Surface Aspartate Residues Are Essential for the Stability of Colicin A P-Domain: A Mechanism for the Formation of an Acidic Molten-Globule[†,‡]

Susan L. Fridd and Jeremy H. Lakey*

School of Biochemistry and Genetics, University of Newcastle, Newcastle-upon-Tyne NE2 4HH, U.K.

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ABSTRACT: The pore-forming domains of members of a family of bacterial toxins, colicins N and A, share > 50% sequence identity, identical folds and yet display strikingly different behavior in acid conditions. At low pH colicin A forms a molten globule state while colicin N retains a native fold. This is relevant to in vivo activity since colicin A requires acidic phospholipids for its toxic activity but colicin N does not. The pI of colicin A (5.25) is far lower than that of colicin N (10.2) because colicin A contains seven extra aspartate residues. We first introduced separately each of these acidic amino acids into homologous sites in colicin N, but none caused destabilization at low pH. However, in the reverse experiment, the sequential replacement of these acidic side chains of colicin A by alanine revealed six sites where this change destabilized the protein at neutral pH. Some of these residues, which each contribute less than 4% to the total negative charge, appear to stabilize the protein via a network of hydrogen bonds and charge pairs which are sensitive to protonation. Other residues have no clear interactions that explain their importance. The colicin A is thus a protein that relies upon acid sensitive interactions for its stability at neutral pH and its in vivo activity.

In general, the in vitro folding of a protein may be divided into three stages: secondary structure formation, intermediate association of secondary structural elements, and finally local adjustment of the intermediate state so that the side chains pack together tightly and van der Waals interactions can be formed (*1*−*3*). Intermediates may be short-lived or absent but the basic premise, supported by widespread evidence that the secondary structure, is regained before tertiary structure during protein refolding experiments (*4*). It is proposed that the observed flexible intermediates are stabilized mainly by hydrophobic interactions and in some cases are equivalent to a "molten globule state".

Proteins that form a stable molten globule state (e.g., α-lactalbumin) are excellent targets for the study of folding pathways (*5*). They are in effect caught in a state that most proteins pass through only transiently on their way from unfolded peptide to fully folded protein. We are studying the molten globule state formed by the pore-forming domain of colicin A (colA-P) at low pH and the inability of the highly homologous colicin N (colN-P) to form a similar unfolded state. This provides a useful system to understand the causes of acidic molten globule formation.

Furthermore, the acidic molten globule state of colicin A is also of great biological interest since it has a clear functional role. Colicins A and B (*6*−*8*) are the only two colicins which form this structure at low pH. The remaining colicin pore-forming domains show either limited pH sensitivity, like colE1-P (*9*), or none at all, like colN-P(*6*). Importantly colicin A requires acidic phospholipids in the target cell membrane whereas colicin N does not (*10*). At this microscopic level, where bulk pH has no meaning and local pH can be decreased by concentrations of acidic groups, acidic lipids could be sufficient to cause molten globule formation (*11*). Thus, the colicin acidic molten globule is of similar in vivo importance to those of SecA (*11*), cytochrome C (*12*) and diphtheria toxin (*13*).

Pore-forming colicins are a group of bacteriocins (Mr = 40−70 kDa) composed of three structural domains (*14*, *15*). They act by binding to and translocating across the outer membrane of target bacteria and thereby inserting their toxic pore-forming domains into the energized inner-membrane. The structural changes occurring during this insertion are extensive and lead to the formation of a voltage gated membrane channel from a previously water soluble protein. In colicin A, the rate of insertion is directly dependent upon the formation of the molten globule like "insertion competent form" (*16*, *17*).

The C-terminal, pore-forming domains of colicins A, B, and N (colA-P, colB-P, and colN-P) share more than 50% primary sequence identity (*18*). The partial crystal structures of colicins A and N have been resolved at 2.4 and 3.1 Å resolution, respectively (*18*−*20*), and show a 1.3 Å C^{alpha} RMSD thus confirming the similarity of the colN-P and colA-P structures. Yet, while colA-P and colB-P form a molten globule state below pH 3.0, colN-P remains in a near native conformation to pH 1 (*6*). colA-P and colB-P form the acidic group of P-domains, with pI values of 5.82 and 5.48, respectively, whereas colN-P (with a pI of 10.25) is part of the larger group of basic colicins (*6*) with which it, however, has lower sequence homology.

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* To whom correspondence should be addressed.

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The pI difference arises because, although the proteins have similar numbers of basic residues, the P-domain of colicin N has seven fewer Asp/Glu residues than colA-P (*6*, *21*). Colicins A and N have similar stabilities with increasing concentrations of urea at pH 7 and also have similar melting temperatures (T_m) at neutral pH (*6*). Since, the near-UV and far-UV CD signals decrease in parallel, we considered that neither colicin A nor N formed a molten globule state (*6*) under these conditions. The colicin A molten globule state appears therefore to be triggered solely by pH.

In a previous paper (*6*) we suggested that, since colN-P is highly positively charged at neutral pH, the increase in positive charge on colA-P at acidic pH does not explain the destabilization. Furthermore, we suggested that some of the acidic residues of colA-P have altered pK_a's in the folded and unfolded forms. This would make folding pH-dependent in the pH range of the pK_a values and their removal would decrease the pH sensitivity. Small ΔpK_a values (<1) have been predicted by the use of Debye−Hückel calculations (*22*), and these may partly explain the pH effect, but surprisingly, the current paper reveals that colA-P is destabilized at neutral pH by six out of seven Asp to Ala replacements.

