The Colicin E1 Insertion-Competent State: Detection of Structural Changes Using Fluorescence Resonance Energy Transfer†

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ABSTRACT: The single cysteine residue (Cys-505) located in the hydrophobic membrane anchor domain in the colicin E1 COOH-terminal channel peptide was labeled with the thiol-specific fluorescent reagent IAEDANS [5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid]. The labeling stoichiometry was nearly 1:1 [AEDANS: peptide (mol:mol)]. Eleven single Trp mutants of the channel peptide were prepared, and the FRET efficiency for each Trp residue (donor) and the AEDANS chromophore (acceptor), covalently attached to Cys-505, was measured. The FRET efficiencies for the various donor–acceptor pairs ranged from 15% to ≈100% for the native peptide in solution (pH 6.0). The FRET efficiency for the W-507 channel peptide–AEDANS adduct approached 100% since this adduct showed no detectable Trp fluorescence. Activation of the channel peptide to the insertion-competent state upon addition of the nonionic detergent octyl β-D-glucoside [10 000:1 detergent: peptide (mol:mol)] resulted in decreased FRET efficiencies. The detergent-activated colicin E1 channel peptide–AEDANS adducts possessed significant *in vitro* channel activity at pH 6.0. The relative changes in the FRET efficiencies upon peptide activation ranged from −1% (W-495 channel peptide–AEDANS adduct) to 48% (W-355 channel peptide–AEDANS adduct). A direct correlation existed between the relative change in FRET efficiency upon channel peptide activation and the position of the Trp (donor) residue within the channel peptide primary sequence (higher relative ΔE the closer the Trp donor was to the NH_2 terminus), except for the W-484 channel peptide–AEDANS adduct, which showed a higher relative ΔE than either W-443 or W-460 channel peptide–AEDANS adducts. In addition, fluorescence anisotropy measurements indicated a change in structure upon activation. These results are consistent with the colicin E1 channel peptide being a compact solution structure which, upon detergent activation, exhibits a specific conformational change at the N-terminus. A model is suggested for the activation of colicin E1 COOH-terminal peptide that proposes the unwrapping of the N-terminal segment of the peptide.

Colicin E1 is a toxin-like protein secreted by strains of *Escherichia coli* that carry a colicin-encoding plasmid. The COOH-terminal[1] channel-forming domain of colicin E1 forms a lethal ion channel which depolarizes the cytoplasmic membrane of target bacterial cells. Before its insertion into the membrane, the colicin E1 channel peptide undergoes a structural change, similar to unfolding, into an "insertion-competent" state. Acidic pH (≤4) or the binding of detergents, i.e., SDS and octyl β-D-glucoside, induces this structural change *in vitro*. Recent data suggest, however, that the insertion-competent state does not involve a large unfolding but an increase in side-chain mobility (Merrill et al., 1990).

It was suggested that the activation of colicin E1 by low pH or detergent (Merrill et al., 1990) results in an intermediate folded structure resembling the molten globule state previously described by Ptsitsyn (1987). A "molten globule" membrane insertion intermediate has been proposed as an important feature of the pore-forming domain of colicin A involved in membrane binding and insertion (van der Goot et al., 1991). Van der Goot et al. (1991) showed a direct correlation between the kinetics of membrane insertion of colicin A and the existence of a molten globule conformation. This idea for the involvement of a protein folding intermediate is not new and has been suggested previously as a mechanism for the translocation of low pH-triggered toxins through the membrane of acidic organelles (Olnes et al., 1988; Parker et al., 1990). Also, the aggregation and pH-independent membrane insertion of the human complement C9 protein proceeds through an intermediate analogous to the thermally unfolded forms of various proteins observed by differential scanning calorimetry (Lohner & Esser, 1991).

Fluorescence resonance energy transfer (FRET) can be used to detect structural changes and to estimate distances between two chromophores within a protein (Amhler et al., 1992; Miki, 1991; Gettins et al., 1990). FRET makes possible the detection of changes in protein structure including increased side-chain mobility within the colicin E1 channel peptide that may occur upon activation. Lakey et al. (1991) performed some FRET measurements on the membrane-bound form of the colicin A COOH-terminal fragment. These researchers used Trp residues as the donor with AEDANS as the acceptor chromophore. However, the actual distances were only average values for the three naturally occurring Trp residues found in the colicin A COOH-terminal fragment.

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[1] Abbreviations: Trp, tryptophan; COOH-terminal, carboxy-terminal; NH_2-terminal, amino-terminal; IAEDANS, 5-[[[(iodoacetyl)amino]-ethyl]amino]naphthalene-1-sulfonic acid; AEDANS, 5-[[(acetylamino)-ethyl]amino]naphthalene-1-sulfonic acid; Cys, cysteine; FRET, fluorescence resonance energy transfer; EDTA, disodium ethylenediaminetetraacetate; MWCO, molecular weight cutoff; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; DMG, 3,3-dimethylglutaric acid; ϵ_M, molar extinction coefficient; BNPS-skatole, 3-bromo-3-methyl-2-[(2-nitrophenyl)thio]-3*H*-indole; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPQ, 6-methoxy-*N*-(3-sulfopropyl)-quinolinium; *E*, fluorescence resonance energy transfer percent efficiency.

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In the present study, Trp residues genetically engineered into the thermolytic colicin E1 channel peptide (MW 23K) have been used as fluorescence donors. These various donors (11, in total) originate from the arsenal of single Trp mutants prepared for the COOH-terminal domain of colicin E1 (Merrill et al., 1993). The acceptor chromophore was the AEDANS chromophore covalently attached to the lone cysteine, Cys-505, in the protein. FRET efficiencies were determined for the soluble colicin E1 channel peptide in both its inactive and active (insertion-competent) states. This approach enabled the mapping of specific structural changes which occurred within the colicin E1 channel peptide upon activation from its water-soluble form to the membrane-binding, active state.

