Colicin E1 forms a dimer after urea-induced unfolding

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Unfolding of the soluble colicin E1 channel peptide was examined with the use of urea as a denaturant; it was shown that it unfolds to an intermediate state in 8.5 M urea, equivalent to a dimeric species previously observed in 4 M guanidinium chloride. Single tryptophan residues, substituted into the peptide at various positions by site-directed mutagenesis, were employed as fluorescent probes of local unfolding. Unfolding profiles for specific sites within the peptide were obtained by quantifying the shifts in the fluorescence emission maxima of single tryptophan residues on unfolding and plotting them against urea concentration. Unfolding reported by tryptophan residues in the C-terminal region was not characteristic of complete peptide denaturation, as evidenced by the relatively blue-shifted values of the fluorescence emission maxima. Unfolding was also monitored by using CD spectroscopy and the fluorescent probe 2-(*p*-toluidinyl)-naphthalene 6-sulphonic acid; the results indicated that unfolding of helices is concomitant with the exposure of protein non-polar

surface. Unfolding profiles were evaluated by non-linear least-squares curve fitting and calculation of the unfolding transition midpoint. The unfolding profiles of residues located in the N-terminal region of the peptide had lower transition midpoints than residues in the C-terminal portion. The results of unfolding analysis demonstrated that urea unfolds the peptide only partly to an intermediate state, because the C-terminal portion of the channel peptide retained significant structure in 8.5 M urea. Characterization of the peptide's global unfolding by size-exclusion HPLC revealed that the partly denatured structure that persists in 8.5 M urea is a dimer of two channel peptides, tightly associated by hydrophobic interactions. The presence of the dimerized species was confirmed by SDS/PAGE and inter-molecular fluorescence resonance energy transfer.

Key words: denaturation, fluorescence, protein, tryptophan.

INTRODUCTION

The colicin E1 protein is a toxin-like antibiotic produced by *Escherichia coli* harbouring the ColE1 plasmid. It is cytotoxic to *E. coli* and other closely related bacteria that do not carry the ColE1 plasmid but do possess the outer membrane receptor for colicin E1. Colicin E1 kills its target cells by forming a dissipative and voltage-dependent channel in the cytoplasmic membrane. It is a monomeric multi-domain protein containing a receptor-binding domain, a translocation domain for crossing the outer membrane of *E. coli*, and a channel domain that forms the cytoplasmic membrane channel. The channel domain can be studied independently because it is resistant to proteolytic degradation and can be isolated from the N-terminal two-thirds of the colicin E1 protein. Lacking its receptor-binding and translocation domains, the channel-forming peptide (channel peptide) cannot penetrate the outer membrane of target bacterial cells. However, the channel peptide forms active channels in osmotically shocked whole cells [1], in membrane vesicles [2] and in planar bilayers [3].

The X-ray structure of the soluble colicin E1 channel peptide has been elucidated [4]. The colicin E1 channel peptide has ten α -helical segments arranged in three layers, with two distinctively hydrophobic antiparallel helices (helices 8 and 9) buried within the core of the structure. It was previously demonstrated for colicin E1 that the buried helices 8 and 9, located in the C-terminal region of the soluble peptide [4–7], insert into the membrane bilayer as a hydrophobic α -helical hairpin that acts as a membrane anchor [8–10]. Furthermore, it has been shown that, before membrane insertion, the channel peptide undergoes a

conformational change into an insertion-competent state that is induced *in vitro* by acidic pH (4 or lower) or on the binding of detergents. The insertion-competent state involves an N-terminal structural rearrangement resulting in the increased exposure of the peptide's hydrophobic core [5,7,11].

Understanding the structural organization and folding of the soluble colicin E1 channel peptide is crucial to the elucidation of the peptide's mechanism of membrane insertion. The unfolding of the colicin E1 channel peptide has been studied previously with guanidinium chloride (GdmCl) as a denaturant [12]. Here we describe the urea-induced denaturation of the colicin E1 channel peptide. Although the molecular structures of urea and GdmCl are similar, GdmCl is a stronger denaturant on the basis of its charge and its ability to mask electrostatic interactions during protein unfolding. Consequently the results of urea-induced denaturation can differ from those of GdmCl-induced denaturation [13]. Global peptide unfolding in urea was monitored with size-exclusion HPLC and with CD and fluorescence spectroscopies. The denaturation of local peptide segments was investigated with the use of single Trp residues as intrinsic fluorescent probes of unfolding.