MATERIALS AND METHODS

Construction of Vectors for Expression of Colicins N and A P-Domains. Pore forming domains were expressed using a modified pET8c vector (*23*) which introduces a methionine, six histidines and two serine linkers at the N-terminus of any gene inserted between the *Xho*I and *Mlu*I restriction sites. The resulting histidine tagged protein is under the control of a T7 promoter on the vector. Inserts, comprising the P-domain of either colicin N or colicin A and the requisite immunity protein, were prepared using PCR. The template was pCHAP4 (*24*), which encodes wild-type colicin N or pcolA (*25*) encoding wild-type colicin A. Primers were designed with 18 bp matching sequences and introducing the restriction sites for *Xho*I and *Mlu*I. The PCR products and vector were each digested with *Xho*I and *Mlu*I, ligated and used to transform competent JM105 cells. Transformants were selected on the basis of ampicillin resistance conferred on the cells by the plasmid. Plasmids were prepared from a number of colonies and their identities were confirmed by restriction enzyme analysis and sequencing. Site-directed mutations were made in the P-domains of colicins N and A using either a 3 primer mutagenesis method (*26*) or "Quickchange" mutagenesis kit (Qiagen).

Protein Purification. Esherichia coli BL21(DE3) were transformed with plasmids encoding the colicin N or colicin A mutant P-domain and grown in LB-media (*27*) with 100 μg/mL ampicillin. At an OD_{600} of 0.7 production of colicin was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) at 1 mM. Cells were harvested by centrifugation at 3000*g* 2 h after induction and resuspended in 20 mM phosphate, 300 mM NaCl, 10 mM imidazole, pH 7. Cells were lysed using a "One Shot cell disrupter" (Constant Systems Ltd.) and the lysate was centrifuged at 100000*g* for 1 h. Colicin P-domain was purified from the supernatant by use of the hexa-histidine tag on Ni-NTA affinity resin (Qiagen). The supernatant was filtered through a 0.2 μm filter and loaded onto the Ni-NTA resin column (bed volume 4 mL) preequilibrated with 20 mM phosphate, 300 mM NaCl, 10 mM imidazole, pH 7. The column was washed with 10 bed volumes of the same buffer and the protein was eluted with 250 mM imidazole, 300 mM NaCl, 20 mM phosphate pH 7.0. Fractions from the column were analyzed by SDS−PAGE and fractions containing pure colicin P-domain pooled.

Circular Dichroism Measurements. CD spectroscopy was carried out using a Jobin Yvon CD6 spectrophotometer. Near UV CD spectra (250−320 nm) were measured in a Hellma 1 cm path length water-jacketed cuvette while far UV CD spectra (190−250 nm) were measured using a Hellma 0.01 cm path length circular cuvette. For acid unfolding experiments, protein was dialyzed for at least 8 h against 10 mM sodium phosphate, 300 mM NaCl adjusted to the appropriate pH with HCl.

Synthesis of Brominated Phospholipids and Vesicle Formation. Brominated DOPG was synthesized and vesicles formed as described previously (*16*, *28*−*30*).

Fluorescence Measurements. Fluorescence measurements were made using an SLM 8100 spectrofluorometer operating in ratio mode with spectral bandwidths of 8 nm for both excitation and emission. The excitation wavelength was set at 280 nm and the measured emission wavelength was set to 332 nm for brominated lipid vesicle experiments. The excitation wavelength was 360 nm and emission was measured between 400 and 550 nm for experiments using the fluorescent probe ANS. Light scattering by the vesicles was minimized by using vertically polarized light for excitation and measuring horizontally polarized emitted light. In the brominated lipid experiments, the protein was used at a concentration of 5 μg in 500 μL total volume in a 0.5 cm path length cuvette. A total of 50 μL of brominated lipid 10 mg/mL Br-DOPG) was added and the decrease in tryptophan fluorescence was followed. The experiments using ANS were carried out in a total volume of 400 μL containing 8μg of protein and 40 μL of ANS stock solution (1mM).

RESULTS

Mutations are numbered according to the alignment in Figure 1, which uses colicin N numbering from full-length colicin N. For colicin A in this paper this scheme is kept to ease comparison between the proteins. To get the correct colicin A numbering see Table 1.

6His-Tagged colN-P. The stability of the pore-forming domains of colicins N and A has been studied previously using pore-forming domains separated from the T and R domains by digestion of the full length protein with thermolysin (thermolytic colicin N and A) (*6*, *16*). Our primary criterion for the formation of a molten globule state by colicin A is the collapse of the tertiary-structure dependent near UV−CD spectra with retention of a near normal amount of secondary structure indicated by far UV−CD (*16*, *31*). By these criteria thermolytic colicin A was shown to lose tertiary structure between pH 2.5 and 3 in 300 mM NaCl while thermolytic colicin N remained folded even to pH 1 (*6*). Our histidine tagged colicin N and A P-domains remove the need for digestion with thermolysin. In order that results obtained using histidine-tagged colicins can be compared to those of previous work with the equivalent thermolytic fragments, the near and far UV−CD spectra were first shown to be superimposable at a range of pH values in 300 mM NaCl

