Different Sensitivities to Acid Denaturation within a Family of Proteins: Implications for Acid Unfolding and Membrane Translocation†

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ABSTRACT: Colicins A, B, and N form a family of membrane pore-forming toxins with >50% sequence identity in their toxic C-terminal domains. The colicin A C-terminal domain has been shown to insert into model membranes via an acidic molten-globule insertion intermediate, and thus this family provides a means to compare acid unfolding of related proteins. Unlike the domains of colicins A and B which are acidic, that of colicin N is very basic with fewer Asp and Glu residues. If surface positive charge density is the crucial factor in acidic molten globule formation, colicin N should begin to unfold at higher pH values than colicins A or B. However, comparison of their CD spectra reveals that colicins A and B both form acidic molten globules but colicin N does not. None of the proteins forms a denaturant-induced molten globule at neutral pH where the proteins exhibit very similar stabilities. The acidic unfolding cannot therefore be due to excess positive surface charge and may be caused by a subset of acidic residues as has been predicted for myoglobin. The difference between the colicins is confirmed by their *in vivo* membrane insertion, with colicins A and B inserting much faster than colicin N. Stopped-flow circular dichroism measurements of colicin A insertion into vesicles confirmed that a molten globule insertion intermediate occurs at the membrane surface.

A number of membrane-associated proteins display acid-sensitive structural changes *in vivo.* In one commonly cited example, diphtheria toxin B subunit forms an acid-denatured state (A state) in the acidic late endosome which allows it to translocate across the membrane into the cytoplasm (Ramsay *et al.,* 1989). In this context it has been shown recently that the pH-dependent formation of vesicles destined for late endosomes relies on a transmembrane pH sensor (Aniento *et al.,* 1996) while the *Escherichia coli* protein secretion chaperone *SecA* has been shown to penetrate lipid bilayers by partial unfolding in the presence of acidic phospholipids (Ulbrandt *et al.,* 1992). Colicin A, a pore-forming toxin active against enterobacterial cells, inserts into membranes in a pH-dependent manner. This was shown to be due to the formation of an acidic molten globule intermediate (González-Mañas *et al.,* 1992, 1993; van der Goot *et al.,* 1991). There are nine known members of this pore-forming colicin family, and the strongest homologies exist in the COOH-terminal pore forming domains (ThCol).[1] ThColA, -B, and -N have sequence identity of more than 50% (Parker *et al.,* 1992), but their calculated p*I*'s (Devereux *et al.,* 1984; Lakey *et al.,* 1994) are 5.82, 5.48, and 10.25, respectively. ThColE1, which has a high p*I* value like colicins N, Ia, Ib, and 28b, has been shown to form a more limited A state than colicin A (Schendel & Cramer, 1994). ThCol domains can thus be divided into the acidic (A and B) and basic (N, Ia, Ib, 28b, E1, K, 10, 5) subtypes.

The suggested reasons why proteins denature at extreme pH include the effects of buried ionizable groups (His, Tyr), salt bridges, and surface charge density (Alonso *et al.,* 1991; Fink *et al.,* 1994; Yang & Honig, 1994). In addition, subtle effects may occur which result in the formation of acidic unfolded states which lose tertiary structure but maintain secondary structure, the so called A states (Fink *et al.,* 1994) or acidic molten globules (Ptitsyn *et al.,* 1990). Fink *et al.* (1994) have classified 20 proteins into three classes according to their acid unfolding characteristics. Upon acidification, class I proteins unfold directly from the native (N) to a completely unfolded (U) state (no secondary or tertiary structure). Further addition of anions can refold these random coils to the A state (native secondary structure and negligible tertiary structure). Class II proteins unfold directly to the A state while class III proteins show little unfolding at low pH and are said to form an N′ state which is largely similar to the native N form. The authors conclude that the different behavior of proteins under acidic conditions is probably the result of subtle changes in the boundaries of the phase diagram for each protein. Each protein's unique structure thus determines its own characteristic response to low pH.

The smeared charge model of protein denaturation at low pH predicts that excess of positive charge at the surface of a protein eventually provides enough repulsion to unfold the native state. In the absence of other effects, this would predict that the basic ThColN would unfold to the A state at a higher pH than that of either the acidic ThColA or ThColB. This paper compares the acid sensitivity of all three proteins.

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[1] Abbreviations: ThColA = thermolytic fragment of colicin A; ThColB = thermolytic fragment of colicin B; ThColN = thermolytic fragment of colicin N; DOPG = dioleoylphosphatidylcholine.