MATERIALS AND METHODS

Preparation of mutants, isolation of colicin E1 and its thermolytic channel peptide and preparation of channel peptide adducts with *N*-acetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine (AEDANS)

Single-Trp mutants of colicin E1 were constructed by site-directed mutagenesis as described previously [6,7]. Colicin E1

Abbreviations used: AEDANS, *N*-acetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine; channel peptide, channel-forming peptide; D_2^1 , denaturant concentration at unfolding transition midpoint; λ_{em}^{max} , emission wavelength maximum; FRET, fluorescence resonance energy transfer; GdmCl, guanidinium chloride; NATA, *N*-acetyltryptophanamide; TNS, 2-(*p*-toluidinyl)naphthalene 6-sulphonic acid.

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was purified from a *lex A⁻ E. coli* strain harbouring the plasmid pSKE1⁻, which encoded the appropriate mutant as detailed previously [6,7]. Trp⁻ peptide–AEDANS adduct was prepared exactly as described previously [7].

Unfolding conditions

A 9 M urea solution was prepared by dissolving Sequanal grade urea (Pierce, Rockford, IL, U.S.A.) in 100 mM NaCl/10 mM dimethylglutaric acid buffer (pH 6.0). The colicin E1 C-terminal thermolytic channel peptide, in 100 mM NaCl/10 mM dimethylglutaric acid buffer (pH 6.0), was mixed with the appropriate amount of 9 M urea to provide solutions from 0 to 8.5 M urea and 0.1 mg/ml channel peptide (0.5 mg/ml for SDS/PAGE) (final concentration). For binding experiments involving 2-(*p*-toluidinyl)naphthalene 6-sulphonic acid, sodium salt (TNS), 10 μ l of 4.5 mM TNS (Molecular Probes, Eugene, OR, U.S.A.) in methanol was added to 1 ml of urea/peptide solution. Peptide concentrations were calculated with the use of the ϵ_{280} values reported earlier [6]. Solutions were incubated at room temperature for at least 30 min before analysis because it was determined that this was sufficient time to reach constant values for fluorescence and CD measurements.

Spectroscopic measurements and size-exclusion chromatography

Tryptophan absorbance and fluorescence spectra and CD spectra were recorded as described previously [12]. Combined tyrosine and tryptophan fluorescence was monitored by scanning from 286 to 450 nm with excitation at 280 nm. The fluorescence emission of TNS was scanned from 390 to 500 nm while the sample was excited at 370 nm. The fluorescence emission of TNS was slightly enhanced in urea and thus a TNS blank was subtracted for each emission spectrum of TNS/peptide in urea. Trp/AEDANS fluorescence resonance energy transfer (FRET) was measured with Trp⁻ peptide–AEDANS adduct fixed at a concentration of 0.05 mg/ml (2.5 μ M) and denatured in 8.5 M urea. The denatured adduct was titrated with wild-type peptide in 0.01 mg/ml (0.05 μ M) increments. Trp/AEDANS emission spectra were recorded with 295 nm excitation. Size-exclusion chromatography was performed as described previously [14] in 0–8.5 M urea.

Data analysis

Unfolding profiles were plotted with a tryptophan fluorescence ratio ($F_{\lambda 1}/F_{\lambda 2}$) as described previously [12]. Unfolding transitions were fitted by using non-linear least-squares analysis (MicroCal Origin; MicroCal Software, Northampton, MA, U.S.A.) and a modified version of the Boltzman sigmoidal equation:

$$X_{[D]} = \{(X_N + m_N[D]) - (X_U + m_U[D])\} / \{1 + \exp[(D - D_{1/2}^2)/dx] + (X_U + m_U[D])\}$$

where $X_{[D]}$ is the value of a given measurement (depending on the technique used to monitor unfolding) at a particular concentration of denaturant D, and the remaining six terms are fitting parameters. The terms X_N and X_U are the values of the measured quantity extrapolated to zero concentration of denaturant, and m_N and m_U are the slopes for the dependence of X_N and X_U on denaturant concentration. The unfolding transition midpoint, a denaturant concentration, is termed $D_{1/2}^2$; dx is the width of the fitting subinterval.

RESULTS

Single-tryptophan mutants and their stability

The wild-type channel peptide has three Trp residues located at positions 424, 460 and 495 (Figure 1). Two of the three Trp residues were replaced by Phe to produce each of the three single-

Figure 1 Sequence of the colicin E1 thermolytic channel peptide, with the use of the numbering system for whole colicin E1

The ten α -helical regions highlighted in black are from the colicin E1 channel peptide crystal structure [4]. Each of the three naturally occurring Trp residues is indicated with an open box and the locations for the insertion of a Trp residue by site-directed mutagenesis are indicated with black boxes. The C-terminal hydrophobic α -helical hairpin domain is indicated by the bold line.

