full-length protein.

**Thermal Transitions of the Isolated Pore-Forming Domain of Colicin B**—Investigation of the isolated ColB PPF indicates that only the heat of transition is pH-dependent, whereas the transition temperature remains unaffected between pH 3.5 and 7. In a similar manner, between pH 5 and 7 the heat of transition of ColA PPF exhibits a small tendency to decrease with increasing pH, at transition temperatures similar to those of ColB PPF (9). At pH < 5, however, both parameters of ColA PPF strongly decrease with pH. The ColB PPF-melting characteristics, therefore, do not provide direct indications of conformational instabilities that might be important during membrane insertion. It should be kept in mind, however, that the slow time scale of the DSC experiment is not sufficiently sensitive to detect the increased membrane insertion kinetics at acidic pH of ColA PPF or ColB PPF (26, 38). Nevertheless, reduced numbers of negative charges at the surface of the protein might be important for low pH interactions with the membrane surface also of this pore-forming domain, in particular when these contain acidic phospholipids (39, 40). Localized electrostatic interactions might help to increase membrane association and to orient the protein with respect to the lipid bilayer. Such interactions thereby open directed avenues for molten globule formation and membrane insertion (26, 38, 41).

**Interactions between R/T and C Domains**—At low pH the transition of the isolated ColB PPF occurs at considerably higher temperatures than transition B of full-length ColB (Tables I and II). Preincubation at temperatures between the transition temperatures of peaks A and B, however, shifts \( T_m \) of peak B from 40 to 62 °C, thereby closely approaching the transition temperature of ColB PPF (Figs. 1D and 3). Similarly, interaction of the PPF with lipid bilayers at low pH results in an increase of \( T_m \) of peak A by >20 °C (Figs. 1D and 6B). These data indicate that interaction between domains results in a reduction of the stability of both cooperative units. The decrease in conformational entropy when two protein domains are tightly coupled to each other is expected to contribute to the destabilization of large protein domains, but more specific interactions between the proteins, such as van der Waals, hydrogen bonding, or electrostatic interactions, can also be important. In a related manner, functional differences in ColA PPF and colicin A have been attributed to interactions between the pore-forming and the receptor binding domains (32, 42).

Although the isolated C-terminal fragment by itself is competent to insert into membranes (16), and pore-forming domains of other colicins exhibit pore-forming activities in vitro (17, 43–45), all three domains are required for recognition, translocation, and pore formation at intact cells in vivo. It appears that mutual interactions between different regions of colicin B help to maintain the translocation and the pore-forming domains in less stable but functionally competent conformations. Intra- and intermolecular interactions thereby reduce the energies involved in conformational changes during protein translocation and insertion (cf. e.g. Ref. 22).

**Thermal Stability of Colicins in the Presence of Lipids**—At pH 3.5 the denaturation profile of ColB shows a decrease in total heat of transition, disappearance of transition B, and stabilization of transition A due to the presence of lipids. Similarly, the heat of transition of membrane-associated ColB PFF is decreased by two orders of magnitude. In addition, CD spectra indicate that a large fraction of its secondary structure is preserved at higher temperatures. These observations are similar to those reported for the pore-forming domains of ColA (9), ColE1 (10), as well as the pore-forming proteins equinatoxin II (46), staphyloccocal α-toxin (12), diphtheria toxin (36), or other transmembrane proteins that appear to be resistant to complete thermal denaturation (11). The hydrogen bonds of helices within hydrophobic environments are considerably stronger than in water (4), therefore, the helical structures of residues buried inside the lipid bilayer are often remarkably stable. In contrast, the least stable regions of membrane proteins are generally those that are extramembranous. As a consequence, these regions seem to unfold in a similar manner than soluble proteins (11).

Proton-decoupled \(^{15}\text{N}\) solid-state NMR spectra of oriented membrane samples indicate that the helices of the ColB and ColE1 C-terminal domains are predominantly orientated parallel to the bilayer surface (16). In addition, neutron scattering experiments show a location of large parts of the ColA PFF close to and beyond the membrane surface (47). We, therefore, suggest that the small but detectable decrease in \( α\)-helix content and the heat of transition observed by calorimetric measurements of the ColB pore-forming fragment in the presence of lipids (Figs. 6 and 7) is associated with unfolding of loop structures, hydrophilic ends of membrane-associated helices, and/or complete unfolding of those helices that are weakly associated with the membrane interface (Ref. 11 and references cited therein).