MATERIALS AND METHODS

Preparation of Single Trp Colicin E1 Mutants. Colicin E1 single Trp mutants were prepared by site-directed mutagenesis as described previously (Song et al., 1991; Merrill et al., 1993). Trp residues were genetically engineered into the colicin E1 COOH-terminal channel domain by replacing either an endogenous Tyr or Phe with a Trp (conservative substitution). This substitution was completed for all of the single Trp channel peptides except the W-424, W-460, and W-495 mutant peptides, which possess an endogenous Trp residue. The mutant colicin E1 proteins were tested for cytotoxicity using a "spot test" on a lawn of colicin-sensitive *E. coli*, strain B (Merrill et al., 1993). *In vitro* channel-forming activity of the colicin E1 COOH-terminal peptides was tested either as described previously (Merrill & Cramer, 1990) or by using a fluorescence assay (described below). For activity measurements, peptides were treated with octyl β-D-glucoside as described previously (Merrill et al., 1990).

Purification of Colicin E1 and Its Thermolytic Channel Peptide. Colicin E1 was isolated from a lexA$^-$ *E. coli* strain, IT3661, harboring the plasmid, pSKE1$^-$, as described (Song et al., 1991). The colicin E1 COOH-terminal thermolytic channel peptides were purified from a digestion (mild conditions) of the various colicin mutant proteins with thermolysin (Boerhinger Mannheim, Laval, Quebec) as described earlier (Merrill et al., 1993).

Preparation of AEDANS–Channel Peptide Adducts. The AEDANS–channel peptide adduct was prepared as described (Merrill et al., 1990) with the following modifications. The COOH-terminal thermolytic peptide was concentrated to 10 mg/mL in 50 mM sodium phosphate buffer, pH 7.0, by centrifugation using a Microcon concentrator (MWCO 10 000; Amicon, Danvers, MA), and 0.1 mL of concentrated peptide was added to 0.3 mL of 8 M urea, 10 mM EDTA (disodium), and 50 mM Tricine (Sigma, St. Louis, MO) buffer, pH 8.0, containing 0.4 mg/mL of IAEDANS (Molecular Probes Inc., Eugene, OR). The mixture was allowed to react while being mixed on a nutator at 4 °C for 20 h. After reaction, the mixture was passed through a small size-exclusion desalting column (5 mL, 5000 MWCO; Pierce, Rockford, IL) previously equilibrated with 200 mM sodium sulfate/10 mM dimethylglutaric acid (DMG) buffer, pH 6.0. Channel peptide–AEDANS adducts eluted in the void volume of the column whereas the free unreacted probe was retarded in its elution from the column. This resulted in a clean separation of the peptide–adduct from the unreacted IAEDANS (data not shown). Protein-containing fractions were pooled and filtered through a Millipore disposable filter (0.2-μm pore size; Mississauga, ON). Peptide and AEDANS–peptide conjugate concentrations were determined using ϵ_M values of 2.29×10^4 (280 nm; Merrill et al., 1993) and 6×10^3 M^{-1} cm^{-1} (337 nm; Hudson & Weber, 1973), respectively. Due to spectral overlap between the absorbance spectra of the peptide and the attached AEDANS chromophore, AEDANS absorbance (280 nm) was subtracted from the total measured absorbance (280 nm) to calculate peptide concentration. The accuracy of this method for determining colicin E1 channel peptide adduct concentration was verified using the Bradford protein assay (Bio-Rad, Richmond, CA) with native, wild-type channel peptide as the protein standard.

Reaction of Thermolytic Peptide with [^{14}C]Iodoacetamide and Cleavage with BNPS-Skatole. Colicin E1 channel-forming peptide (MW ≈23 K), W-460, was reacted with [^{14}C]-iodoacetamide (NEN, Boston, MA) under similar conditions used for the preparation of AEDANS–peptide adduct. The labeled peptide adduct was cleaved as described by Merrill and Cramer (1990) using the Trp-specific protein cleavage reagent, 3-bromo-3-methyl-2-[(2-nitrophenyl)thio]-3*H*-indole (BNPS-Skatole; Pierce, Rockford, IL). The peptide cleavage fragments were subjected to SDS–PAGE (Laemmli, 1970) and resolved into individual bands visualized by Coomassie Blue R-250 staining. The resolved protein fragments were excised from the gels and solubilized with H_2O_2, and their radioactivity was determined by scintillation counting (Merrill & Cramer, 1990).

Measurement of AEDANS–Channel Peptide Adduct in Vitro Activity. Asolectin (Sigma, St. Louis, MO) vesicles were prepared as previously described (Shiver et al., 1988) and were loaded with 100 mM KCl/10 mM DMG buffer, pH 6.0, and the Cl$^-$-sensitive fluorophore, 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ, 16 mM). The vesicles were dialyzed against 100 mM KCl/10 mM DMG buffer, pH 6.0, immediately before use. Chloride efflux from vesicles was measured according to Illsley and Verkman (1987) with the following modifications. An aliquot of dye-loaded vesicles was added to 3 mL of 100 mM $NaNO_3$/10 mM DMG buffer of the appropriate pH and allowed to equilibrate for 1 min with stirring. A *trans*-negative membrane potential was induced by the addition of valinomycin (15 nM final concn). The fluorescence signal was recorded with λ_{ex} (347 nm) and λ_{em} (445 nm) at 20 °C (excitation and emission slits, 4 nm). Total encapsulated Cl$^-$ was determined by adding Triton X-100 (0.1% final concn).

Spectroscopic Measurements. (1) Absorption Measurements. Absorption spectra were recorded on a Perkin-Elmer λ6 scanning absorbance spectrometer interfaced to a computer with both the sample and reference cells thermostated at 10 °C.