Figure 2 Unfolding of wild-type channel peptide in urea monitored by far-UV CD spectroscopy

The curve shown is the sigmoidal fit to the data. The unfolding profile ($[\theta]_M$ at 222 nm as a function of urea concentration) depicts the loss in helical content with increasing concentration of denaturant. The CD spectra of the native (broken line) and denatured (solid line) peptides are shown in the inset. Conditions were as described in the Materials and methods section.

Table 1 Urea $D_{1/2}^1$ values of wild-type and single-Trp mutant peptides

$D_{1/2}^1$ values were obtained from non-linear least-squares regression (see the Materials and methods section) and represent the denaturant concentration at the unfolding transition midpoint. Single-Trp mutant peptide $D_{1/2}^1$ values were obtained from tryptophan fluorescence on denaturation. Values are means $\pm$ S.E.M. 'Wild-type (TNS)' is the $D_{1/2}^1$ for TNS binding after urea-induced unfolding; 'wild-type (CD)' is the $D_{1/2}^1$ for urea-induced unfolding as measured by CD spectroscopy; 'wild-type' is the $D_{1/2}^1$ obtained from urea-induced unfolding measured by tryptophan fluorescence.

Peptide	$D_{1/2}^1$ (M)
Wild-type (TNS)	6.2 ± 0.1
Wild-type (CD)	6.4 ± 0.1
Wild-type	6.7 ± 0.1
Trp-355	4.9 ± 0.1
Trp-367	4.9 ± 0.1
Trp-404	4.8 ± 0.1
Trp-413	5.7 ± 0.1
Trp-424	6.1 ± 0.1
Trp-431	5.8 ± 0.1
Trp-443	5.5 ± 0.2
Trp-460	6.1 ± 0.1
Trp-484	4.6 ± 0.1
Trp-495	5.3 ± 0.1
Trp-507	5.7 ± 0.1

Trp mutants Trp-424, Trp-460 and Trp-495 [6]. A series of single-Trp mutants of the channel domain has previously been reported and characterized [6]. The secondary structure of the mutant peptides ($47 \pm 5\%$ α -helix), determined by CD spectroscopy, is analogous to that of wild-type peptide [6] and the channel-forming activities of the channel peptide mutants both *in vivo* and *in vitro* are comparable to that of the wild-type peptide [5,6]. These results indicated that the mutations do not perturb the structure of the native peptide to any significant degree. Furthermore, it was previously demonstrated that the mutations do not alter the peptide's global unfolding equilibria [12].

Unfolding of wild-type peptide monitored by CD spectroscopy

The collapse of secondary structure during unfolding was observed by CD spectroscopy. An unfolding profile depicting the loss in helical content with increasing concentration of denaturant was generated by plotting urea concentration against $[\theta]_M$ at 222 nm (Figure 2). No significant decrease in helical content occurred in urea solutions at 5.5 M or lower. However, between 5.5 and 7.5 M urea, a large decrease in secondary structure occurred in a single transition, with only a residual amount of helix content remaining at 8.5 M urea. A non-linear least-squares fit to the CD unfolding profile of the wild-type peptide provided a $D_{1/2}^1$ of 6.4 ± 0.1 M (Table 1).

Binding of TNS to wild-type peptide during unfolding

The TNS fluorophore was used to examine the exposure of hydrophobic regions of the peptide after being unfolded by urea. When this fluorophore binds to exposed hydrophobic patches on protein surfaces it concomitantly undergoes a blue shift in its fluorescence emission wavelength maximum (λ_{em}^{max}) [15]. The shift in the TNS fluorescence λ_{em}^{max} in the presence of peptide as a function of urea concentration was used to produce an unfolding profile (Figure 3). The unfolding profile had a single major transition, depicting a sharp increase in TNS binding (blue shift) that occurred between 5.5 and 7 M urea with a $D_{1/2}^1$ of

Figure 3 Binding of TNS to wild-type channel peptide monitored by the fluorescence ratio F_{419}/F_{426} as a function of urea concentration

The curve shown is the sigmoidal fit to the data. The channel peptide was unfolded as described in the Materials and methods section. The fluorescence emission of TNS was scanned from 390 to 500 nm while the sample was excited at 370 nm. The excitation and the emission bandpasses were 4 nm.