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MATERIALS AND METHODS

Protein Purification. Preparation of thermolytic COOH-terminal fragments for colicins A and B was carried out as described previously (Cavard & Lazdunski, 1979; Tucker *et al.,* 1986). ThColN was prepared using a modification of that described by Massotte and Pattus (1989). The protease inhibitors benzamidine and PMSF (1 mM) replaced procaine while a single ion exchange purification using S-Sepharose (Pharmacia) and a 0.3−0.6 mM NaCl gradient yielded pure colicin N as judged by SDS−PAGE. The C-terminal peptides were purified by filtration on a Sephadex G50 column. The thermolytic fragment concentration was determined from the absorbance at 280 nm using calculated molar absorption coefficients for ThColA ($\epsilon_{280} = 2.1 \times 10^4$ M^{-1} cm^{-1}), ThColB, and ThColN (2.35×10^4 M^{-1} cm^{-1}). Acetylation of ThColN lysine residues using acetic anhydride was carried out as previously described (Azzi *et al.,* 1975).

Synthesis of Brominated Phospholipids and Vesicle Formation. The brominated phospholipid was synthesized as already reported (East & Lee, 1982). DOPG (100 mg in chloroform) at −20 °C and 40 μL of bromine were added, and the reaction was allowed to proceed for 30 min. Excess bromine was eliminated by placing the reaction mixture in a silicic acid column pre-equilibrated with chloroform and eluting with 250 mL of chloroform and 250 mL of 10% (v/v) methanol in chloroform. Subsequent elution with 250 mL of 50% methanol in chloroform released the brominated phospholipid. Both brominated and native DOPG vesicles were produced by ultrasonication followed by centrifugation to remove large aggregates (González-Mañas *et al.,* 1993). Lipid concentrations were determined as previously described (González-Mañas *et al.,* 1992). Dioleoylphosphatidylglycerol was from Sigma. Tris(hydroxymethyl)aminomethane (Tris), reagent grade, was obtained from Sigma. Bromine and analytical grade solvents (chloroform, ethanol, and acetic acid) were from Merck. The silica gel used was Kieselgel 60 (Fluka) with a particle size range of 0.04−0.063 mm (230-400 mesh ASTM).

Fluorescence Measurements. Fluorescence kinetics were recorded at 35 °C using an SLM 8100 spectrofluorometer operating in the ratio mode with spectral bandwidths of 8 nm for both excitation and emission. Excitation wavelength was set to 280 nm. The scattering contribution of the vesicles to the spectrum was minimized by exciting the sample with vertically polarized light, and measuring horizontally polarized emitted light. The inner filter effect was minimized by using 0.5 cm path length cuvettes. The optical density at the excitation wavelength of the protein samples never exceeded 0.03. The Raman scatter contribution was eliminated by subtraction of appropriate blanks.

Insertion of the Pore-Forming Domain into Vesicles. The quenching of the intrinsic fluorescence of ThColA can be followed kinetically (González-Mañas *et al.,* 1992). Addition of Br-DOPG vesicles to a protein suspension results in an exponential decrease of the tryptophan fluorescence emission that follows first order kinetics. At time $t = 0$, all the protein is free and the fluorescence we observe is F_0, whereas at time $= \infty$ all the protein is inserted and $F = F_\infty$. At any time, the observed fluorescence is proportional to the fraction of noninserted colicin.

$$F(t) = F_\infty + (F_0 - F_\infty)e^{-kt}$$

Thus, if we plot $\ln((F(t) - F_\infty)/(F_0 - F_\infty))$ versus time, we obtain a straight line whose slope is $-k$. This rate constant (k_{ins}) has been shown to be a highly quantitative measure of the insertion rate of the COOH-terminal fragment into vesicles (González-Mañas *et al.,* 1992; van der Goot *et al.,* 1991). Each labeled mutant was placed in a cuvette at a concentration of 20 μg/mL in pH 5.0 Tris−acetate (50 mM) buffer. No salt was added to counteract the insolubility of colicin N since the working concentrations were low. Brominated DOPG vesicles were added at $t = 0$ to give a lipid to protein ratio of 450. The decrease in tryptophan fluorescence was measured as a function of time and analyzed.