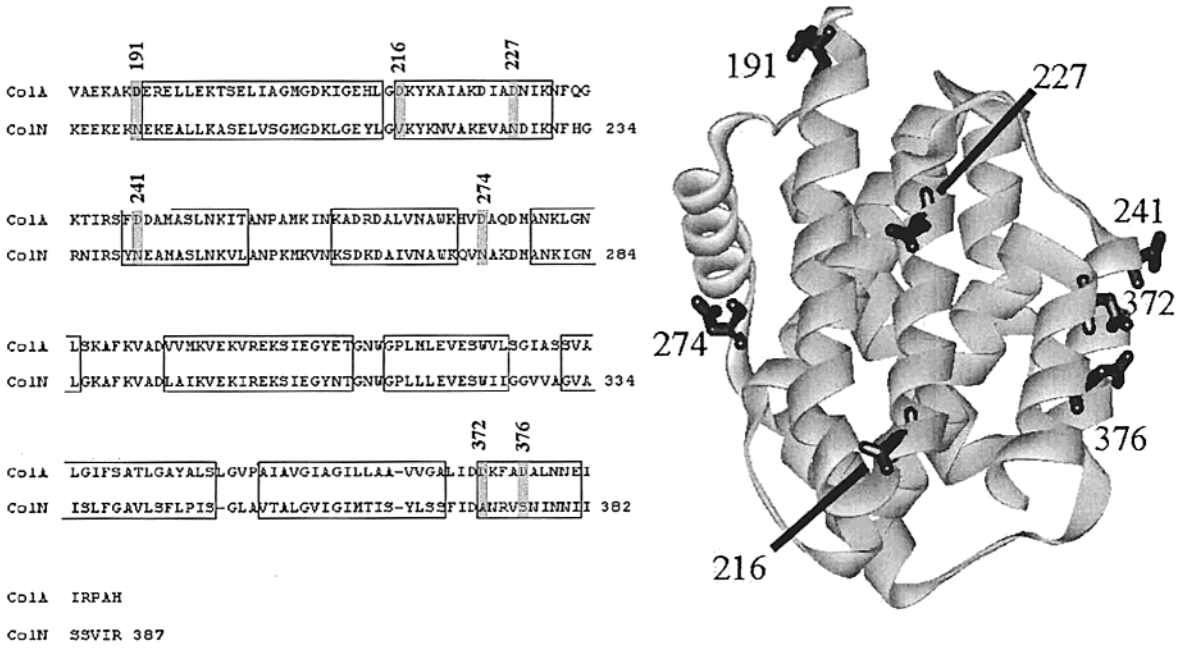

FIGURE 1: Alignments made using Clustalw of the pore-forming domains of colicins A (*32*) and N (*24*) showing the high degree of sequence identity between these 2 molecules. The mutations of are indicated with colicin N numbering. Colicin A alanine substitutions were at the same sites, for numbering see Table 1. Double mutants in colicin N were N227D/A372D and N241D/A372D. Helices derived from the colicin A X-ray structure 1COL (*18*) are shown boxed and this structure is used to represent the positions of the mutations in the ribbon diagram.

Table 1: Accessibility, ΔpK_a, and Fraction of Unfolding for the Mutants[a]

mutation		% access	ΔpK_a (*22*)		*f*WT at 294 nm		*f*WT at 270 nm		*f*WT at 223 nm	
colN	colA	colA a/b	colN	colA	pH 7.0	pH 1.0	pH 7.0	pH 1.0	pH 7.0	pH 1.0
N191	D395	65/47		0.05	0.35	1.14	0.28	1.34	0.89	0.88
D208	D404	37/63	−0.65	−0.58						
V216	D420	96/73		0.28	0.86	2.80	0.94	3.00	0.99	0.91
E224	D428	34/53	−0.19	0.34						
N227	D431	63/65		−0.15	nd	nd	nd	nd	nd	nd
N241	D445	75/79		−0.32	nd	nd	nd	nd	nd	nd
E242	D446	55/51	−0.51	−0.27						
D262	D466	8/20	0.43	0.32						
D264	D468	48/33	0.07	0.15						
N274	D478	67/65		−0.16	nd	nd	nd	nd	nd	nd
D277	D481	49/55	−0.64	−0.24						
D293	D497	67/54	−0.21	−0.23						
D371	D576	55/41	−0.77	−0.46						
A372	D577	29/36		−0.37	0.55	1.12	0.36	1.64	0.63	0.70
S376	D581	44/41		−0.29	0.54	1.14	0.84	1.27	0.47	0.61
Colicin A WT at pH 2.5 (*16*)					0.13		0.21		0.83	

[a] The table shows data for all the aspartate residues of colicin A and indicates their equivalent in colicin N. Mutants in bold were studied in this work. Percent access (%Access) indicates calculated side-chain accessibility calculated using Quanta where 100% is that of a Gly-X-Gly tripeptide. The high-resolution structure of colicin A pore-forming 1COL (*18*) has two molecules in the asymmetric unit and the values for the a/b monomers are shown separately and were calculated using isolated monomer structures. Warwicker calculated the structure induced changes in side chain pK_a values using a Debye−Huckel method as, ΔpK_a = native ΔpK_a − unfolded ΔpK_a ($\Delta pK_{a,NU}s = \Delta pK_{a,N} - \Delta pK_{a,U}$) (*22*). These values are our best indicator of the likely effect of structure on the behavior of individual side chains. *f*WT indicates the relative intensity of the mutant/wild-type CD signals at the designated wavelength where 1 equals the normalized wild-type protein CD intensity and a zero value equates to the buffer baseline. Bottom row shows *f*WT values for colicin A where in this case *f*WT = CD pH 2.5 divided by CD pH 7.0. This indicates that D191A most closely resembles the colicin A molten globule state.