6.2 ± 0.1 M (Table 1) but showed no significant change in TNS binding below 5 M urea.

Site-specific unfolding monitored by tryptophan fluorescence

The effect of urea on the fluorescence λ_{em}^{max} of tryptophan in solution was deemed negligible by monitoring the fluorescence of *N*-acetyltryptophanamide (NATA) as a function of urea concentration (results not shown). Furthermore, as a control, the tyrosine fluorescence of a Trp⁻ channel peptide was monitored after unfolding (with excitation at 280 nm): no wavelength shift was detectable (results not shown).

The fluorescence λ_{em}^{max} of the wild-type channel peptide, which contains three tryptophan residues located in the C-terminal half of the peptide (Trp-424, Trp-460 and Trp-495), underwent a 15 nm red shift (Table 2) on unfolding. However, no change in $F_{\lambda 1}/F_{\lambda 2}$ (see the Materials and methods section) occurred until very high urea concentrations (more than 6 M) were reached (Figure 4), resulting in a high $D_{1/2}^1$ value for the unfolding profile of wild-type peptide (6.7 ± 0.1 M; Table 1).

To examine the unfolding of specific segments within the peptide, changes in the fluorescence λ_{em}^{max} for 11 single-Trp mutant proteins were measured. The wavelength shift in the tryptophan fluorescence λ_{em}^{max} of the various mutant peptides between their folded (0 M urea) and unfolded (8.5 M urea) states are shown in Table 2. When fully folded, each single-Trp mutant peptide had a characteristic tryptophan fluorescence λ_{em}^{max} that can be attributed to the unique degree of exposure of each tryptophan to the aqueous medium and the chemical environment of these residues in the native peptide. These results are analogous to the fluorescence λ_{em}^{max} , as determined earlier [6]. The fluorescence λ_{em}^{max} of each Trp changed on titration with denaturant; with the exception of Trp-443, each single-Trp

Table 2 Fluorescence $\lambda_{em}max$ for mutant peptides in their folded and partly denatured states in both urea and Gn-HCl

Abbreviations: F, folded peptides in buffer; UF_{UR}, partly unfolded peptides in 8.5 M urea; I_{GdmCl}, partly denatured intermediate structure present in 4 M GdmCl [12].

Peptide	$\lambda_{em}max(F)$ (nm)	$\lambda_{em}max(UF_{UR})$ (nm)	$\lambda_{em}max(I_{GdmCl})$ (nm)
Trp-355	329	345	344
Trp-367	318	347	346
Trp-404	334	345	345
Trp-413	328	342	344
Trp-424	324	340	339
Trp-431	330	337	337
Trp-443	337	330	330
Trp-460	319	338	338
Trp-484	322	328	329
Trp-495	323	329	329
Trp-507	322	337	336
Wild-type	321	336	337

Figure 4 Relative wavelength shift ($F_{\lambda_1}/F_{\lambda_2}$) for wild-type channel peptide and mutant single-Trp channel peptides as a function of urea concentration

The curves shown are the sigmoidal fits to the data. Unfolding conditions were as described in the Materials and methods section. Samples were excited at 295 nm and their emission was scanned from 303 to 450 nm. The excitation and the emission bandpasses were 4 nm.

mutant peptide exhibited a characteristic red-shifted fluorescence $\lambda_{em}max$ in 8.5 M urea. The largest shift in fluorescence $\lambda_{em}max$ (29 nm) was reported by Trp-367, whereas Trp-484 and Trp-495 reported small shifts (6 nm). The fluorescence $\lambda_{em}max$ of Trp-443 underwent a 7 nm blue shift on being unfolded by 8.5 M urea (Table 2). Only peptides Trp-355, Trp-367 and Trp-404 had

fluorescence $\lambda_{em}max$ values near (within 5 nm of) those observed for NATA in solution (350 nm). For each peptide at a given urea concentration, the fluorescence intensities at the wavelengths given in Table 2 were used to calculate the ratio of fluorescence ($F_{\lambda_1}/F_{\lambda_2}$) as described in the Materials and methods section to provide unfolding profiles

Figure 5 Size-exclusion HPLC elution chromatograms of colicin E1 channel peptide in urea

The peptide's elution (elution volume, V_e) was detected by UV absorbance at 280 nm. The column was equilibrated to the appropriate urea concentration (M) as indicated in the figure and calibrated with protein standards as described in the Materials and methods section. The calculated Stokes radii are shown (in Å) for each peak.

