pH-dependent Stability and Membrane Interaction of the Pore-forming Domain of Colicin A*

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Thermal stability of the pore-forming domain of colicin A was studied by high sensitivity differential scanning calorimetry and circular dichroism spectroscopy. In the pH range between 8 and 5, the thermal denaturation of the protein in solution occurs at 66–69 °C and is characterized by the calorimetric enthalpy of approximately 90 kcal/M. At pH below 5, there is a rapid pH-dependent destabilization of the pore-forming domain resulting in the lowering of the midpoint denaturation temperature and a decrease in the calorimetric enthalpy of denaturation. Circular dichroism spectra in the near and far ultraviolet show that the thermotropic transition is associated with collapse of the native tertiary structure of the pore-forming domain, although a large proportion of the helical secondary structure remains preserved. The present data indicate some similarity also between acid-induced and temperature-induced denaturation of the pore-forming domain of colicin A. Association of the pore-forming domain with phospholipid vesicles of dioleoylphosphatidylglycerol results in total disappearance of the calorimetric transition, even at pH values as high as 7. Since lipid binding also induces collapse of the near ultraviolet circular dichroism spectrum, these data indicate that interaction with the membrane facilitates a conformational change within the pore-forming domain to a looser (denatured-like) state. These findings are discussed in relation to the recent model (van der Goot, F. G., Gonzalez-Manas, J. M., Lakey, J. H., Pattus, F. (1991) Nature 354, 408–410) which postulates that a flexible "molten globule" state is an intermediate on the pathway to membrane insertion of colicin A.

Colicin A is a 592-residue proteinaceous toxin produced by and active against sensitive *Escherichia coli* cells. The mode of action of colicin involves three major steps (Pattus *et al.*, 1990): (i) binding to the specific receptor on the outer membrane; (ii) translocation across the outer membrane; and (iii) insertion into the cytoplasmic membrane and formation of hydrophilic transmembrane channels. Efflux of ions through these channels is believed to be responsible for depolarization and deenergization of affected cells (Konisky, 1982; Lazdunski *et al.*, 1988).

The pore-forming activity of colicin A is associated with the 20-kDa C-terminal domain that can be isolated and purified after thermolysin digestion of the protein (Martinez *et al.*, 1983). This polypeptide provides an interesting example of a protein that, depending on environmental conditions, can exist in either a water-soluble or a membrane-associated state. X-ray crystallography reveals that the pore-forming domain of colicin A consists of a bundle of 10 α-helices which are arranged in three layers containing a hydrophobic helical hairpin buried within the protein (Parker *et al.*, 1989, 1992). Based on this structure, a model for colicin insertion into the lipid bilayer has been proposed recently (Parker *et al.*, 1989, 1990). This model postulates that upon initial electrostatic binding to negatively charged lipids, insertion into the bilayer is triggered by opening of the compact protein molecule, allowing exposure and hydrophobic penetration of the originally buried helical hairpin.

The notion of a transition to a "insertion-competent" conformation as a prerequisite for membrane insertion of the pore-forming domain of colicin A has been supported by studies with model lipid membranes (Lakey *et al.*, 1991a, 1991b; Gonzalez-Manas *et al.*, 1992). As with some other bacterial toxins (Olsnes *et al.*, 1988; Zhao and London, 1988; Jiang and London, 1990; Farahbakhsh and Wisnieski, 1989; Menestrina *et al.*, 1989; Merrill *et al.*, 1990), the triggering event that promotes penetration of colicin A into the membrane *in vitro* is exposure to low pH (Pattus *et al.*, 1983; van der Goot *et al.*, 1991). Based on CD data, the low pH folding intermediate of the pore-forming domain of colicin A has been characterized recently as a "molten globule" state (van der Goot *et al.*, 1991). However, a detailed description of this intermediate is still lacking. In this study, we have used differential scanning calorimetry and CD spectroscopy to characterize further the pH-dependent structural stability of the pore-forming domain in solution and in a membrane-bound form.