The residual change in heat of transition observed in denaturation profiles of lipid-associated C-terminal domains of ColB (this study), ColA (9), and ColE1 (10), therefore, strongly suggest that a fraction of the membrane-inserted protein is located

---

**Table III**

| pH | \( T_m^A \) | \( T_m^B \) | \( T_m^C \) | \( T_m^D \) | \( \Delta H_{tot} \) |
|----|-----------|-----------|-----------|-----------|-------------|
| 7.0 | 51.1 ± 0.2 | 54.9 ± 0.1 | 56.5 ± 0.2 | 58.5 ± 0.2 | 5 kcal/mol |
| 3.5 | 53.2 ± 0.3 | 55.0 ± 0.1 | 56.7 ± 0.0 | 58.8 ± 0.3 | 6 kcal/mol |

All enthalpies are in kcal/mol. The values for \( T_m \) are means ± S.D. (n = 3).
in extramembranous or interfacial regions. The reproducible fine structure encompassing at least four peaks in the DSC traces of ColB PFF is to our knowledge observed for the first time for membrane-associated proteins and could derive from, for example, independent helical structures at the membrane interface (Fig. 6, C and D).

In summary, this study provides thermal denaturation profiles of proteins in solution and in their membrane-associated
states. The same protein reveals pronounced differences in stability and thermodynamic properties when investigated in solution or in the membrane. The membrane-inserted parts of the proteins seem to a large extent resistant to thermal denaturation and exhibit an interesting fine structure in DSC experiments. In contrast, those regions exposed to the aqueous environment lose much of their native conformation. The domain structure of colicin B is also reflected in two well-separated thermal transitions in aqueous buffer. Nevertheless, at low pH the interaction between these domains results in their mutual destabilization and transition temperatures well below those of the domains when uncoupled from each other. Intramolecular interactions may, therefore, play an important role during conformational changes that are associated with translocation and insertion into the inner E. coli membrane.

Acknowledgments—We thank Dr. Michael Bärmann and Prof. Erich Sackmann of the Department of Physics, E22, Technische Universität München, Germany, for discussions and for allowing us to use the DSC instrument of their laboratory; Luis Moroder for providing access to CD and fluorescence spectrophotometers; Volkmar Braun for the colicin B strain; Mechthild Linnemann for technical help during protein purification; Leopold Ilag, Ulrike Harzer, Bas Vogt, Pieter Jaapserse, and Satish Hasigae for their help and discussions; Lissy Wahey for the helpful advise during UV-CD measurements; and Dr. Miriam Gitler of NOVA Research Company, United States, for the review of this manuscript. The comments by Jeremy Lakey were very valuable and helped improve the manuscript.