Figure 6 Dimerization of urea-denatured wild-type channel peptide detected by SDS/PAGE

Lane 1, peptide incubated in buffer; lane 2, peptide incubated in 4 M urea; lane 3, peptide incubated in 6.5 M urea; lane 4, peptide incubated in 8.5 M urea (see unfolding conditions in the Materials and methods section). Band a shows dimerized channel peptide (molecular mass 36 kDa); band b shows monomeric channel peptide (molecular mass 18 kDa). The positions of molecular mass standards are indicated (in kDa, top to bottom) at the left: phosphorylase b, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme.

Figure 7 Dimerization of urea-denatured channel peptide detected by intermolecular Trp/AEDANS FRET

Upper panel: AEDANS-labelled Trp⁺ peptide was titrated with wild-type peptide and the increase in AEDANS acceptor ($\lambda_{em,max}$ 480 nm) fluorescence was measured (8.5 M urea; see the Materials and methods section). Lower panel: dimerization, rather than the aggregation of higher order, was confirmed by the linearity ($r = 0.98$) of the plot.

(Figure 4). All of the single Trp residues exhibited single transition sigmoidal unfolding profiles in urea. To compare the unfolding profiles of each local peptide segment, the midpoint ($D_{1/2}$) of each unfolding transition was calculated (Table 1) by non-linear least squares regression (see the Materials and methods section).

Reversibility of unfolding

The urea-induced unfolding of wild-type channel peptide was not reversible. Neither rapid dilution, stepwise dilution (0.25 M increments) nor dialysis of an 8.5 M urea solution containing wild-type peptide could restore the fluorescence $\lambda_{em,max}$ of the denatured peptide to that of the native peptide (results not shown). Furthermore, the urea-denatured peptide could not be refolded in the presence of salt at high concentration (4 M NH_4Cl ; results not shown). These findings contrast, however, with the previous observation that the GdmCl-induced unfolding of the colicin E1 channel peptide was reversible [12].

Size-exclusion chromatography and SDS/PAGE

The peptide's denaturation in urea was also monitored by size-exclusion HPLC (Figure 5). From 0 to 4 M urea, the channel

peptide eluted as a single peak with a calculated Stokes radius between 20 and 25 Å (Figure 5). At 6.5 M urea the presence of aggregated protein was evident by a shift to smaller elution volumes. However, at 8.5 M urea only a single peak was observed, at both peptide concentrations, corresponding to a protein with a Stokes radius of 72 Å. Although the channel peptide does contain a single cysteine residue, aggregation was unaffected by the inclusion of dithiothreitol in urea solutions.

To examine the nature of the aggregated species detected by chromatography, urea-denatured peptide was subjected to SDS/PAGE under reducing conditions. Great care was taken to ensure the sufficient reduction of any intermolecular disulphide bonds that might have formed. Electrophoresis revealed that the two peaks observed in size-exclusion HPLC represented monomeric (Figure 6, band b) and dimeric (Figure 6, band a) species of the channel peptide.

FRET

To confirm peptide dimerization in solution (8.5 M urea), Trp (donor) to AEDANS (acceptor) intermolecular FRET was measured between wild-type channel peptide and AEDANS-labelled mutant channel peptide devoid of Trp (Trp⁻). Intermolecular Trp/AEDANS FRET was not detected between the two protein species in the native state (0 M urea; results not shown). Intermolecular Trp/AEDANS FRET (Figure 7, upper panel) was observed in 8.5 M urea and was shown to increase on titration of acceptor (Trp⁻ peptide–AEDANS adduct, 2.5 µM) with donor (wild-type peptide, 0 to 3 µM). The linearity of the plot of the mole fraction of acceptor against acceptor fluorescence (Figure 7, lower panel) verified that the peptide forms a dimer in 8.5 M urea and not higher-order aggregates [16,17].