Circular Dichroism Measurements. Circular dichroism spectra and the temperature dependence of CD were measured on a Jobin Yvon CD6 spectrometer attached to a Haake water bath. Near-UV spectra (250−320 nm) were measured using a Hellma 1 cm path length cuvette, and far-UV spectra (190−250 nm) were measured using a Hellma 0.02 cm water jacketed cuvette. Temperatures were measured with a Whatman electronic thermometer and a thermocouple attached to (far-UV) or inserted into (near-UV) the cuvette. pH effects were determined in 30 mM phosphate−citrate/300 mM NaCl buffer. Urea denaturation was performed using a 10 M urea/30 mM MOPS stock solution (Pace *et al.,* 1989) diluted with 30 mM MOPS and protein in 30 mM MOPS. Urea concentrations were measured by a refractometer. Protein concentrations were 330 μg/mL.

Stopped-Flow Circular Dichroism Measurements. An Applied PhotoPhysics (Leatherhead, U.K.) SX.17MV stopped-flow spectrometer was used, equipped with the CD measurement accessory. CD was measured at 267 nm (Figure 6). Syringes loaded with protein and vesicle solutions were mixed in a 1:1 ratio. The total reaction volume was 200 μL. The transient signals were recorded and analyzed on a Acorn A5000 computer. The time constant of CD change was fitted using a single exponential floating end point function.

RESULTS

Our criterion (van der Goot *et al.,* 1991, 1992) for the formation of the molten globule state is the complete disappearance of the tertiary-structure dependent near-UV CD spectrum when the far-UV CD spectrum is still indicating a near-normal amount of secondary structure. Although the primary sequences are similar, and all the colicins have three tryptophans in identical positions, ThColB and ThColN have two more tyrosines (in different positions) than ThColA. Presumably because of these differences, the near-UV CD spectra differ significantly (Figures 1, 2, and 7) and thus the wavelengths at which the near-UV CD changes are monitored differ for each protein. Since ThColN is insoluble at low salt concentrations, we first confirmed that the acid unfolding of ThColA (van der Goot *et al.,* 1991) was the same in 300 mM NaCl (results not shown). Under these conditions, the acid unfolding of ThColB was very similar to that of ThColA since the near-UV signal is lost between pH 2 and 3 in solution (Figure 1) with a 15% loss of secondary structure (results not shown). This is not the case for ThColN which loses little near-UV CD even at pH 2 (Figure 2(b)). Muga *et al.* (1993) have shown that temperature can increase the amount of ThColA in the molten

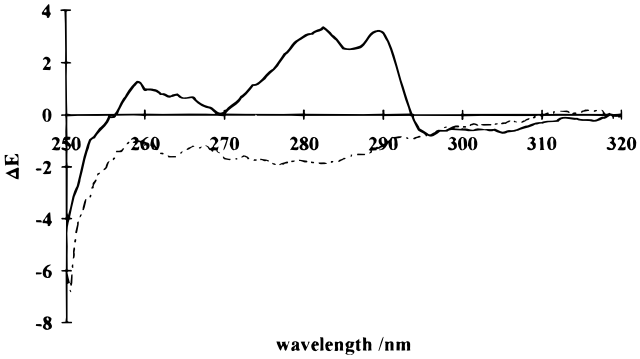

FIGURE 1: The near-UV CD spectra of ThColB at pH 2.0 and 5.0. Solid line: pH 5.0. Dashed line: pH 2.0. Buffer 30 mM phosphate−citrate/300 mM NaCl, temperature 20 °C. path length 1 cm, Δ*E* calculated using protein molar concentration, bandwidth 2 nm. The steep decline at 255 nm is due to remaining secondary structure which is similar in both cases (data not shown).