MATERIALS AND METHODS

Sample Preparation—Colicin A and its thermolytic fragment were obtained as described previously (Cavard and Lazdunski, 1979; Tucker *et al.*, 1986). The thermolytic fragment was purified by Sephadex G-50 chromatography, dialyzed against water, and lyophilized. The lyophilized protein was redissolved in 30 mM Na_2HPO_4, and the pH of the solution was adjusted to the desired value with citric acid. Before use, samples were centrifuged at 14,000 × g for 10 min to remove undissolved protein aggregates. Concentration of the protein was determined spectrophotometrically as described previously (Massotte *et al.*, 1989).

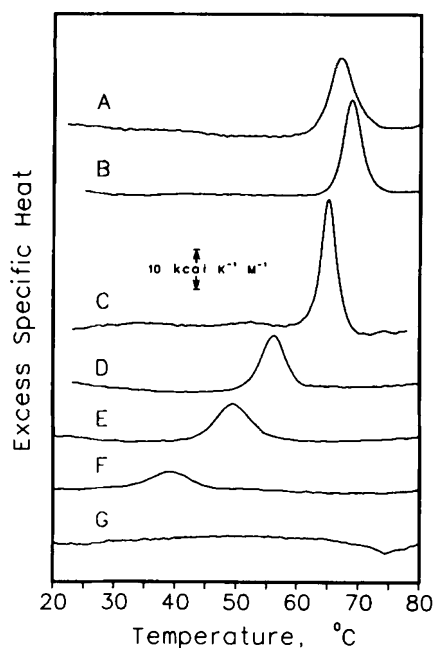To prepare samples of membrane-associated colicin A, unilamellar

FIG. 1. **Differential scanning calorimetry thermograms for the pore-forming domain of colicin A in solution at pH 8.0 (A), 7.0 (B), 5.0 (C), 4.0 (D), 3.5 (E), 3.0 (F), and 2.5 (G).**

phospholipid vesicles were used. For the latter, approximately 40 mg of DOPG[1] (Avanti Polar Lipids, Birmingham, AL) was dissolved in chloroform, the solvent was evaporated under nitrogen, and the lipid film was dispersed in 1 ml of 30 mM phosphate/citric acid buffer, pH 5.0. This was followed by sonication to optical clarity (10 min, 4 °C) with a probe-type Branson sonifier. Residual multilamellar liposomes and titanium particles released from the probe were removed by centrifugation at 14,000 × *g* for 20 min. Unilamellar vesicles were then mixed with the thermolytic protein fragment solution in the same buffer (lipid to protein molar ratio of approximately 500:1), and samples were incubated for at least 1 h before the measurements.

Differential Scanning Calorimetry—The thermal stability of the thermolytic fragment of colicin A at different pH values was studied by high sensitivity differential scanning calorimetry using a Microcal MC-2D instrument at a scanning rate of approximately 50 °C/h. Calorimetric data were filtered, converted to heat capacity *versus* temperature functions, and analyzed with software provided by Microcal, Inc. Base lines were subtracted by using a cubic splines interpolation procedure.

CD Spectroscopy—CD spectra were obtained on a JASCO J-600 spectropolarimeter equipped with a variable temperature accessory. The instrument was calibrated with ammonium-*d*-camphorsulfonate. Spectra in the near UV region were measured in a 1-cm quartz cylindrical cuvette at a protein concentration of 0.7 mg/ml. Those in the far UV region were acquired using protein concentration of 0.4 mg/ml and a 0.02-cm path length cell.

RESULTS

Differential Scanning Calorimetry—Thermal stability of the thermolytic fragment of colicin A was studied in the pH range between 8 and 2.5. Under these conditions, the calorimetric denaturation was found to be irreversible. After rapid cooling from the first run, no transition could be detected in the second heating cycle. This apparent irreversibility, which may arise from post-transitional aggregation of the pore-forming domain, complicates thermodynamic analysis of the calorimetric data (see *e.g.* Sanchez-Ruiz *et al.*, 1988). Nevertheless, two parameters can be derived in a straightforward manner from the symmetric excess heat capacity *versus* temperature curves of the protein (Fig. 1): the midpoint transition temper-

[1] The abbreviations used are: DOPG, dioleoylphosphatidylglycerol; θ_{222}, ellipticity at 222 nm.

ature, T_m, and the calorimetric enthalpy of denaturation, ΔH_{cal}, which is proportional to the area under the transition curve. These parameters provide a measure of relative protein stability under conditions of different pH.