DISCUSSION

Data on unfolding obtained by CD spectroscopy (Figure 2) implied that the peptide was significantly denatured in 8.5 M urea. (Although the presence of a heterogeneous protein solution consisting of partly folded monomers and dimers might complicate the interpretation of the unfolding data obtained by CD spectroscopy, general conclusions about the approximate secondary structure content can be made.) However, the tryptophan fluorescence $\lambda_{\text{em}}^{\text{max}}$ of the wild-type peptide was blue-shifted (Table 2) relative to a fully solvent-exposed Trp residue (NATA $\lambda_{\text{em}}^{\text{max}}$ 350 nm). Furthermore, the binding of the hydrophobic probe TNS to the peptide indicated the existence of structure at high concentrations of urea. In the presence of peptide, the TNS fluorescence $\lambda_{\text{em}}^{\text{max}}$ exhibited a blue shift from 426 to 419 nm in urea concentrations ranging from 5.5 to 7 M (Figure 3), depicting the exposure of hydrophobic surface on unfolding. In 8.5 M urea, the TNS fluorescence $\lambda_{\text{em}}^{\text{max}}$ reached a plateau at 419 nm, indicating that the exposed non-polar region remained intact even at high denaturant concentrations. Notably, on unfolding, the loss of helical content was concomitant with the exposure of the hydrophobic surface detected by TNS binding. The midpoint of the TNS binding transition correlated with the midpoint of the transition reported by CD spectroscopy (Table 1). Collectively, these results show that the channel peptide was not completely denatured in 8.5 M urea and that the partly unfolded species that exists in 8.5 M urea has a small amount of secondary structure and consists of a large cluster of hydrophobic residues. The channel peptide is, however, fully denatured in 7 M GdmCl [12].

Even though urea is expected to increase protein solubility, the results of size-exclusion HPLC suggested that the peptide aggregates on denaturation, forming a dimerized species in 8.5 M

urea. The structure that persists in 8.5 M urea has an elution volume of 23 ml, corresponding to a Stokes radius of 72 Å (Figure 5). However, a random coil of approx. 180 amino acid residues has a radius of gyration of approx. 40 Å [18]. Therefore the 178-residue channel peptide, only partly denatured in 8.5 M urea, would be expected to have a Stokes radius of at most 40 Å as opposed to the observed radius of 72 Å. Indeed, the experimental Stokes radius of the peptide denatured in 7 M GdmCl is 39 Å [16]. The large Stokes radius observed in urea thus represents two tightly associated and partly unfolded peptide monomers.

The presence of the dimerized species was confirmed by SDS/PAGE (Figure 6) and FRET (Figure 7). The magnitude of the interactions that give rise to the dimerized species was demonstrated by the inability of SDS to separate the dimer fully under reducing conditions. Although size-exclusion chromatography established that only dimerized peptide is present in 8.5 M urea (Figure 5), SDS did dissociate the dimer somewhat because some monomeric peptide was detected in the sample denatured in 8.5 M urea (Figure 6).

The channel peptide contains a single buried cysteine residue (Cys-505; Figure 1) and it is plausible that dimerization results from the formation of an intermolecular disulphide bond between two unfolded monomeric peptides. However, the inclusion of a large excess of dithiothreitol in urea solutions had no effect on the results of size-exclusion chromatography. In addition, the dimerized peptide was detected by SDS/PAGE in the presence of 2-mercaptoethanol. Additionally, dimerization was observed in solution by FRET. Intermolecular Trp/AEDANS FRET (Figure 7) was observed in 8.5 M urea and was shown to increase linearly on the titration of acceptor with donor. The ability of denatured AEDANS-labelled peptide to exchange with wild-type peptide added after unfolding also demonstrates that dimerization is non-covalent.

The channel peptide is unusually hydrophobic [19] and the region of the peptide that retains structure in the partly denatured state contains only hydrophobic residues [12]. Therefore dimerization of the channel peptide after urea-induced unfolding probably results from very strong hydrophobic interaction between partly denatured monomers. Furthermore, maximum TNS binding occurs in 8.5 M urea, demonstrating the hydrophobic nature of the partly unfolded and dimerized structure.

It is interesting to note that the fluorescence $\lambda_{\text{em}}^{\text{max}}$ of the single-Trp mutant peptides in 8.5 M urea corresponded remarkably to their fluorescence $\lambda_{\text{em}}^{\text{max}}$ in the intermediate partly unfolded structure that exists in 4 M GdmCl (Table 2). Also, analogously to the urea-unfolded structure described here, the GdmCl-induced intermediate (4 M GdmCl) possessed little secondary structure [12]. It therefore seems that in 8.5 M urea the channel peptide is unfolded to an intermediate state that corresponds to the partly unfolded structure described earlier [12]. Indeed, previous results from our laboratory have shown that the GdmCl intermediate is a dimerized species [16].