(2) Fluorescence Measurements. Fluorescence excitation and emission spectra were recorded on a PTI Alphascan-2 spectrofluorometer (PTI, South Brunswick, NJ) equipped with a jacketed cell holder set at 10 °C. For experiments to obtain excitation spectra, the excitation wavelength was scanned from 250 to 400 nm (2-nm excitation and emission bandpass), with fluorescence emission monitored at 490 nm. For quantum yield measurements, a wedge depolarizer (Oriel Corp., Stratford, CT; 95% *T*) was placed on the exit side of the excitation monochromator, and exciting light (295 nm, 2-nm slit) was used while the fluorescence emission was detected at right angles. Fluorescence emission was scanned from 300 to 450 nm (2-nm slit). Corrections were made for the appropriate blanks and for the wavelength-dependent bias of the optical and detection systems.

Fluorescence anisotropy measurements were conducted employing Glan-Thompson prism polarizers and using a "T-format" detection system, with the excitation being vertically

polarized. The excitation and emission wavelengths for these anisotropy measurements were 337 and 490 nm, respectively (4-nm slit widths). The "G" instrumental factor, I_{HV}/I_{HH}, was determined using horizontally polarized excitation. Each anisotropy value was the mean of 3 or more separate measurements, with each measurement including a 30-s time scan which was averaged to calculate the anisotropy from the respective horizontal and vertical fluorescence emission components.

(3) Calculation of Donor–Acceptor FRET Efficiencies and Apparent Distances in the Colicin E1 Channel Peptide. The efficiency of FRET (E) between the donor and acceptor chromophores relates to the inverse sixth power of the distance between the chromophores (R):

$$R = R_0(E^{-1} - 1)^{1/6}\ (\text{Å}) \qquad (1)$$

where R_0 is the distance at which the efficiency of transfer is 50%. The efficiency of transfer (E) was calculated from the excitation spectrum of the energy acceptor (AEDANS) using the fluorescence intensity (F) and molar extinction coefficients (ϵ) of the donor (Trp) and acceptor (AEDANS) at 290 and 337 nm, respectively:

$$E = (F_{290}/F_{337} - \epsilon_{A290}/\epsilon_{A337}) \times (\epsilon_{A337}/\epsilon_{D290}) \qquad (2)$$

Therefore, in the excitation method the AEDANS fluorescence intensity [λ_{em}(max), 490 nm] upon excitation in the range of 275–290 nm, due to energy transfer from Trp (donor) to the AEDANS (acceptor), was used to calculate E.

The distance at which the efficiency of transfer is 50% (R_0), can be calculated using the following equation, where J is the spectral overlap integral, κ^2 is the orientation factor, Q_D is the fluorescence quantum yield of the donor, and η is the refractive index of the medium between the chromophores:

$$R_0 = (J\kappa^2 Q_D \eta^{-4})^{1/6} \times (9.7 \times 10^3)\ (\text{Å}) \qquad (3)$$

The spectral overlap integral (J) was determined using the integral [4] where F_D is the fluorescence intensity in the presence of the donor only, ϵ_A is the molar extinction coefficient of the acceptor, and λ is the wavelength:

$$J = \int_{F_D}(\lambda)\ \epsilon_A(\lambda)\lambda^4\ \delta\lambda \Big/ \int_{F_D}(\lambda)\ \delta\lambda \qquad (4)$$

The spectral overlap integral was calculated from the spectral data using a computer program designed solely for that purpose. The orientation factor, κ^2, describes the relative rotational movement of the donor and acceptor chromophores with respect to each other. If both the donor and acceptor rotate freely in a time period shorter than the excited-state lifetime of the donor, then κ^2 can be assumed to be $^2/_3$ (Stryer, 1978). However, the greatest source of inaccuracy in these types of measurements is the magnitude of the orientation factor, i.e., the measure of the relative dipole orientation. The orientation factor, κ^2, can adopt values between 0 and 4, but if the donor and acceptor rotate freely in a time period shorter than the excited-state lifetime of the donor (hence the transfer time), then $\kappa^2 \approx {}^2/_3$. The refractive index of the medium between the donor and acceptor chromophores (η) was taken as 1.4 (Wu & Stryer, 1972; Fairclough & Cantor, 1978; Kawata & Hamaguchi, 1991). The donor fluorescence quantum yield (Q_D) was measured spectrophotometrically as previously described (Hutnik & Szabo, 1990).

RESULTS

(1) Determination of Reaction Stoichiometry and Localization of AEDANS (Acceptor) Chromophore. Eleven single-Trp channel peptides were used to prepare the corresponding AEDANS–peptide adducts. Previously, *in vivo* cytotoxicity and *in vitro* channel activity were tested and found to be comparable with the wild-type peptide for this repertoire of single-Trp mutants of colicin E1 (Merrill et al., 1993). In addition, Merrill et al. (1993) used circular dichroism to determine the secondary structure content for each of the single-Trp thermolytic channel peptides of colicin E1 and established that all of the mutants were comparable in secondary structure content to the wild-type protein.