As shown in Fig. 1, the thermal stability of the pore-forming domain of colicin A is strongly pH-dependent. In the pH range between 8 and 5, the thermotropic transition occurs at 66–69 °C and is characterized by the calorimetric enthalpy of approximately 90 kcal/M. Further decrease in pH results in a rapid destabilization of the protein. This is indicated by an abrupt drop (between pH 5 and 3) of both the temperature and enthalpy of denaturation. No calorimetric transition could be detected at pH 2.5 and below. The T_m or ΔH_{cal} *versus* pH plots (Fig. 2) indicate midpoint of acid-induced protein destabilization at pH 3.5–3.7.

The differential scanning calorimetry data of Fig. 1 can be used to construct pH stability isotherms (Ramsay *et al.*, 1989). To this end, the fractional degree of denaturation, P_d, at a given temperature t is calculated as $P_d = \Delta H_t/\Delta H_{total}$, where ΔH_t is the fractional enthalpy of denaturation up to temperature t and ΔH_{total} represents the total enthalpy of denaturation. The P_d values are then plotted as a function of pH. The pH stability curves for the thermolytic fragment of colicin A at a few selected temperatures are shown in Fig. 3.

The pore-forming domain of colicin A is known to interact with and insert into membrane vesicles containing acidic phospholipids (Pattus *et al.*, 1990 and references within). Fig. 4 compares differential scanning calorimetry traces obtained at pH 5 for the thermolytic fragment of colicin A in solution (*upper trace*) and for the protein associated with DOPG vesicles (*lower trace*). The striking difference between these traces is the complete disappearance of the calorimetric transition for the lipid-associated protein. Repeated experiments have been performed with different preparations of the thermolytic fragment-DOPG complexes at pH 5; for each preparation no calorimetric transition could be detected up to 100 °C. Similarly, no calorimetric transition was observed for the membrane-associated protein at pH 7 and 4 (data not shown).

CD Spectroscopy—To gain insight into the nature of the structural/conformational changes that give rise to calorimetric transitions, CD spectra of the thermolytic fragment of colicin A were studied as a function of temperature. In accord
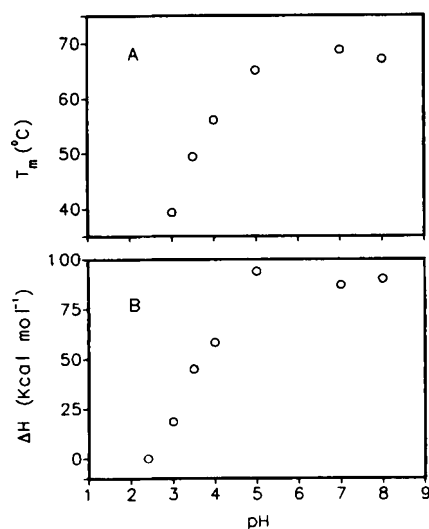

FIG. 2. **pH dependence of the temperature (A) and calorimetric enthalpy (B) of thermal denaturation for the pore-forming domain of colicin A.**
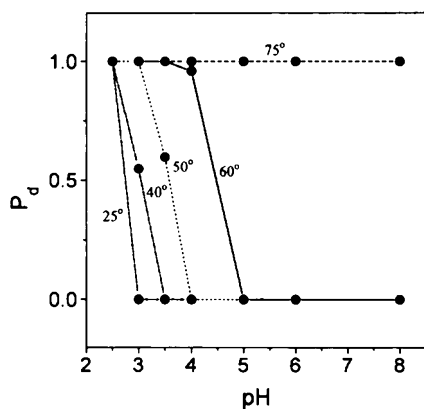
FIG. 3. pH/temperature stability diagram for the pore-forming domain of colicin A. The *curves* represent the fractional degree of denaturation as a function of pH at the indicated temperatures (see "Results"). Because of the aggregation of the denaturated species (and thus the apparent lack of reversibility), the diagram should be read in the denaturation direction only (Ramsay *et al.*, 1989).
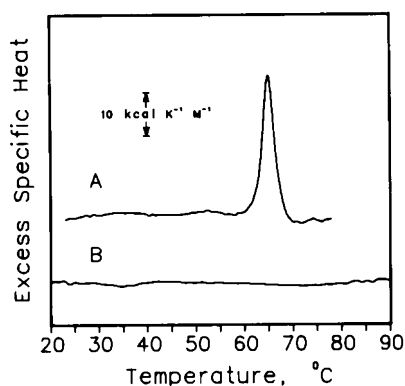