Denaturation of the channel peptide in urea was not reversible. However, denaturation of the channel peptide in GdmCl was reversible [12]. Poor reversibility of the protein-folding process in urea as opposed to GdmCl has been reported elsewhere [20]. At high urea concentrations the channel peptide seems irreversibly trapped in a partly denatured state consisting of a tight cluster of hydrophobic residues. Although it might be that dimerization of this non-polar intermediate leads to irreversible unfolding, the peptide has been shown to form a similar dimerized species in GdmCl, where unfolding is reversible. One possibility for explaining this difference is that the high ionic strength of GdmCl masks non-specific charge–charge interactions that might other-

Figure 8 Model illustrating the urea-induced denaturation of the colicin E1 channel peptide to a partly unfolded and dimerized intermediate that entails the C-terminal portion of the peptide

The ten helical segments are numbered and the hydrophobic α -helical hairpin (helices 8 and 9) is shown in black. (A) The peptide in its native state. Only the general topological positions of the helices are implied. Tryptophan positions are numbered within a rectangle and shaded rectangles represent tryptophans that remain shielded from solvent in the partly unfolded dimer structure. (B) The non-polar and dimerized residual structure involving the hydrophobic α -helical hairpin domain is shown in bold. Tryptophan residues that are involved in the residual structure are numbered in the figure. Regions that are fully unfolded are shown as thin lines; tryptophan residues located within these peptide segments are not labelled.

wise inhibit refolding. Nevertheless the urea-denatured peptide could not be refolded in the presence of 4 M NH_4Cl (results not shown). On protein denaturation, charge masking by GdmCl probably occurs in concert with its chaotropic effect and interaction with the protein exterior. As such, the addition *in trans* of a salt such as NH_4Cl might not effectively mimic the charge-masking effect of the denaturant.

Reversible unfolding of the peptide in GdmCl (but not in urea) might be attributed to the ability of GdmCl to solvate the partly denatured hydrophobic intermediate better. Urea has a tendency to penetrate the non-polar protein core, whereas the charged guanidinium molecule binds only to the protein surface [21]. Therefore, for the denatured intermediate of the colicin E1

peptide, GdmCl might act better as a bridge between the hydrophobic surface of the intermediate and the surrounding aqueous solvent. Fundamentally, this bridging effect has been demonstrated in which GdmCl was shown to increase the water solubility of a non-polar 'solute' (benzene) by acting as a water-solute bridge [22].

The site-specific profiles of urea-induced unfolding obtained from single-Trp mutant peptides were all monophasic and, with the exception of the Trp-443 profile, depicted red shifts in fluorescence $\lambda_{\text{em}}^{\text{max}}$ (Table 2; Figure 4). The blue shift in the fluorescence $\lambda_{\text{em}}^{\text{max}}$ reported by Trp-443 could possibly arise from the residue's actually becoming less exposed to solvent. If residue Trp-443 were located at the dimer interface it would be expected to report a blue shift in its $\lambda_{\text{em}}^{\text{max}}$ on dimerization. However, although the fluorescence $\lambda_{\text{em}}^{\text{max}}$ of Trp-443 is significantly blue-shifted at 4 M urea (Figure 4), dimer formation does not occur in 4 M urea (Figure 5). Therefore, rather than resulting from dimer formation, the blue shift in the fluorescence $\lambda_{\text{em}}^{\text{max}}$ of residue Trp-443 most probably results from the alleviation of intrinsic quenching of the Trp after denaturation. Investigation of the X-ray structure data suggests that Trp-443 might be quenched by the nearby residues Tyr-478 and/or Lys-470 [4]. A blue shift in the fluorescence $\lambda_{\text{em}}^{\text{max}}$ of Trp-443 was also reported for unfolding in GdmCl [12]. Notably, Royer *et al.* [23] observed a similar intrinsic quenching process in *trp* apo-repressor, in which the intrinsic quenching of a Trp residue by a nearby Asn side chain was alleviated on unfolding.

Because the denaturation of the channel peptide in urea was not reversible, the calculation of equilibrium constants and free energies of unfolding would not be valid. Therefore the unfolding profiles were fitted with the use of a modified sigmoidal fitting function (see the Materials and methods section) which calculates the $D_{1/2}$ of the denaturation profile. The $D_{1/2}$ values obtained serve only as a means for comparing the various unfolding profiles obtained for the single-Trp mutant peptides.