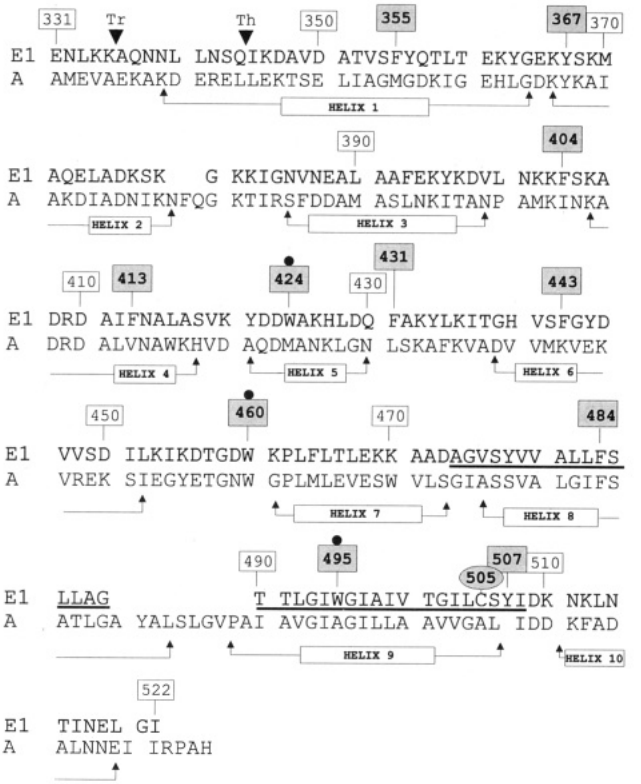

FIGURE 1: Aligned sequences of the channel-forming domains of colicins E1 and A using the numbering system for colicin E1 (Yamada et al., 1982). The ten α-helical regions shown were defined in the colicin A crystal structure (Parker et al., 1992). The hydrophobic α-helical hairpin is indicated by the bold line (Song et al., 1991). The N-termini of the tryptic (Tr) and thermolytic (Th) channel peptides of colicin E1 are shown. For the colicin E1 channel peptide sequence, the sites where a Trp residue has been substituted for either a Phe or Tyr residue are indicated by the larger boxes and the number corresponding to the amino acid sequence position is in boldface. The three naturally occurring Trp residues are marked with a dot above the box. The attachment site for the AEDANS probe (Cys-505) is circled, with the corresponding number in boldface type.

The positions of the various Trp residues (donors) within the colicin E1 channel peptide sequence are shown in Figure 1. The IAEDANS (acceptor) chromophore was reacted with the lone Cys in the peptide (Cys-505). Careful analysis of the absorption spectra of the various adducts indicated that the reaction stoichiometry was close to 1:1 AEDANS:peptide (0.94–1.16). To determine the stringency of the reaction between the single Cys residue and the IAEDANS reagent, the single Trp peptide, W-460, was reacted with [^{14}C]-iodoacetamide and then cleaved at Trp with BNPS-skatole. The W-460 peptide cleavage products, after resolution by SDS–PAGE, are shown in Figure 2. These peptide cleavage fragments have been sequenced, identified, and characterized as reported previously (Merrill & Cramer, 1990). The radiolabeled acetamide was found associated exclusively with the COOH-terminal fragment, K-461–I-522 (band c, Figure 2), indicating that the thiol- specific reagent, iodoacetamide,

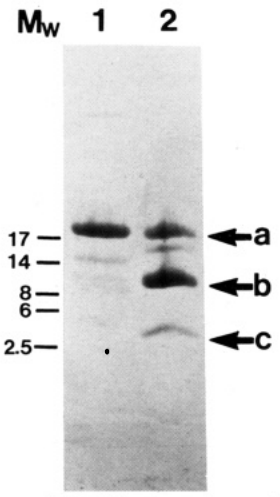

FIGURE 2: Cleavage of W-460 channel peptide–[^{14}C]acetamide adduct with BNPS-skatole. Lanes: 1, W-460 channel peptide; 2, W-460 channel peptide cleaved with BNPS-skatole: (a) uncleaved W-460 channel peptide, (b) Ile-345 to Trp-460 channel peptide cleavage product, (c) Lys-461 to Ile-522 channel peptide cleavage product containing the [^{14}C]acetamide label at Cys-505. Molecular weight standards (top to bottom): myoglobin polypeptide backbone (17 000), myoglobin fragment I-II (14 000), myoglobin fragment I (8000), myoglobin fragment II (6000), myoglobin fragment III (2500).

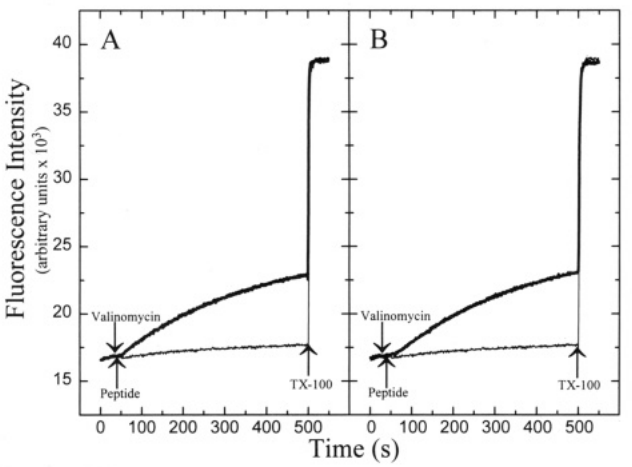

FIGURE 3: Fluorescence emission of asolectin vesicles in 100 $NaNO_3$ and 10 mM DMG, pH 6.0, containing 100 mM NaCl, 10 mM DMG, pH 6.0, and 16 mM SPQ at 20 °C. (A) WT channel peptide, 12 ng/mL (bold line); lysozyme, equimolar (thin line). (B) W-355 channel peptide–AEDANS adduct, 14 ng/mL (bold line); lysozyme, equimolar (thin line). Excitation λ, 347 nm; emission λ, 445 nm; ex and em slits 4 nm.

had reacted with the Cys-505 residue and not Met-370.