FIG. 4. Differential scanning calorimetry thermograms for the pore-forming domain of colicin A at pH 5.0 in solution (*A*) and after association with the vesicles of DOPG (*B*).
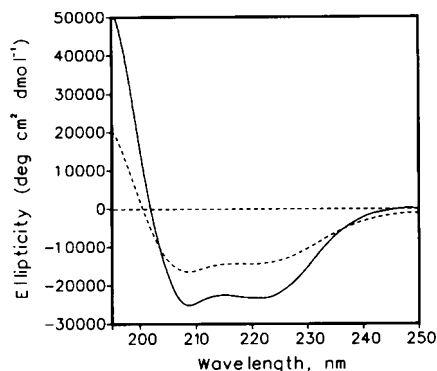

FIG. 5. Far ultraviolet CD spectrum for the pore-forming domain of colicin A in solution (pH 5.0) at 25 °C (*solid line*) and at 90 °C (*dashed line*).

with previous results (Lakey *et al.*, 1991a), the far ultraviolet CD spectrum of the protein in solution at room temperature (Fig. 5, solid line) displays strong minima at 208 and 222 nm. This spectrum is characteristic of a highly α-helical structure. Temperature-induced changes in helicity can be followed by monitoring ellipticity at 222 nm. The melting curve of the thermolytic fragment at pH 5 (Fig. 6) indicates thermotropic transition at approximately 67 °C, *i.e.* at a temperature very close to that determined by differential scanning calorimetry.
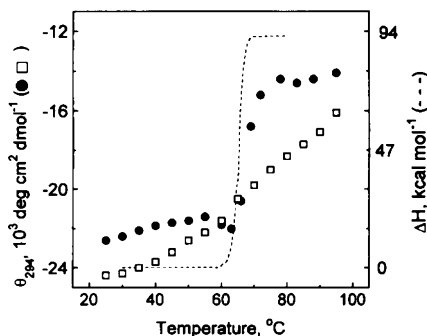

FIG. 6. Temperature dependence of the ellipticity at 222 nm for the pore-forming domain of colicin A at pH 5.0 in solution (●) and after association with the vesicles of DOPG (□). For comparison of the differential scanning calorimetry and CD data, calorimetric transition for the protein in solution at pH 5.0 is replotted as a *dashed curve*. This *curve* was obtained by integration of the excess specific heat *versus* temperature function of Fig. 1*C*.
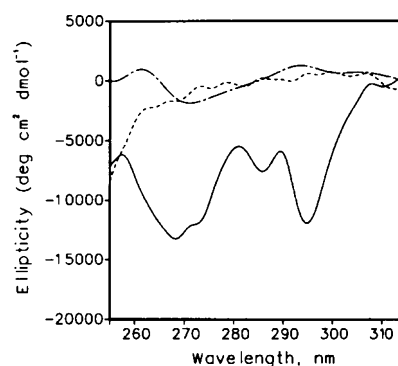

FIG. 7. Near ultraviolet CD spectrum for the pore-forming domain of colicin A in solution (pH 5.0) at 25 °C (———) and at 90 °C (— — —). Trace (— · —) represents 25 °C spectrum of the protein associated with vesicles of DOPG (pH 5.0).
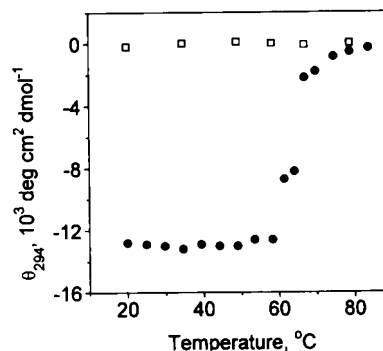

FIG. 8. Temperature dependence of the ellipticity at 294 nm for the pore-forming domain of colicin A at pH 5.0 in solution (●) and after association with the vesicles of dioleoyl-phosphatidylglycerol (□).