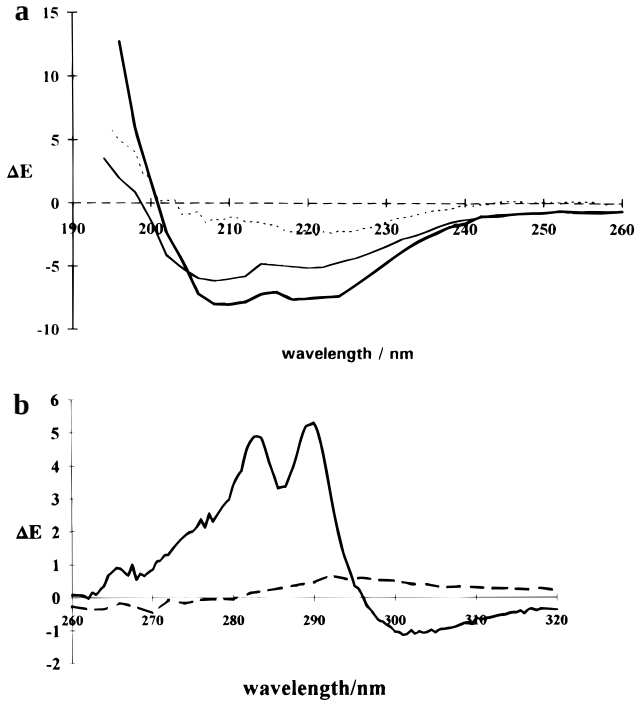

FIGURE 2: The far and near-UV CD spectra of ThColN at pH 2.0 and 7.0. (a) Far-UV CD. Bold solid line: pH = 2.0, temp = 20 °C. Thin solid line: pH 2.0, 60 °C. Dashed line: pH 7.0, 75 °C. Buffer 30 mM phosphate−citrate/300 mM NaCl, path length 0.2 mm, Δ*E* calculated using amino acid residue molar concentration, bandwidth 2 nm. (b) Near-UV CD. Solid line: pH = 2.0 at 20 °C. Dashed line: pH 2.0 at 60 °C. Path length 1 cm, Δ*E* calculated using protein molar concentration, bandwidth 2 nm. The pH 7.0 results at 20 °C for both near- and far-UV spectra were similar to those for pH 2.0 at 20 °C (see Figure 4).

globule state, and this is also true for ThColN at pH 2 where the near-UV signal is cooperatively lost at 55 °C with a 12% loss of secondary structure (Figure 2). This temperature (T_m), at which the near-UV CD signal is lost, is pH dependent for all three colicins, and at neutral pH the stability of all three is similar (Figure 3). In acidic conditions the T_m for ColB and ColA appears to fall below the ambient temperature, and thus a stable acidic molten globule is seen to occur. Hence all three proteins show an effect of pH upon their tertiary structure stability, but the effect is much more pronounced for colicins A and B. In all cases, the loss of secondary structure at T_m (pH 7) is much larger than at acidic pH's showing that the conditions for molten globule formation are not met for any colicins at neutral pH. Interestingly, colicin N is even more resistant to acid denaturation than colicin E1 which shows ≈20% of A state at pH 2.0 (Schendel & Cramer, 1994).

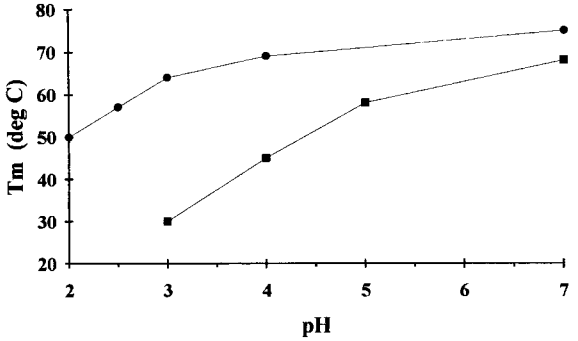

FIGURE 3: The effect of pH upon T_m. The T_m (melting temperature of the protein) is defined here as the temperature required to cause a reduction of 50% in the near-UV CD at 294 nm. ThColN (●), ThColA (■). Path length 1 cm, Δ*E* calculated using protein molar concentration, bandwidth 2 nm. Buffer 30 mM phosphate−citrate/300 mM NaCl, path length 0.2 mm, Δ*E* calculated using protein molar concentration, bandwidth 2 nm.

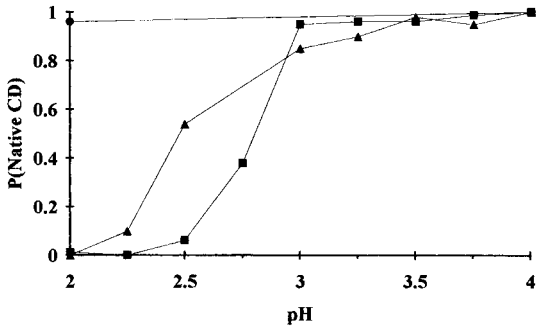

FIGURE 4: The proportion of native near-UV CD signal at different pH values. *P*(Native CD) = CD - (CD pH 2.0)/(CD pH 7.0) − (CD pH 2.0), where CD is the signal at 294 nm for ThColA (■) and ThColN (●) or 290 nm for ThColB (▲). Buffer 30 mM phosphate−citrate/300 mM NaCl, path length 0.2 mm, Δ*E* calculated using protein molar concentration, bandwidth 2 nm.