(2) Activity of AEDANS–Channel Peptide Adducts. It was previously demonstrated that the low pH-induced *in vitro* activity of the AEDANS–channel peptide adduct (wild-type peptide) was comparable to the activity of native wild-type peptide (Merrill et al., 1990). The octyl β-D-glucoside-induced activity of the channel peptide adducts was also found comparable to that of the wild-type protein (Figure 3). The ability of the channel peptide adducts to permeabilize the liposome bilayer was measured by a fluorescence assay developed specifically for the determination of Cl^- efflux from vesicles. This assay exploits the ability of Cl^- to quench the fluorescent dye, SPQ (see Materials and Methods). At 10–15 ng/mL detergent-treated native channel peptide or peptide adduct concentrations, significant rates of Cl^- release from vesicles were observed at pH 6.0. At this relatively high pH, and without treatment with amphipathic molecules such as

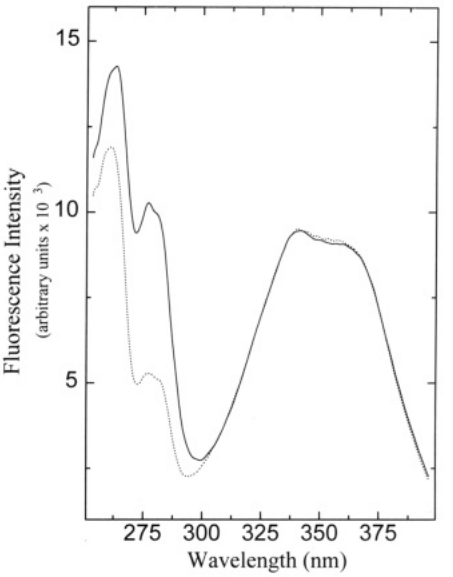

FIGURE 4: Fluorescence excitation spectrum of W-355–AEDANS adduct in the native state, pH 6.0 (—), and the active state, octyl β-D-glucoside (···). The adduct was in 200 mM $Na_2(SO_4)$ and 10 mM DMG, pH 6.0 at 10 °C. Excitation λ, scanned from 250 to 400 nm; emission λ, 490 nm; ex and em slits 2 nm.

SDS or octyl β-D-glucoside, colicin-induced Cl^- release was negligible as previously shown (Merrill et al., 1990). Furthermore, the addition of an equimolar concentration of detergent-treated lysozyme caused no significant release of Cl^- from vesicles.

(3) Efficiency of FRET. The FRET efficiency between the AEDANS chromophore (acceptor) and each of the 11 single Trp (donors), prepared by genetically engineering Trp residues within the colicin E1 channel peptide primary sequence, was calculated from excitation spectra using Förster's theory (see Materials and Methods). A second method, which involves the measurement of fluorescence lifetimes of the donors, was also used to determine donor–acceptor FRET efficiencies. This second method served as a comparison to the excitation method for donor–acceptor energy-transfer measurements. The two methods gave similar results; e.g., for the W-404 adduct the efficiency (E) of FRET was 21% and 23% for the excitation and lifetime methods, respectively. A third method, involving the measurement of fluorescence quantum yields, was not used because of the difficulty in the accurate determination of quantum yields in samples which cause some light scattering. The excitation method was deemed superior for these particular measurements of donor–acceptor distances since light scattering caused by detergent micelles was a problem. Therefore, the excitation method was chosen as the FRET technique to use for the determination of donor–acceptor distances within the channel peptide in the presence and absence of detergent.

Typical excitation spectra are shown in Figure 4 for W-355 in the presence and absence of octyl β-D-glucoside. The addition of octyl β-D-glucoside resulted in a significant reduction in the AEDANS fluorescence intensity (490 nm) upon excitation in the range of 275–290 nm (due to Trp excitation). This reduction in AEDANS fluorescence intensity indicated that the two chromophores had moved further apart as a result of detergent treatment of the peptide.

The FRET efficiencies (Table 1) between each single Trp donor chromophore and the attached AEDANS acceptor chromophore varied from a minimum of 8% (W-355 in the presence of octyl β-D-glucoside) to a maximum of almost 100% (W-507, in the absence of octyl β-D-glucoside). Interestingly,

Table 1: The Efficiency of FRET and the AEDANS–Trp Distances of AEDANS–Channel Peptide Adducts in the Native and Insertion-Competent States

peptide	$\langle E \rangle$ (%)[a]	$\langle E \rangle_{OG}$ (%)[b]
W-355	15.3 ± 1.1 (19)	7.9 ± 0.5 (46)
W-367	28.0 ± 2.6 (10)	15.9 ± 0.6 (19)
W-404	21.1 ± 2.1 (14)	12.6 ± 0.3 (28)
W-413	38.6 ± 0.4 (≪10)	25.8 ± 0.4 (12)
W-424	30.3 ± 2.4 (9)	20.2 ± 2.3 (15)
W-431	42.4 ± 4.9 (10)	29.9 ± 3.3 (10)
W-443	31.3 ± 2.1 (8)	25.7 ± 1.2 (11)
W-460	30.5 ± 0.6 (9)	25.9 ± 1.6 (12)
W-484	48.0 ± 7.8 (≪10)	38.4 ± 3.4 (≪10)
W-495	64.7 ± 0.7 (≪10)	65.5 ± 0.8 (≪10)
W-507	≈100	≈100

[a] The FRET efficiency in the native state (pH 6.0). [b] The FRET efficiency in the insertion-competent state (octyl β-D-glucoside). Errors are the standard deviation from three or more experiments. The estimated Förster distances, $R(^2/_3)$, are shown in parentheses.