(results not shown). The unfolding of this His-tagged colicin A was identical to the natural fragment despite an increase in overall pI to 6.48. This adds to our previous data (*6*) which showed that when the lysine residues of colicin N are acetylated its pI changes to 4.5 but its stability at low pH is unaffected. Thus, the acid dependent unfolding is due to local effects rather than overall pI. The acid unfolding of colA-P was again shown to be independent of salt concentration and all experiments were performed in 300 mM NaCl as colN-P is insoluble at low ionic strength. This insensitivity to salt further suggests that the pH modified interactions are hydrogen bonds rather than charge effects.

Insertion of Aspartate Residues into colN-P. To test the hypothesis that carboxylic acid side chains, present in colicin A but not in colicin N, destabilize the protein at low pH, we replaced existing colicin N residues by aspartyls at sites where these occur in colicin A P-domain. This was done on the basis of a sequence alignment (see Figure 1) based on

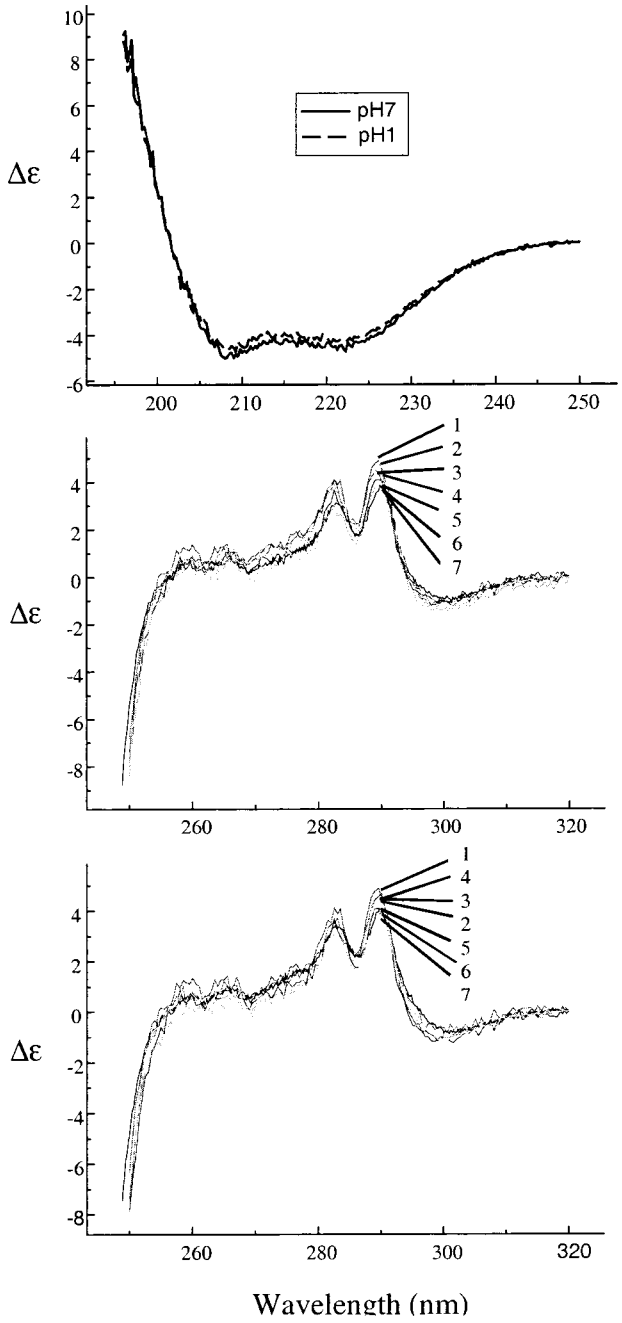

FIGURE 2: (Upper) Far UV CD spectra of wild-type colicin N P-domain at pH 7.0 and 1.0 confirm that the secondary structure is also folded and shows the expected result for a highly α-helical protein. Far UV signals for each of the mutant proteins were superimposable on the wild-type signal (data not shown). The near UV CD spectra of each of the colicin N P-domain mutant and wild-type proteins at pH 7 (middle) and at pH1 (lower). All of the mutant proteins, like the wild-type, show fully folded tertiary structure at both pH values. Numbered spectra are 1(V216D), 2(N191D), 3(N227D), 4(A372D), 5(N274D), 6(N241D), 7(WT).

over 50% sequence identity and the crystal structures that are superimposable (*18*−*20*). The near and far UV−CD spectra of each of the colicin N single aspartic acid mutants showed no unfolding of the tertiary or secondary structure at neutral or at low pH (pH 1) (see Figure 2). Double mutations combining pairs of the above Asp insertions (N227D/A372D and N241D/A372D) also showed wild-type stability.

Removal of Aspartate Residues from colA-P. Since the insertion of "colicin A" aspartate residues into colicin N had no destabilizing effects, we wished to understand the role of these acidic side chains in the stability of colicin A. A series of single alanine substitutions (Asp→Ala) mutants at the same sites as above (Figure 1) were prepared and purified.