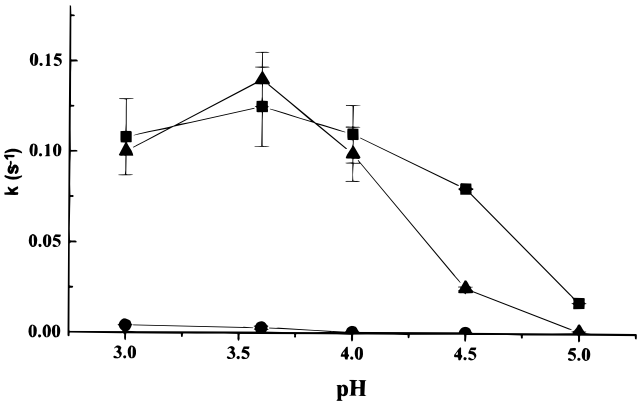

FIGURE 5: The effect of pH upon the insertion kinetics. The protein was added at a ratio of 450 lipids per protein to Br-DOPG vesicles; pH as indicated in 50 mM tris−acetate buffer. The insertion rate constant (*k*) was calculated from the decrease in tryptophan fluorescence with time, as described in the text and González-Mañas *et al. (*1992). ThColA (■), ThColN (●), or ThColB (▲).

As a result, when the loss of near-UV CD is plotted against pH, it shows that ThColB unfolds about 0.5 pH unit lower than ThColA but that ThColN shows no significant unfolding (Figure 4). This behavior is exactly reflected in the insertion kinetics into lipid bilayers by all three colicins (Figure 5).

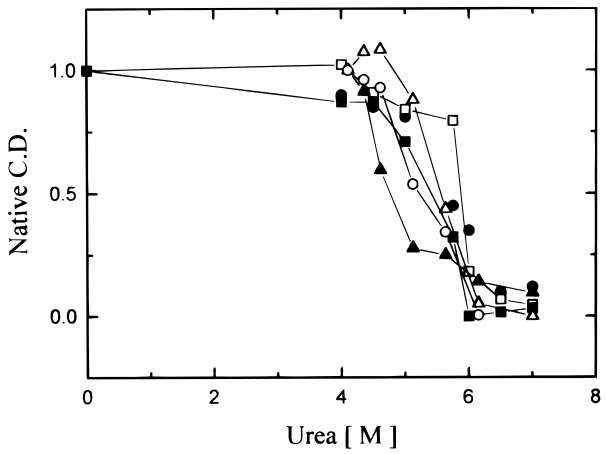

FIGURE 6: The effect of urea on the near-UV and far-UV spectra of ThColA, -B, and N. ThCol samples were mixed with 10 M urea/30 mM MOPS stock solution (Pace *et al.,* 1989) diluted with 30 mM MOPS and protein in 30 mM MOPS to give the required urea concentrations and a protein concentration of 330 μg/mL. After incubation at room temperature for 1 h, the samples were measured for CD signal strength in the far-UV (wavelength = 222 nm for ThColA (●), ThColN (■), and ThColB (▲); path length 0.2 mm) and near-UV (294 nm for ThColA (○) and ThColN (□) or 290 nm for ThColB (△); path length 1 cm).

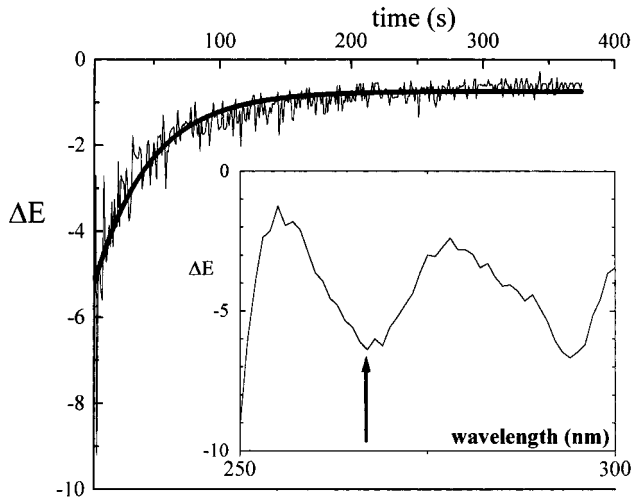

FIGURE 7: Stopped-flow circular dichroism measurements of the insertion of ThColA into lipid vesicles. Inset: Steady-state near-UV circular dichroism spectrum recorded in the stopped-flow spectrometer of 0.5 mg/mL colicin A thermolytic fragment in 50 mM tris−acetate, pH 5.0. Path length 1 cm. The signal at 267 nm used in stopped-flow mode is marked with an arrow. Main figure: Disappearance of CD signal at 267 nm following 1:1 mixing of colicin (final concn 0.5 mg/mL) with DMPG at 450 lipids per protein. Buffer as above. Temp 30 °C. Following the initial unresolved rapid decrease, the slow phase was fitted to a single exponential with a rate constant of 2.2 (±0.1) × 10^{-2} s^{-1}.