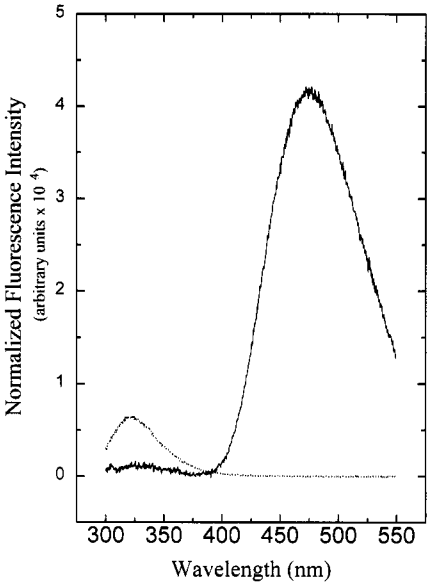

FIGURE 5: Fluorescence emission spectrum of W-507 channel peptide (···) and W-507–AEDANS adduct (—). The protein samples were in 200 mM $Na_2(SO_4)$ and 10 mM DMG, pH 6.0 at 10 °C. Excitation λ, 295 nm; emission λ, scanned from 300 to 550 nm; ex and em slits 2 nm.

the fluorescence emission spectrum of W-507 channel peptide–AEDANS adduct (Figure 5) confirmed that the FRET efficiency between adjacent chromophores was almost 100% since no Trp fluorescence emission could be detected in this adduct. In the absence of the AEDANS acceptor, W-507 exhibited a characteristic Trp fluorescence emission spectrum [quantum yield (Q) = 0.085; λ_{em}(max) = 324 nm, data not shown]. This result gave further credence to the conclusion drawn from experiments designed to localize the AEDANS chromophore to Cys-505 within the channel peptide. These data indicated that the AEDANS chromophore was indeed covalently attached to Cys-505 (only two residues from Trp-507, Figure 1).

The three Trp residues (W-484, W-495, and W-507) located in the hydrophobic anchor domain of the colicin E1 channel peptide showed the highest FRET efficiencies (% E, native adduct) of any of the 11 single channel peptide–AEDANS adducts which were studied (48 to ≈100%, Table 1). This finding is consistent with the proposed location of these 3 Trp residues in the highly nonpolar segment of the channel peptide (A-474–I-508), which has been shown to be buried in the soluble colicin E1 channel peptide structure (Merrill et al., 1990; Bishop et al., 1985). The centrally located Trp residues in the channel peptide (W-413, W-424, W-431, and W-460) exhibited intermediate % E values for energy transfer to the AEDANS acceptor (32–42%). The remaining NH_2-terminal Trp residues (W-355, W-367, and W-404) showed % E values which were considerably lower, ranging from 15 to 28%. Upon detergent activation of the channel peptide, the same general pattern within the colicin E1 peptide for the FRET efficiencies between the Trp donors and the AEDANS acceptor was seen (Table 1). However, there were marked differences in the relative changes in the FRET efficiencies among the various Trp donors (Figure 6). Figure 6 shows the percent change in FRET efficiency plotted against Trp residue position. These values (% $\Delta E_{[native-OG]}$) ranged from 48% (W-355) to −1% (W-495). There was a direct correlation between the magnitude of the relative change in FRET efficiency and the position of the Trp donor residue within the channel peptide primary sequence (higher relative ΔE the closer to the NH_2 terminus the Trp residue was located), except in the case of the W-484 channel peptide–AEDANS adduct, which showed a greater change than either W-443 or W-460 channel peptide–AEDANS adducts.

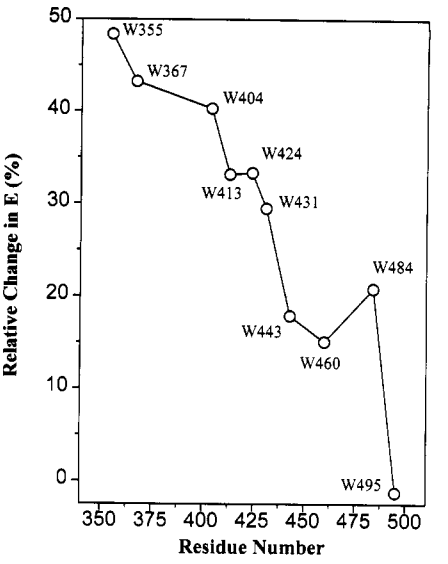

FIGURE 6: Relative change (%) in donor–acceptor efficiencies for the various single- Trp channel peptide–AEDANS adducts. Each point is the relative change (%) between the Trp–AEDANS efficiency in the activated state (octyl β-D-glucoside) and in the native state (pH 6.0), where relative percent change $(\Delta E)\% = [(E_{native} - E_{OG})/E_{native}] \times 100$. The error (standard deviation from 3 or more experiments) was less than 8% for all measured FRET efficiencies.

(4) Characteristic Distances between Cys-505 and Trp Residues: Structural Changes Invoked upon Activation. The determination of an absolute measure of distance between a fluorescence donor and acceptor pair is not feasible given the uncertainty in the FRET measurement, particularly with respect to the magnitude of the orientation factor, κ^2. However, characteristic distances (estimates) between the AEDANS acceptor chromophore (Cys-505) and each of 11 donor Trp residues ranged from ≈8 to ≈19 Å for the native adducts (no octyl β-D-glucoside; Table 1) except for W-413, W-431, W-484, W-495, and W-507. This latter group of peptide adducts showed Trp donor–AEDANS acceptor distances which were significantly less than 10 Å. In these cases, Förster's theory is no longer valid due to the high probability of electron exchange processes predominating when molecular orbitals are in close proximity (Truro, 1978). These results suggest that the three N-terminal Trp residues, W-355, W-367, and W-404, are relatively distant from the buried Cys-505

Table 2: Average AEDANS Fluorescence Anisotropy of AEDANS–Channel Peptide Adducts in the Native State and in the Insertion-Competent State[a]

peptide	$\langle r \rangle$[b]	$\langle r \rangle_{OG}$[c]
W-355	0.129 ± 0.003	0.114 ± 0.005
W-367	0.138 ± 0.002	0.117 ± 0.004
W-404	0.141 ± 0.001	0.119 ± 0.000
W-413	0.131 ± 0.010	0.115 ± 0.008
W-424	0.135 ± 0.010	0.108 ± 0.006
W-431	0.131 ± 0.009	0.112 ± 0.005
W-443	0.138 ± 0.007	0.115 ± 0.008
W-460	0.137 ± 0.001	0.114 ● 0.003
W-484	0.134 ± 0.004	0.111 ± 0.006
W-495	0.136 ± 0.001	0.113 ± 0.004