All but one (D216A) of these mutants were less folded than the wild-type colA-P at neutral pH (see Figure 3). CD spectra could not be recorded for some of the mutants (D227A, D241A, D274A) as they were prone to aggregation. The near and far UV CD spectra of the other mutants revealed that they had lost tertiary, and to a lesser extent secondary, structure. D372A and D376A lost the most secondary structure and thus D191A, which lost most tertiary structure and least secondary structure, fits most closely to our definition of a molten globule state These mutants lost their remaining tertiary structure at low pH (like the wild-type), indicating that pH dependence was retained at the remaining sites.

To further investigate whether these colicin A mutants form a partially unfolded state at pH 7, we used the hydrophobic fluorescent probe, ANS. This has been used previously to investigate molten globule states since it binds to exposed hydrophobic regions of a protein which generally occur in partially folded states (*33*). Both the intensity and maximum emission wavelength of the ANS fluorescence change when it inserts into the hydrophobic parts of a protein. The maximum emission wavelength of ANS with histidine tagged colicin A P-domain was shown to be 500 nm. ANS in solution with colicin A P-domain mutants D372A, D191A, and D376A shows slightly increased fluorescence intensity and different degrees of blue shift in maximum emission wavelengths (see Figure 4). The highly destabilized mutant D227A caused the largest blue shift (30 nm) in the maximum emission wavelength of ANS. Mutant D216A was identical to the wild-type protein.

Further investigation of the properties of the partially unfolded states of the colicin A mutants followed the insertion of the P-domains into brominated DOPG vesicles. Insertion has been shown to be proportional to the degree of molten globule state formation (*16*). The intrinsic fluorescence of the tryptophans in the molecule decreases as the P-domain inserts into the brominated lipid vesicles through quenching by the bromine. The rate of insertion into brominated DOPG vesicles of wild-type and histidine tagged colicin A P-domain was first investigated, and this confirmed that both inserted into brominated DOPG vesicles in the same pH dependent way as previously shown for thermolytic colicin A (*16*, *29*, *30*).

Insertion rates for the mutant colicin A P-domains were then compared to wild-type histidine-tagged P-domain at pH 5 (see Figure 5). At this pH, the different insertion rates could be compared with that of the WT, which does not insert at neutral pH. The mutant D216A was shown to insert into brominated lipid vesicles at a similar rate wild-type colicin A. However, analysis of mutants D372A and D376A was complicated by their complex insertion kinetics. Hence, during the mixing time they appeared to have largely inserted faster than wild-type into the brominated DOPG vesicles and this was followed by kinetics that resembled WT. The latter two mutants showed higher levels of residual fluorescence than WT, which may indicate that some of the protein had

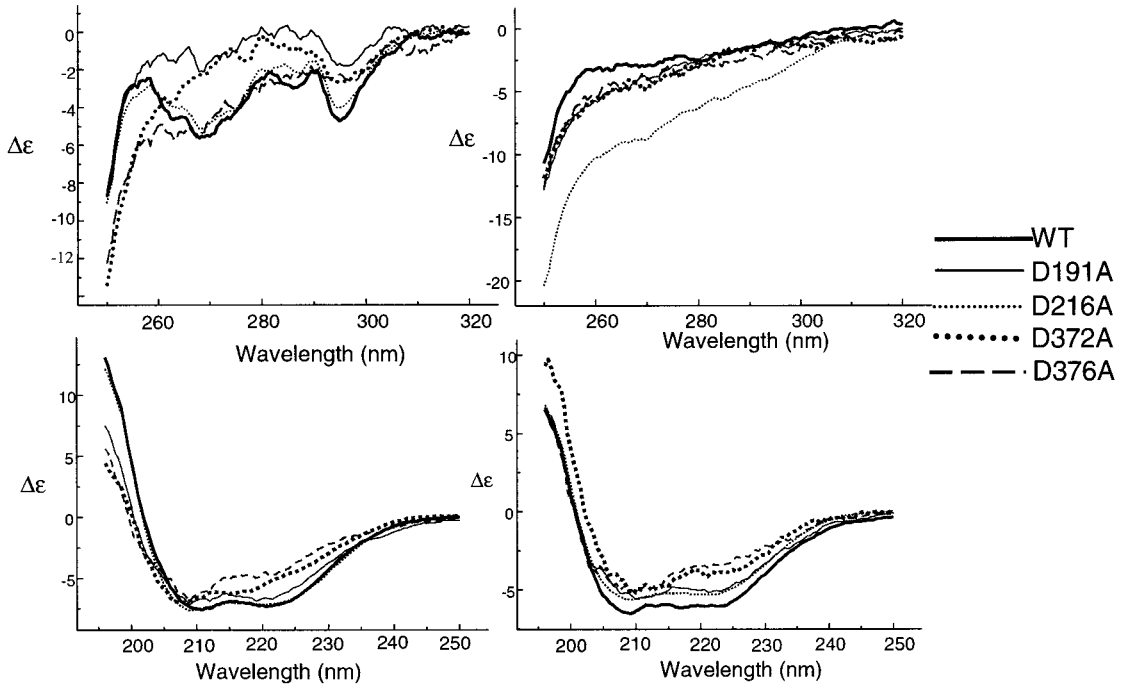

FIGURE 3: Near UV CD spectra of each of the colicin A mutant P-domain proteins at pH 7 (upper left) and at pH 1 (upper right). The mutant proteins show differing degrees of loss of tertiary structure at neutral pH when compared to the wild-type protein, which is fully folded. At pH 1, each of the proteins shows complete loss of tertiary structure. Far UV CD spectra of the same mutants at pH 7 (lower left) and pH 1 (lower right) show slight losses of secondary structure in the mutant proteins, which are similar at neutral and low pH.