The $D_{1/2}$ values reported by each single Trp for urea-induced unfolding are listed in Table 1. The unfolding profiles provided by the N-terminal residues Trp-355, Trp-367 and Trp-404, which report full denaturation in 8.5 M urea, have low $D_{1/2}$ values, indicating that this region of the peptide is most susceptible to urea denaturation. Higher $D_{1/2}$ values for Trp residues located in the peptide's C-terminal region indicated that that locale is less susceptible to urea-induced denaturation (Table 1). It should be noted that the urea $D_{1/2}$ values were not dependent on peptide concentration (results not shown). In addition, after 30 min of incubation in urea (see the Materials and methods section), the $D_{1/2}$ values remained constant with increasing time.

In contrast with other residues located in the C-terminal portion of the peptide, Trp-484 provided a low $D_{1/2}$ value analogous to the $D_{1/2}$ values of the N-terminal Trp residues (Table 1). Residue Trp-484 is located in a buried and hydrophobic region of the peptide beneath helix 1 [4] and was expected to give a higher $D_{1/2}$ value, analogous to that of residues Trp-460, Trp-495 and Trp-507. Because Trp-484 is located beneath helix 1, the fluorescence $\lambda_{\text{em}}^{\text{max}}$ of Trp-484 would probably be sensitive to movement of the peptide's N-terminal helices, which unfold at lower concentrations of denaturant. Therefore it might be that the lower $D_{1/2}$ value of Trp-484 results largely from the movement of helix 1 (B. A. Steer, unpublished work).

Single Trp residues in the C-terminal two-thirds of the peptide possessed fluorescence $\lambda_{\text{em}}^{\text{max}}$ values that were substantially blue-shifted (Table 2) relative to a fully solvent-exposed tryptophan residue. These results, along with the finding discussed above, that the C-terminal residues report higher $D_{1/2}$ values for unfolding, indicate that it is the C-terminal portion of the peptide

that retains structure in 8.5 M urea. The results of site-specific unfolding analysis by Trp fluorescence demonstrated that the partly unfolded and dimerized structure that is composed of clustered hydrophobic residues involves the C-terminal region of the peptide. Indeed, the C-terminal one-third of the channel peptide contains a remarkably pronounced stretch of hydrophobic residues [18] (Figure 1). This hydrophobic C-terminal region of the channel peptide is functionally significant because it contains the hydrophobic α -helical hairpin domain that has been proposed to penetrate the cytoplasmic membrane bilayer of the target bacterium initially on channel formation [8]. This domain is buried in the soluble native structure of the channel peptide [4] and contains residues Trp-484, Trp-495 and Trp-507, which remain considerably buried in the peptide unfolded by urea (Table 2).

An outline of the combined results of this study is shown in an illustrative model that describes the channel peptide's denaturation in urea (Figure 8). The peptide is denatured to a partly unfolded and dimerized structure that has little helical content but entails clustered residues of the peptide's C-terminal hydrophobic α -helical hairpin domain. This domain is believed to insert into the membrane as two tightly associated antiparallel helices [8–10]; this study has shown that although its secondary structure is diminished, this region of the peptide remains intact and tightly clustered at high concentrations of urea. Exposure of this highly non-polar core region of the peptide leads to dimerization by hydrophobic association.

Interestingly, on extraction of the colicin E1 channel peptide from membrane vesicles, the formation of a dimeric peptide species, which is resistant to dissociation and provides the same SDS/PAGE banding pattern observed in this study, has been observed in our laboratory (B. A. Steer, unpublished work). Although it is a possibility that the colicin E1 membrane channel is a dimer, no studies have been able to show that the colicin E1 membrane channel is dimeric. Cell survival experiments [24] and studies on the kinetics of ion release from vesicles [2,25,26] point towards a monomeric membrane channel. The membrane-bound form of the colicin E1 peptide retains helical structure [27] but its structure is significantly different from that of the soluble native channel peptide. Consequently, on membrane insertion the peptide must undergo significant structural rearrangement or unfolding. Thus, in view of the results of this study, it is likely that dimerization of the peptide on extraction from the membrane involves the association of the peptide's exposed hydrophobic α -helical hairpin domain.

Protein aggregation after chemical denaturation in both GdmCl [28] and urea [29] has been observed elsewhere. It has been proposed that denaturant-induced protein aggregation involves the molten globule state [30] because the molten globule involves a native-like structure but with a greater-than-native fraction of non-polar residues exposed to solvent [31]. Although the residual structure of the urea-denatured colicin E1 channel peptide does encompass a substantial portion of the peptide (the

hydrophobic anchor region), it does not resemble the molten globule-like structure described earlier [30] because it does not retain any significant amount of secondary structure. The residual structure most probably does not resemble the native protein folding but instead consists of a large non-native assembly of hydrophobic residues.

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