[a] Conditions for the anistropy measurements were as described in Materials and Methods (see Spectroscopic Measurements), and sample conditions were as described in Figure 4 legend. [b] Fluoresence anisotropy in the native state (pH 6.0). Errors are the standard deviation from 3 or more experiments. [c] Fluorescence anisotropy in the insertion-competent state (octyl β-D-glucoside). Errors are the standard deviation from 3 or more experiments.

residue (Bishop et al., 1985). The latter of these three N-terminal Trp residues is located at or near the surface of the soluble channel peptide as determined by fluorescence quenching experiments with the nonionic quencher, acrylamide (Merrill et al., 1993). Upon activation to the insertion-competent state (binding of octyl β-D-glucoside) the characteristic donor–acceptor distances for the N-terminal Trp residues in the channel peptide exhibited the largest increases (Table 1).

(5) AEDANS Fluorescence Anisotropy. The AEDANS fluorescence anisotropy values of the mutant peptides were similar and highly reproducible (Table 2). The anisotropy values ranged from 0.129 to 0.141 in the native (inactive) structure whereas the anisotropy values ranged from 0.108 to 0.119 in the activated protein structure. A small but significant decrease in the anisotropy values was observed for the AEDANS chromophore in each adduct, showing that there was a general loosening of the channel peptide structure (near Cys-505) upon detergent activation. Also, the steady-state anisotropy values were similar for the AEDANS chromophore in all of the single Trp peptide adducts in the absence of detergent. Since the AEDANS (acceptor) chromophore was conjugated to the same residue (Cys-505) in each mutant, this implies that the folded protein structure must be comparable for the various adducts.

DISCUSSION

The channel-forming colicins (A, B, E1, Ia, Ib, and N) like many toxins require low pH for *in vitro* activity. The physiological relevance of the low pH-induced activity for the colicins has been questioned. However, Merrill et al. (1990) demonstrated that low concentrations of detergent, such as SDS or octyl β-D-glucoside, can mimic the *in vitro* activation while at neutral or near-neutral pH values. The nonionic detergent octyl β-D-glucoside was used for this study as channel peptide activator since low pH resulted in a reduction in the solubility of the peptide–AEDANS adducts (data not shown). This detergent-induced activation resulted in comparable levels of *in vitro* channel-forming activity for native, wild-type channel peptide and for the AEDANS–channel peptide adducts, which also corresponded favorably with previously reported levels of activation (Merrill et al., 1990). Thus, this method for activating the channel peptide was deemed suitable to provide protein for FRET structural studies.

Förster theory for the dipole–dipole energy-transfer process, which postulates that the efficiency of transfer depends on the inverse sixth power of the distance between the donor and acceptor, has been verified by numerous fluorescence studies (Stryer & Haughland, 1967; Fairclough & Cantor, 1978). However, the limitations of the theory constrain the working distances for measurement in biological molecules to the range of 10–60 Å (Stryer & Haughland, 1967). Thus, a distance cannot be assigned to chromophores which are separated by less than 8–10 Å. Accordingly, this was observed for the W-413, -431,-484, -495, and -507–AEDANS adducts, where the transfer efficiency was greater than 38%. Trp-507 is located very close (2 residues) to the acceptor attachment site, Cys-505. This region of the colicin E1 channel peptide (near W-507) is most probably α-helical, on the basis of secondary structure predictive methods (Cramer et al., 1989) and as implied from sequence alignment of the colicin E1 and the colicin A primary structures. The latter approach involved using the secondary structure data available from the refined 3-D colicin A crystal structure (a functionally similar colicin with between 30% and 40% sequence homology; Parker et al., 1992). Also, Cys-505 is predicted to be near the COOH-terminal end of helix 9 (Cramer et al., 1990). Therefore, on the basis of the dimensions of the standard α-helix (3.6_{13} helix), Cys-505 and Trp-507 would be 3.0 Å apart if the chromophores were merely points in space. The obliteration of the Trp-507 fluorescence in the presence of the Cys-505–AEDANS acceptor verifies the location of the AEDANS chromophore exclusively at Cys-505 and adds credence to the FRET method for the determination of donor–acceptor distances in proteins.

The magnitude of the orientation factor (κ^2) in energy-transfer measurements is a definite source of error that needs to be considered when evaluating the accuracy of the FRET method and hence the reliability of results obtained on macromolecular structure using FRET. The limiting anisotropy (r_{lim}), which is an estimate of the polarization anisotropy of any relatively immobilized fluorophore of a donor–acceptor pair, was previously reported by Lakey et al. (1991) for a similar channel-forming colicin, colicin A. On the basis of their measurements, it is estimated that the error in using $\kappa^2 \approx {}^2/_3$ rather than the actual unknown value is less than 20%. In addition, the mixed polarizations occurring in the IAEDANS excitation polarization spectrum are likely to reduce the error in the assumption of κ^2 even further (Cheung, 1991). Furthermore, the low polarization values of the Trp residues in the adducts (0.05–0.15) indicated that the donor orientation was quite isotropic (data not shown). Further supporting evidence for the use of $\kappa^2 = {}^2/_3$ can be seen in the value of R_0 reported herein for the various channel peptide adducts (Trp–AEDANS, donor–acceptor pair), since R_0 reflects the changes occurring in the orientation factor (see Materials and Methods). The values ranged from 20–26 Å, which was in good agreement with the R_0 value (22 Å) reported for the Trp–AEDANS pair (Fairclough & Cantor, 1978).