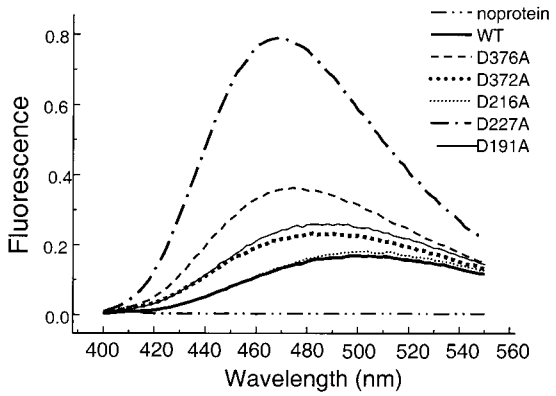

FIGURE 4: Fluorescence spectra of ANS at 0.1 mM in solution with each of the colicin A P-domain mutants at 30 μg/400 μL. The excitation wavelength was 360 nm. All spectra were taken in a buffer containing 300 mM NaCl, 10 mM sodium phosphate at pH 7. Changes in the fluorescence intensity and maximum emission wavelength of the ANS indicate the degree of unfolding of the proteins.

aggregated. Mutant D227A inserted at a slower rate than wild-type colicin A P-domain. D191A, which by CD shows the clearest pH 7.0 molten globule state, had the most wild-type insertion kinetics, although it too had a higher residual fluorescence than the WT or D216. This mutant was thus examined at pH 7.0 and pH 6.0. The data shows that, unlike the WT, the mutant does insert at neutral pH and that insertion at pH 7 and pH 6 is very similar.

DISCUSSION

Protein denaturation at acidic pH has long been viewed according to the Linderström−Lang model (*34*), in which the protein is treated as a sphere whose net surface charge is zero at its pI and increases at extremes of pH. The charge is evenly spread over the surface of the sphere and seen as repulsive, so that as it increases the molecule is forced to unfold. This theory predicts that colicin N would unfold at a higher pH than colicin A since the pI of colicin N is much higher than that of colicin A (10.25 and 5.82, respectively, for the P-domains). Instead colicin A unfolds to a molten globule state between pH 2 and 4 while colicin N remains native below pH 1 (*6*). Nevertheless, charge repulsion has been observed by Fink et al. (*35*) to contribute to some examples of unfolding at low pH. It is proposed that protonation of acidic residues leads to a build up of positive charge as the pH decreases and the repulsive forces created push the molecule apart. This can be counteracted at very low pH when all acidic groups have been protonated, as further addition of acid introduces anions which mask the repulsive positive charges. This is the explanation for the observation that some proteins can unfold as pH decreases and then refold to a molten globule as the pH decreases yet further (*36*).

For other cases, Yang and Honig (*37*) have shown that charges at the surface are largely stabilizing. Thus, protonation of aspartic acid groups that have the same pK_a in both folding states is likely to be neutral in terms of protein stability particularly when desolvation effects are involved (*38*). The candidacy for side chains whose protonation leads to the unfolding of the protein below pH 4.0 therefore falls upon carboxylic acid side chains have a reduced pK_a in the folded form and are possibly involved in hydrogen bonds or salt bridges. A specific case is the Asp−His ion pair in T4 lysozyme which stabilizes the protein by 3−5 kcal/mol and results in pK_as for the Asp and His of 0.5 and 9.1, respectively. Here the acid unfolding is due to protonation and, thus, stabilization of the unfolded form (*39*). Apart from these clear salt bridge effects, carboxylates with low pK_as may arise from strong hydrogen bonding (e.g., Asp 66 in hen egg-white lysozyme), and these are likely to arise in