REFERENCES

1. Privalov, P. L. (1982) Adv. Protein. Chem. 35, 1–104
2. Murphy, K. P., Bhakuni, V., Xie, D., and Freire, E. (1992) J. Mol. Biol. 227, 293–306
3. Engelmann, D. M., Steitz, T. A., and Goldman, A. (1986) Ann. Rev. Biophys. Biophys. Chem. 15, 321–353
4. White, S. H., and Wimley, W. C. (1999) Annu. Rev. Biophys. Biomol. Struct. 28, 319–365
5. Gousaux, E. (1997) Structure 5, 313–317
6. Lazdunski, C., Bouveret, E., Rigal, A., Journet, L., Lloubes, R., and Benedetti, H. (1998) J. Bacteriol. 180, 4993–5002
7. Straw, R. M., Seiling, K., Wiener, M., and Freymann, D. (1998) Curr. Opin. Struct. Biol. 8, 525–533
8. Parker, M. W., Tucker, A. D., Tsernoglou, D., and Pattus, F. (1990) Trends Biochem. Sci. 15, 126–129
9. Muga, A., Gonzalez-Manas, J. M., Lakey, J. H., Pattus, F., and Surewicz, W. K. (1993) J. Biol. Chem. 268, 1553–1557
10. Zakharov, S. D., Lindeberg, M., Griko, Y., Salamon, Z., Tollin, G., Prendergast, F. G., and Cramer, W. A. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 4282–4287
11. Haltia, T., and Freire, E. (1995) Biochim. Biophys. Acta 1241, 295–322
12. Veesey-Senjen, B., Knapp, S., Molloy, R., and van der Goot, F. G. (1999) Biochemistry 38, 4296–4302
13. Muga, A., Mantsch, H. H., and Surewicz, W. K. (1991) Biochemistry 30, 7219–7224
14. Butko, P., Hunag, F., Pustai-Carey, M., and Surewicz, W. K. (1997) Biochemistry 36, 12862–12868
15. Pressler, U., Braun, V., Wittmann-Liebold, B., and Benz, R. (1986) J. Biol. Chem. 261, 2654–2659
16. Lambotte, S., Jasperse, P., and Bechinger, B. (1998) Biochemistry 37, 16–22
17. Baty, D., Knüchel, M., Verheij, H., Pattus, F., Shire, D., Bernadac, A., and Lazdunski, C. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 1152–1156
18. Wallace, B. A., and Moa, B. (1984) Anal. Biochem. 142, 317–328
19. Parker, M. W., Postma, J. P., Pattus, F., Tucker, A. D., and Tsernoglou, D. (1992) J. Mol. Biol. 224, 639–657
20. Elkins, P., Bunker, A., Cramer, W. A., and Stauffacher, C. V. (1997) Structure 5, 443–458
21. Vetter, I. R., Parker, M. W., Tucker, A. D., Lakey, J. H., Pattus, F., and Tsernoglou, D. (1998) Structure 6, 863–874
22. Wiener, M., Freymann, D., Ghosh, P., and Straw, R. M. (1997) Nature 385, 461–464
23. Lakey, J. H., and van der Goot, F. G., and Pattus, F. (1994) Toxiconology 87, 85–108
24. Privalov, P. L. (1980) in Biological Microcalorimetry (Breezer, E. A., ed) pp. 413–451, Academic Press, London
25. Lakey, J. H., Massotte, D., Heitz, F., Dasseux, J. L., Faucon, J. F., Parker, M. W., and Pattus, F. (1991) Eur. J. Biochem. 196, 699–707
26. van der Goot, F. G., Gonzalez-Manas, J. M., Lakey, J. H., and Pattus, F. (1991) Nature 354, 408–410
27. Privalov, P. L., Privalov, O. B., and Birshtein, T. M. (1969) Biopolymers 8, 559
28. Privalov, P. L. (1979) Adv. Protein Chem. 33, 525–533
29. Yu, Y., Makhatadze, G. I., Pace, C. N., and Privalov, P. L. (1994) Biochemistry 33, 3312–3319
30. Bullock, J. O., Armstrong, S. K., Shear, J. L., Lies, D. P., and McIntosh, M. A. (1990) J. Membr. Biol. 114, 79–95
31. Pattus, F., Martinez, M. C., Dargent, B., Cavard, D., Verger, R., and Lazdunski, C. (1983) Biochemistry 22, 5698–5703
32. Frenette, M., Knibiehler, M., Baty, D., Geli, V., Pattus, F., Verger, R., and Lazdunski, C. (1989) *Biochemistry* **28**, 2509–2514
33. Mel, S. F., and Stroud, R. M. (1993) *Biochemistry* **32**, 2082–2089
34. Kienker, P. K., Qiu, X., Slatin, S. L., Finkelstein, A., and Jakes, K. S. (1997) *J. Membr. Biol.* **157**, 27–37
35. Bullock, J. O. (1992) *J. Membr. Biol.* **125**, 255–271
36. Ramsay, G., Montgomery, D., Berger, D., and Freire, E. (1989) *Biochemistry* **28**, 529–533
37. Choe, S., Bennett, M. J., Fujii, G., Curmi, P. M., Kantardjieff, K. A., Collier, R. J., and Eisenberg, D. (1992) *Nature* **357**, 216–222
38. Evans, L. J., Goble, M. L., Hales, K. A., and Lakey, J. H. (1996) *Biochemistry* **35**, 13180–13185
39. Lakey, J. H., Parker, M. W., Gonzalez-Manas, J. M., Duché, D., Friend, G., Baty, D., and Pattus, F. (1994) *Eur. J. Biochem.* **220**, 155–163
40. van der Goot, F. G., Didat, N., Pattus, F., Dowhan, W., and Letellier, L. (1993) *Eur. J. Biochem.* **213**, 217–221
41. Merrill, A. R., Steer, B. A., Prentice, G. A., Weller, M. J., and Szabo, A. G. (1997) *Biochemistry* **36**, 6874–6884
42. Collarini, M., Amblard, G., Lazdunski, C., and Pattus, F. (1987) *Eur. Biophys. J.* **14**, 147–153
43. Martínez, M. C., Lazdunski, C., and Pattus, F. (1983) *EMBO J.* **2**, 1501–1507
44. Ghosh, P., Mel, S. F., and Stroud, R. M. (1993) *J. Membr. Biol.* **134**, 85–92
45. Liu, Q. R., Cruzel, V., Levinthal, F., Slatin, S., Finkelstein, A., and Levinthal, C. (1986) *Proteins* **I**, 218–229
46. Poklar, N., Fritz, J., Macek, P., Vesnaver, G., and Chalikian, T. V. (1999) *Biochemistry* **38**, 14999–15008
47. Jeanteur, D., Pattus, F., and Timmins, P. A. (1994) *J. Mol. Biol.* **235**, 898–907
48. Sanchez-Ruiz, J. M., Lopez-Lacomba, J. L., Cortijo, M., and Mateo, P. L. (1988) *Biochemistry* **27**, 1648–1652