Colicins B and A show significant and similar increases in spontaneous insertion into brominated DOPG bilayers when the pH is lowered (González-Mañas *et al.,* 1992, 1993). The discrepancy between these pH values and those occurring at the acidic membrane surface is about −1.6 pH units (van der Goot *et al.,* 1991) so they are in agreement with the results for the loss of near-UV CD in solution. Once again, ThColB requires a lower pH than ThColA for the same activity. ThColN does show a pH-dependent increase in insertion rate, but like its change of CD signal, this is more than an order of magnitude less than for the other two colicins. In order to ensure that the A state is not dependent upon an acidic p*I*, we acetylated the lysine residues of colicin N (Azzi *et al.,* 1975). This acetylated ThColN had a p*I* on IEF gel (Phast System, Pharmacia) of 4.5 and showed no difference in solution spectroscopy or insertion rate compared to colicin N. However, it was soluble in low salt buffers.

The stability of all three colicins was measured as a function of the concentration of the denaturant urea at neutral pH. Each of the colicins had similar stability in this test, and the near- and far-UV signals were equally sensitive to increasing concentrations of denaturant (Figure 6). These data confirm the conclusion from the similarity of T_m (pH 7) values that all three proteins have a similar stability at pH 7. Hence the ability to lose tertiary structure without significant loss of secondary structure is entirely pH-dependent. It also shows that colicins cannot form a denaturant-induced molten globule such as occurs with β-lactamase or α-lactalbumin (Ptitsyn *et al.,* 1990).

In the stopped-flow spectrophotometer operating in steady-state spectrum mode, we were able to measure a near-UV signal for ThColA which exactly matched that obtained from the dedicated steady state machine (Lakey *et al.,* 1991; van der Goot *et al.,* 1991) (Figure 7.) The peak at 267 nm was chosen as the signal to be followed in time resolved mode. When mixed with DMPG vesicles at a ratio of 450 lipids per protein, there was a very fast and unresolved decrease of 15% in the circular dichroism signal. Following this, the signal declined with a first order rate constant of 2.2 (± 0.1) × 10^{-2} s^{-1}, which compared with a value of 1.7 × 10^{-2} s^{-1} obtained for insertion using brominated lipid quenching (González-Mañas *et al.,* 1992). When the ThColA was injected into a solution at pH 3.6 which mimicked the pH occurring at the surface of the vesicles in pH 5.0 buffer the 267 nm signal declined with a rate constant of 3.52 s^{-1}. This implies that in vesicles a rapid loss of signal to an equilibrium value is followed by a further slow decline which is linked directly to membrane insertion.

DISCUSSION

The results presented here have interest for two reasons: they provide insight into both the nature of the acid unfolded state of proteins and the nature of protein translocation across membranes.

The Acid Unfolded State. Using the classification of Fink *et al.* (1994), these closely related colicins fall into two separate classes, with A and B in either class I or II and N clearly in class III. Although ThColA and -B appear to be in class II (those proteins which directly form an A state), all our measurements were in 300 mM salt or with high concentrations of citrate ions. Thus we may be observing anion stabilized class I proteins.

It is not surprising that ThColN should behave differently to the other colicins since it has a strikingly different p*I*. However, many explanations of acid unfolding use the smeared charge model in which electrostatic repulsion at the surface acts to unfold the protein unless balanced by strong hydrophobic or other stabilizing forces. This predicts that proteins are most stable at their p*I*. Since we know that these colicins are closely related and have similar stabilities at pH 7.0, then the smeared charge theory would predict that ThColN would form an A state at pH values higher than that required by ThColA. Similarly, the A state of colicin A should be inhibited by 300 mM NaCl. Neither prediction is correct, and the alternative explanation provided by Yang

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   1                                                  51
a  VAEKAKDERELLEKTSELIAGMGDKIGEHLGDKYKAIAKDIADNIKNFQGK
n  KEEKEKNEKEALLKASELVSGMGDKLGEYLGVKYKNVAKEVANDIKNFHGR
b  KKEQENDEKTVLTKTSEVIISVGDKVGEYLGDKYKALSREIAENINNFQGK