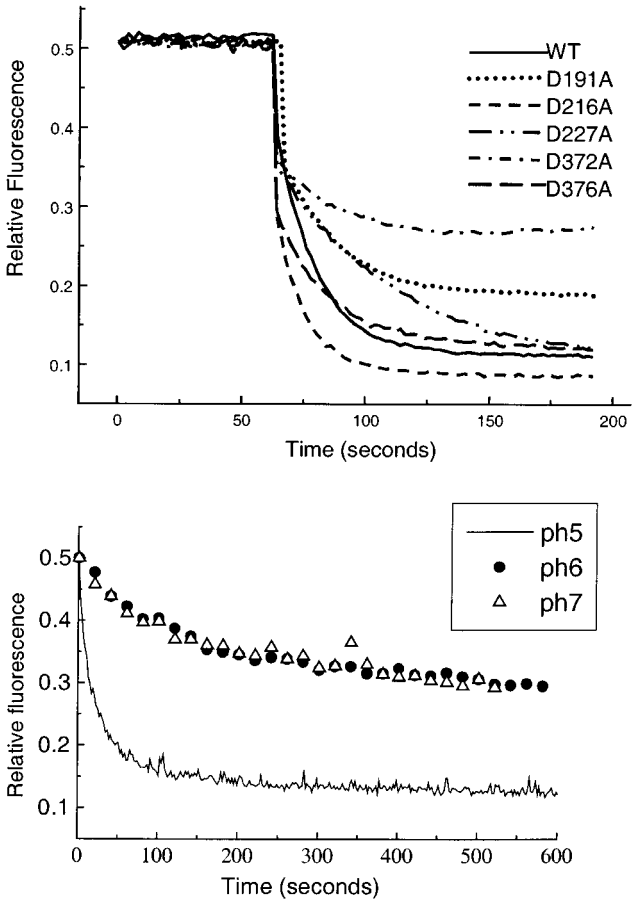

FIGURE 5: Fluorescence of the tryptophan residues in the P-domain of the colicin mutant decreases as it inserts into the brominated DOPG lipid vesicles. (Upper) Each experiment was carried out in 300 mM NaCl, 50 mM Tris-HCl at pH 5.0. Baseline of unquenched fluorescence for 70 s precedes the addition of brominated phospholipid vesicles, Addition and mixing takes a maximum of 5 s during which data collection is halted. Normally the data shows clear first order kinetics which can yield good quality quantitative data on insertion. However, in this case many of the mutants showed biphasic insertion possibly due to aggregation and further analysis was not attempted. (Lower) Comparison of "D191A" mutant of colicin A (D395A) at different pH values. At pH 5 (at a lipid to protein ration of 450) the insertion rate is 33.3×10^{-3} s^{-1}, whereas the rates at pH 6.0 and pH 7.0 were 7.2×10^{-3} and 6.6×10^{-3} s^{-1}, respectively. The values for colicin A WT were measured as 27.4, 0.35 and 0×10^{-3} s^{-1} respectively. Hence, the mutation introduces a new basal pH independent insertion rate allowing slow insertion even at pH 7.0. However, the rate is still much less than when all aspartates are protonated at pH 5.0.

hot spots on the proteins surface (*37*). However, the effects leading to reduced pK_as are likely to be complicated involving a balance of desolvation effects, hydrogen bonding, and charge−charge interactions (*38*).

For colicin A, suspicion centered on the seven aspartyl residues not present in the pH insensitive colicin N. If protonation of up to seven aspartic acid side chains can cause unfolding of colicin A to a molten globule state, then introduction of the same as single or paired aspartic acid residues into colicin N might cause a measurable degree of unfolding at low pH. However, these mutations had no effect. When the mutants were investigated using circular dichroism, their spectra were identical to wild-type at neutral and very low pH. It was clearly not possible to recreate the precise "colicin A" hydrogen-bonding environment of each aspartate. Nevertheless, considering the sequence homology and the high pI of colicin N, it was considered likely that if these residues had a reduced pK_a in colA-P it could occur also in colN-P. Hence one or more of the insertions would cause measurable pH sensitivity against such a clear baseline of pH stability. It can be seen that the near and far UV−CD spectra are very reproducible and are therefore a very reliable indicator of the complete lack of acidic unfolding. The transfer of specific charged regions of colicin A into colicin N would probably be required to clarify whether colicin N can be made pH sensitive.

Since it appears from this part of the study that the unfolding effects of these aspartyls cannot be simply transplanted to colicin N, the second part of the study involved their removal from colicin A. If their altered pK_as in unfolded and folded states are responsible for the pH sensitivity, this should be decreased by their removal. Surprisingly, this study revealed that six of the seven were required for tertiary structure stability at neutral pH. This is somewhat surprising as they are all surface residues and the experiments were carried out in 300 mM NaCl to reduce nonspecific electrostatic effects. Furthermore, since there is a total of 28 acidic residues in colA-P, each deletion removes less than 4% of the total negative charge. In these mutants, we have entirely removed the polarity of the side chains by alanine substitution, whereas protonation would not be so extreme. Hence it could be argued that asparagine, glutamine, serine, or threonine might be better substitutes which could mimic the true low pH effect. However, alanine insertion shows unequivocally the extreme sensitivity to polarity at these sites.

On the basis of the CD data, mutant D191A loses most of its tertiary structure while retaining most of the secondary structure, which fulfils the criteria (*16*) for formation of a molten globule state of colicin A. D372A and D376A show a smaller but significant change in tertiary structure but a larger loss of secondary structure. This may indicate that, at pH 7.0, these latter mutants form a less tightly packed form of the molten globule than is formed by colicin A at low pH. CD data could not be obtained for the mutants D227A, D241A, and D274A because they were prone to aggregation.

It was previously reported that the acid-induced molten globule state of colicin A is too compact for ANS to bind and no increase in fluorescence is seen in these experiments (*16*). The mutants D372A, D376A, and D191A were shown to have some degree of exposure of the hydrophobic interior of the protein. Interestingly, the mutant that could not be investigated by CD, D227A, is probably more unfolded than the two mutants discussed above. It caused a large increase in fluorescence of the hydrophobic probe ANS and a considerable blue shift in its maximum emission wavelength, indicating that large sections of hydrophobic interior of the molecule are exposed to the solvent. This may be the reason it aggregates easily. D216A as expected showed a low degree of ANS binding, which was similar to WT colicin A.