However, a notable result of the CD experiments is the observation that the drop in θ_{222} associated with this transition is very moderate: between 60 and 75 °C the ellipticity decreases by only 30%. Furthermore, even at 90 °C the CD spectrum (Fig. 5, *dashed line*) shows features characteristic of α-helical proteins. The estimated helical content of the pore-forming fragment of colicin A at temperatures above the transition region is as high as 50–60%.

The θ_{222} *versus* temperature plot for the membrane-associated thermolytic fragment of colicin A shows a gradual decrease in ellipticity over the entire temperature range between approximately 30 and 90 °C (Fig. 6). However, in contrast to

colicin in solution, there is no indication of any cooperative transition for the membrane-associated protein. This is consistent with the absence of calorimetric transition for the membrane-bound protein (Fig. 4).

The CD spectrum in the near ultraviolet region provides information about the conformation and orientation of aromatic residues and has been used as a sensitive probe of the tertiary structure of colicin A pore-forming fragment (Lakey *et al.*, 1991a; van der Goot *et al.*, 1991). The room temperature spectrum in solution at pH 5 (Fig. 7) is well structured and displays pronounced minima at 294 and 268 nm. This spectrum is characteristic of a native conformation of the protein with a fixed orientation of aromatic residues. According to recent crystallographic data, these residues form a well defined cluster made up of Trp-86 which stacks against Tyr-161 and Trp-130 which packs perpendicular to Tyr-125 (Parker *et al.*, 1992). The spectrum remains virtually unchanged up to approximately 60 °C. However, further increase in temperature results in an abrupt collapse of the near UV CD spectrum (Fig. 7), which indicates the loss of the native tertiary structure. The midpoint transition temperature determined from the ellipticity at 294 nm *versus* temperature plot (Fig. 8) is approximately 65 °C.

The room temperature CD spectrum in the near UV region of the thermolytic fragment associated with DOPG vesicles lacks the fine structure observed in the spectrum of the protein in solution (Fig. 7). The general shape of this spectrum is very similar to that of a "collapsed" solution spectrum at post-transition temperatures or at acidic pH (van der Goot *et al.*, 1991). Similar collapse of the near UV CD spectrum has been found upon association of the protein with dimyristoyl-phosphatidylglycerol (Lakey *et al.*, 1991a). The spectrum of the DOPG-associated pore-forming fragment of colicin A remains essentially unchanged upon increase in temperature up to 90 °C (Fig. 8).

DISCUSSION

The results of this study clearly demonstrate that exposure to acidic environment (pH below 5) results in a pronounced destabilization of the pore-forming domain of colicin A. This destabilization is indicated by a pH-dependent lowering of the temperature of the thermotropic transition (denaturation) and the accompanying reduction in the enthalpy of this transition. Approximately 80% of the enthalpy of thermal denaturation is lost upon lowering the pH from 5 to 3. Finally, at pH 2.5 and below, the thermodynamic state of the protein at all temperatures appears to be indistinguishable from that of the denatured species, as indicated by the total disappearance of the calorimetric transition.

Calorimetric data extend recent spectroscopic observations on pH-dependent conformational changes in the pore-forming domain of colicin A (van der Goot *et al.*, 1991). From the results of CD and fluorescence measurements, it has been postulated that at acidic pH the pore-forming domain undergoes transition to a molten globule state. In the latter state the protein is still relatively compact and retains a large proportion of its native secondary structure. However, the tertiary structure of the molten globule protein is considerably loosened (Dolgikh *et al.*, 1981). This results in the loss of the characteristic asymmetric environment of the aromatic residues and leads to the collapse of the near ultraviolet CD spectrum. Previous CD measurements have indicated that at room temperature the pK of the native state to molten globule transition is at pH 2.7. This is fully consistent with the calorimetric data. Our pH/stability diagram (Fig. 3) shows

that at 25 °C acid-induced denaturation occurs in the pH range between 3 and 2.5.

The present data point also to some similarity between acid-induced and temperature-induced denaturation of the pore-forming domain of colicin A. In both instances, drastic changes in the near ultraviolet CD spectrum are accompanied by only a modest drop in ellipticity at 222 nm. The calorimetric enthalpy of thermal denaturation appears thus to represent, at least to a large extend, the collapse of native tertiary structure of the pore-forming domain. Such an interpretation of the calorimetric transition is further supported by experiments with the membrane-associated protein. Upon membrane binding the pore-forming domain shows no detectable calorimetric transition. CD spectroscopic studies indicate that, even at room temperature, the membrane-inserted protein fragment lacks a well defined tertiary structure but retains the helical secondary structure.