   52                                                 102
a  TIRSFDDAMASLNKITANPAMKINKADRDALVNAWKHVDAQDMANKLGNLS
n  NIRSYNEAMASLNKVLANPKMKVNKSDKDAIVNAWKQVNAKDMANKIGNLG
b  TIRSYDDAMSSINKLMANPSLKINATDKEAIVNAWKAFNAEDMGNKFAALG

   103                                                153
a  KAFKVADVVMKVEKVREKSIEGYETGNWGPLMLEVESWVLSGIASSVALGI
n  KAFKVADLAIKVEKIREKSIEGYNTGNWGPLLLEVESWIIGGVVAGVAISL
b  KTFKAADYAIKANNIREKSIEGYQTGNWGPLMLEVESWVISGMASAVALSL

   154                                                204
a  FSATLGAYALSLGVPAIAVGIAGILLAAVVGALIDDKFADALNNEIIRPAH
n  FGAVLSFLPIS.GLAVTALGVIGIMTISYLSSFIDANRVSNINNIISSVIR
b  FSLTLGSALIAFGLSATVVGFVGVVIAGAIGAFIDDKFVDELNHKIIK
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FIGURE 8: Sequence conservation among the three colicin COOH-terminal domains. The sequences were aligned using UWGCG programs (Devereux *et al.,* 1984). Sequence data are from the following: colicin A (Morlon *et al.,* 1983), colicin B (Schramm *et al.,* 1987), and colicin N (Pugsley 1987).

and Honig (1994) appears to fit better. They predict, using sperm whale myoglobin as an example, that the electrostatic forces at the surface are attractive even at extremes of pH.

This prediction for the A state of myoglobin is based upon the abnormally low pK_a values of some histidine and carboxylic acid groups in the folded structure. The histidines are involved in a denaturation step that occurs at pH 5.0 while the carboxylic acids are involved in a separate unfolding below pH 3.0. There is no unfolding of ThColA, -B, or -N at pH 5, and it has been shown by chemical modification that histidines do not play a role in the pH-dependence of membrane insertion by ThColA (Massotte, 1991). Thus the role of carboxylic acid side chains needs to be studied. ThColN has 21 Asp + Glu and 28 Arg + Lys while ThColA and -B have 28/25 and 25/23, respectively (Figure 8). Are the unconserved carboxylates responsible for the A state of colicins B and A?

High resolution structures are available for colicins A (Parker *et al.,* 1989, 1992) and N (I. Vetter, personal communication) while a model of ThColB has been built using the coordinates of ThColA (Lakey *et al.,* 1994). ThColA crystallized as a dimer in the asymmetric unit, and coordinates are available for both monomers a and b. Using these, we determined whether the missing side chains might be expected to have anomalous pK_a values by looking for salt bridge formation or for burial of charged side chains. Only D189 and D193 formed a clear salt bridge (with K65) with O:N distances of 2.9 (a monomer) and 2.7 (b monomer) and 3.1 and 3.2 Å for D189 and D193, respectively. The fractional surface exposure was calculated using Quanta (Molecular Simulations Inc.), and this was around 50% for each side chain compared to the exposure for a Gly-X-Gly model peptide. This approximates to an arbitrary threshold for burial of the side chains, and visual examination showed that the charged groups were exposed on a Connolly surface (Connolly, 1983) calculated with a probe radius of 1.4 Å. Thus for these residues, only their participation in a theoretical salt bridge indicates a possible change in pK_a. In addition, a glutamate (E13), not involved in salt bridges and absent in ThColN, is partially buried in ThColA structure. These may be candidates to explain the particular sensitivity of colicins A and B to pH.

In summary, carboxylate groups with altered pK_a values in the folded structure *may* cause destabilization of some colicins when the ambient pH approaches the pK_a because the folded versus unfolded equilibrium becomes pH sensitive (Yang & Honig, 1994). Since this may be localized to one side chain, it may explain why the effects are limited to molten globule formation rather than complete unfolding.

Membrane Insertion. The importance of the insertion competent state for colicins arises from our wish to understand the structural reorganizations which occur in these and other molecules during protein translocation through membranes (van der Goot *et al.,* 1992). The discovery of the A state for ThColA was a major breakthrough in this work since it provided physicochemical data to support the previously proposed idea that molten globules could be the basis for insertion or translocation competent states (Bychkova *et al.,* 1988).

The recognition of the A state as the insertion competent state arose from the correlation between the proportion of colicin in the A state and the rate of insertion into lipid vesicles. By measuring the pH at the lipid interface, a direct comparison could be made between solution circular dichroism studies and the insertion process. In this paper, we have confirmed these earlier results in two ways, by the use of different colicins and by the direct visualization of the circular dichroism changes at the lipid interface.