Modeling of the hydrogen-bonding network of colicin A P-domain showed that D372 and D376 were involved in a charge chain involving K373, K248, and E223 that links helices 2, 3, and 10 (see Figure 6). Residue D216 could form a hydrogen bond with the backbone oxygen of K223 and D191 is the N-terminal residue of the first helix. D227 is highly exposed and shows no close association with other

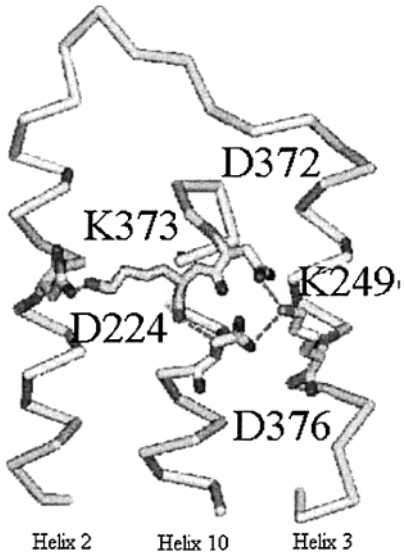

FIGURE 6: Hydrogen-bonding network of helices 2, 3, and 10 of colicin A (*18*) modeled using Quanta. The side chain of D224 on helix 2 makes a hydrogen bond with K373 on helix 10 while neighboring side chains of D372 and D376 on helix 10 hydrogen bond with K249 on helix 3. The model uses coordinates from monomer A of PDB file 1COL.

residues, and thus, the reason for its particular instability is unclear. The residues also show varying levels of surface exposure; D216 is very exposed, but so are D241, D227, and D274, while D372/376 are less exposed. A more quantitative description of the environments of the residues can be obtained from predicted changes in pK_a compared to the values obtained in free solution. Warwicker (*22*) has used a simplified (without dielectric boundaries) Debye−Hückel calculation which uses the electrostatic interactions in the unfolded protein as a baseline. This has the benefit of having predicted a greater stability for colN-P compared to colA-P at low pH, although the difference is less than that observed by experiment. This method predicts the ΔpK_a values shown in Table 1. The common feature among the residues that decrease secondary structure as well as tertiary structure is that they have negative values for ΔpK_a whereas for D216 this is strongly positive. Residue D191, our best model for the pH 7.0 molten globule is very slightly positive.

The rate of insertion of the colicin A P-domain mutants into brominated DOPG vesicles supports the findings of the other experiments that most of the colicin A mutants are less tightly folded than wild-type colicin A. D191A, the "best" molten globule (Table 1), showed clear kinetics and was studied at neutral pH, and this showed that a basal pH independent insertion was introduced by the mutation. At pH 5.0, the insertion is 5-fold faster and similar to the WT, showing that protonation of the remaining aspartates is still required for the full insertion rate. Hence, no single residue mutation can achieve the membrane insertion rate provided by acidic condition.

The apparent formation of a molten globule at pH7 by mutants of colicin A whose hydrogen-bonding network has been broken should be compared with work on colicin E1 which inserts into membranes at low pH and has been found to have a pH-sensitive trigger mechanism (*40*). The trigger in colicin E1 proposed to be formed by three hydrogen bonds: between K406 (colN═K260) and D410 (D264), between S405 (N259) and D408 (D262), and between K420 (N274) and D410 (D264). Disruption of one or all of these H bond links may cause a cascade of structural events that leads to the movements observed in a series of tryptophan residues, both naturally occurring and introduced into the P-domain of colicin E1 by site-directed mutagenesis (*40*, *41*). The hydrogen bonds of the proposed pH trigger of colicin E1 are between side chains on the same helix rather than between helices and are not a trigger to form a molten globule state since this does not occur in colicin El (*9*). The increased insertion rate of colE1-P into membranes at low pH is thus probably due to some limited local unfolding as evidenced by small changes in near UV−CD (*9*). Thus a common feature is that K420 is the analogue of the destabilizing 274 site in our study (N274 in colN and D478 in colA).

It is therefore clear that the characteristic acidic molten globule of colA-P is a result of the protein's extreme sensitivity to the charge on individual aspartate residues. While involvement in charge pairs and hydrogen bonds may explain the role of D372A and D376A the reasons for the observed behavior of the mutants D191A and D227A is not at all clear from inspection and or even calculated shifts in pK_a (*22*). Why is colicin A so dependent upon surface negative charges for its stability, and second, what process unfolds colicin N to allow it to insert into the target cell membrane? It appears evident that these closely related colicins (A and B on one side and N on the other) have divergently evolved two quite separate insertion mechanisms. If we can understand what the differences are, then we may begin to understand how these water-soluble colicin proteins translocate across two membranes.

The sensitivity of colA-P to Asp to Ala substitutions reveals a rare example of the contribution of surface charge to protein stability. In most cases where alanines replace charged side chains small effects are seen and these are often stabilizing (*42*, *43*). The cases where significant destablization occurs upon Asp removal seem restricted to strong salts bridges such as in T4 lysozyme (*39*). Meeker and colleagues carried out a comprehensive study of charged to Ala mutations in Staphylocccal nuclease and concluded that most of the small effects observed were due to consequences of mutation (packing and bonding) other than the charge removal (*44*). Hence, the effects of truncation beyond the β-carbon do not depend on charge and for example the average stability loss for aspartate and asparagine substitutions is almost identical (*44*). The situation in colicin A is thus atypical and our future work will be aimed at defining precisely the role of aspartyl residues in stabilizing the native structure. It should be remembered that this work was concerned solely with the tertiary structure changes associated with membrane penetration whereas the literature examples are concerned with changes in the free energy of folding based upon secondary structure content. The results indicate that surface aspartate residues of colicin A have a more widespread relevance for protein stability.

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NOTE ADDED IN PROOF

Readers are also recommended to read the study by Perl and Schmid of Electrostatic Stabilization of a Thermophilic Cold Shock Protein. (2001) *J. Mol. Biol. 313*, 343−357.

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