Colicin A belongs to an intriguing group of proteins that, depending on pH and/or other environmental conditions, can exist either in water-soluble or membrane-embedded forms. The feature of colicin A, which renders it uniquely well suited for studying structural and mechanistic aspects of the membrane insertion process, is the availability of high resolution crystallographic data for the pore-forming domain (Parker *et al.*, 1989). This domain comprises a bundle of 10 α-helices. Two of these helices form a highly hydrophobic helical hairpin which, in the water-soluble structure, remains buried in the interior of the protein molecule. A model for the membrane insertion of colicin A (Parker *et al.*, 1989, 1990) postulates that the initial electrostatic binding to negatively charged groups of acidic phospholipids places the protein on the membrane surface, with the hydrophobic hairpin oriented perpendicularly to the membrane plane. In the next phase, the hairpin is believed to penetrate spontaneously into the membrane, during which process a conformational change occurs within the protein molecule leading to its opening in an umbrella-like fashion. The rate of membrane penetration by colicin A is strongly pH-dependent (Pattus *et al.*, 1983; van der Goot *et al.*, 1991; Gonzalez-Manas, 1992). It is very slow at neutral pH and accelerates rapidly under acidic conditions. The present calorimetric data allow us to rationalize, in energetic terms, the observed pH dependence of the membrane insertion of colicin A. Destabilization of the pore-forming domain at acidic pH is likely to facilitate transition to a looser and more flexible structure. In this less stable structure, the energy barrier required to expose the originally buried helical hairpin becomes significantly reduced. The exposure (even transient) of some nonpolar residues could trigger the onset of hydrophobic interactions between protein and the lipid bilayer. This would facilitate further unmasking of hydrophobic helices, making membrane insertion a thermodynamically favorable process. A similar general mechanism is likely to be operational in the pH-dependent membrane insertion of colicin E1 (Merrill *et al.*, 1990), diphtheria toxin (Zhao and London, 1988; Ramsay *et al.*, 1989) and, possibly, some other bacterial toxins (Jiang and London, 1990; Menestrina *et al.*, 1989).

The results of this study support and expand the recent hypothesis that the flexible molten globule state is an intermediate on the pathway to membrane insertion of colicin A (van der Goot *et al.*, 1991). This hypothesis was based on experimental data indicating that the variation of the rate of membrane insertion of the pore-forming domain as a function of an *interfacial* pH correlates with the formation of acid-induced molten globule state of the protein. However, as pointed out by Pain (1991), the question remains as to

whether and to what extent the correlations established using pH at the membrane surface remain valid for the protein protruding by as much as 3 nm from the membrane surface and being thus partly exposed to the bulk pH which is approximately 1.6 units higher. In this context, calorimetric data are essential as they demonstrate that substantial destabilization of the pore-forming domain (indicated by drop in T_m and ΔH_{cal}) can occur at a pH about 1 unit higher than that required to produce spectroscopically detectable transition to a molten globule state (see Fig 2 in this paper and Fig. 3 in the report of van der Goot *et al.* (1991)). Further loosening of this partly acid-destabilized (but still native-like structure) may be facilitated by factors other than local acidity. We postulate that formation of a flexible insertion-competent folding intermediate of the pore-forming domain is assisted (in addition to low pH) by the initial electrostatic interaction of the protein with the membrane surface. This is supported by calorimetric and CD experiments which show that, even under moderately acidic or neutral conditions (*i.e.* at a pH considerably higher than that required to induce the molten globule transition in bulk solution), the lipid-bound pore-forming domain has loose (molten globule-like) tertiary structure. Phospholipid-induced conformational destabilization has been recently demonstrated also for cytochrome *c* (Muga *et al.*, 1991) and for an artificial mitochondrial precursor protein consisting of the cytochrome oxidase subunit IV presequence fused to mouse dihydrofolate reductase (Endo *et al.*, 1989; Endo and Oya, 1989). Furthermore, a similar effect has been postulated for human complement protein C9 (Lohner and Esser, 1991).

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