The results from ThColB and in particular ThColN show that there is a direct correlation between the ability to form an A state and the ability to insert into lipid membranes. The half pH unit difference in the formation of the ThColB state is exactly correlated with the change in the insertion rate constant. ThColN does show a much smaller pH-dependent increase in insertion which correlates with its slight change in stability. This correlation between solution spectroscopy and insertion kinetics shows that the A state is important for membrane insertion. Although there is no available kinetic data, it is reasonable to assume that the 20% A state seen in colicin E1 at pH 2−3 (Schendel & Cramer, 1994) is sufficient to explain its insertion properties in terms of our model presented here. Hence all the colicins studied so far fit to the original model where insertion *in vitro* is dependent upon an insertion competent A state.

By measuring the surface pH of acidic vesicles, we can correlate the pH-dependence of the solution A state and the surface bound insertion competent state (van der Goot *et al.,* 1991). Surface pH is however an unsatisfactory concept and is simply an indicator of how the complex interfacial electrostatic conditions affect pK_a values. We should nevertheless be able to show by stopped-flow CD that the sequence of events predicted by such correlations does occur at the membrane interface. This predicts that the rate of insertion *k* correlates with the proportion of protein in the A state.

Since the inserted state has a near-UV CD signal equivalent to the A state, the sequence:

$$[\text{Native}_{\text{sol}}] \xrightarrow{K_1} [\text{Native}_{\text{assoc}}] \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} [\text{MG}_{\text{assoc}}] \xrightarrow{K_3} [\text{Inserted}]$$

will appear on the CD trace as

$$[\text{Native}_{\text{sol}} + \text{Native}_{\text{assoc}}] \Rightarrow [\text{MG}_{\text{assoc}} + \text{Inserted}]$$

We have previously correlated the CD data in solution with

that at the vesicle surface by measuring the surface pH ofthe acidic vesicles. In pH 5.0 solution, the surface pH is 3.6 and K_1 has a value of 47 s^{-1} (Lakey *et al.,* 1994). $K_2 = 3.52$ s^{-1} when measured as [Native] to [MG] in solution. It is thus probable that [$Native_{sol}$] to [MG_{assoc}] accounts for the initial rapid loss of 15% of CD following mixing. This is followed by a slow rate of disappearance of the CD signal ($K_3 \approx 2 \times 10^{-2}$ $s^{-1} = k$) which is due to the increase in [Inserted] which depopulates [$Native_{assoc}$] via [MG_{assoc}]. This implies that some equilibrium is maintained between [$Native_{assoc}$] and [MG_{assoc}], i.e., (K_2/K_{-2}), which correlates with the equilibrium molten globules seen in solution. Since 15% of the colicin is MG and 85% native, the equilibrium constant is 0.17, and therefore K_{-2} is approximately 21 s^{-1}. Other results possible from this measurement would be the complete disappearance of the native state at a faster rate than accounted for by insertion (this would have indicated that the molten globule state was not the direct precursor of insertion and that some subsequent reorganization was required). Alternatively, native state loss could occur at the same rate as insertion but without the rapid early decline; this would mean that a molten globule is not an insertion intermediate at all and that tertiary structure loss only occurs upon insertion.

We previously concluded that, due to the lack of an acidic compartment on the translocation pathway (van der Goot *et al.,* 1992), the *in vivo* insertion intermediate of colicins is not an acidic molten globule. (In addition, it should be noted that the 100% acidic lipid content of the vesicles used here is never found *in vivo.*) Nevertheless, it was recently shown that colicin A activity *is* dependent upon the level of acidic lipids in the cytoplasmic membrane whereas colicin N activity is not (van der Goot *et al.,* 1993). Thus colicin A does react to acidic conditions *in vivo*, and this may correlate with its special *in vitro* behavior. A recent study of colicin E1 also concluded that there is a partly unfolded state at acidic pH that is neccesary for activity (Schendel & Cramer, 1994) but that it retained more tertiary structure than ThColA. Colicin E1 C-terminal domain has lower sequence homology with the ABN group and has a p$I_{(calc)}$ of 9.81 while the other two members of its homology group Ia and Ib have p$I_{(calc)}$ values of 10.29 and 10.11, respectively. Thus it may be that colicins A and B are unusual in their p*I*, their ability to form a complete A state, and (for ThColA at least) the dependence upon acidic lipids *in vivo*. If they do form an A state *in vivo*, it is unclear what drives the formation of the *in vivo* insertion competent state in the colicins with basic C-terminal domains.

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