The characteristic distances estimated from the measured FRET efficiencies for the various Trp residues within the native channel peptide and the AEDANS probe (Cys-505) all ranged from being adjacent to the acceptor chromophore to as distant as 19 Å away. However, one caveat to this method is that the calculated distance is from the electronic center of the donor and acceptor chromophores, which contributes at least 4–5 Å of variability to the donor–acceptor distances. Nonetheless, in spite of the uncertainty in the distances calculated from FRET measurements, these results are consistent with the known hydrodynamic radius of the colicin E1 channel peptide (22.4 Å, pH 6.0; Merrill et al., 1990) and suggest that the COOH-terminal hydrophobic domain of the

channel peptide is buried with the N-terminal portion of the channel peptide wrapped around the hydrophobic core. The relatively high FRET efficiencies measured for the AEDANS (acceptor) and the 3 Trp (donor) residues located in the hydrophobic anchor domain of the channel peptide are again consistent with the idea that this nonpolar domain of the peptide is completely buried in the soluble peptide (Merrill et al., 1990; Bishop et al., 1985). Furthermore, the FRET distances estimated for the various Trp (donor) and AEDANS (acceptor) pairs in the colicin E1 channel peptide are surprisingly consistent with the colicin A crystal structure (C. Stauffacher, personal communication) given the uncertainty of the distance calculations due to the size of the donor and acceptor chromophores and the uncertainty of the orientation factor (κ^2).

Despite the many problems associated with the accurate determination of FRET efficiency, useful information concerning structural changes in macromolecules can be obtained. Kawata and Hamaguchi (1991) used FRET to characterize the degree of compactness of the constant fragment of an immunoglobulin light chain in the early stage of protein folding. This fragment had only one Trp residue (W-158). The distance between Trp-158 and an AEDANS group (covalently attached to a Cys in the protein), as estimated from FRET data, corroborated structural data previously obtained by X-ray crystallographic analysis. In another study, Divita et al. (1993) applied FRET to assist in the structural mapping of the yeast mitochondrial F_1-ATPase catalytic site and to determine its orientation with respect to the α-subunit of this ATPase. These investigators measured a high FRET efficiency occurring between Trp-257 (donor) and the 2′,3′-*N*-methylanthraniloyl (acceptor) group and obtained a calculated distance of 10.5 Å.

The interactions between calmodulin and the Trp residues of synthetic peptides corresponding to the calmodulin binding domains of skeletal muscle light chain kinase and the plasma membrane calcium pump were examined by Chapman et al. (1992). FRET from peptidyl Trp residues to an AEDANS moiety attached to Cys-26 of spinach calmodulin was measured. The data indicated that the indole ring of each peptide inserted 32–35 Å away from Cys 26 and may, therefore, interact with the COOH-terminal lobe of calmodulin in its "bent" conformation. Also, the interchange of Trp-3 and Phe-21 of the calcium pump peptide increased the FRET efficiency to the AEDANS moiety ca. 12-fold, reducing the calculated distance to 20 Å. These authors concluded that Phe-21 of the calcium pump peptide interacts with the hydrophobic cleft in the amino-terminal lobe of calmodulin.

The applicability of the FRET technique was furthermore demonstrated by the report of Gardner and Matthews (1991). These researchers examined the energy transfer between two Trp residues and an AEDANS moiety attached to Cys-140 in the wild-type lactose repressor. Energy transfer between Trp residues and the AEDANS moiety(ies) in the wild-type *lac* repressor occurred with an efficiency of 6.7 ± 1.9% in the absence and 7.8 ± 1.6% in the presence of inducer. The distance between the Trp donor(s) and the acceptor in the wild-type was estimated to be 35 Å. It was concluded that the FRET occurred between subunits in the native tetrameric repressor.

FRET was applied to the study of the membrane-bound topology of the colicin A COOH-terminal fragment (Lakey et al., 1991, 1993). Lakey et al. (1991) used the three naturally occurring Trp residues as fluorescence donors to AEDANS (acceptor chromophore) covalently attached to a single Cys

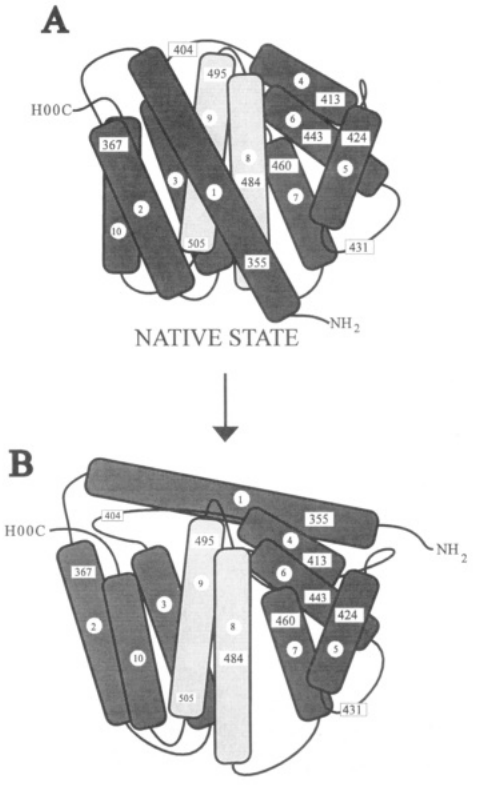

FIGURE 7: Extension cord model for the activation of the colicin E1 channel peptide. The hydrophobic α-helical hairpin is shown in light gray (helices 8 and 9). The attachment site for AEDANS (Cys-505) and the approximate positions of donor tryptophyls are shown. Only the general topological position of the helices and labeled residues is implied. (A) The compact colicin E1 channel peptide structure (native state, pH 6.0). (B) The molten globular channel peptide (active state, octyl β-D-glucoside).

in the colicin A channel peptide. Three single Cys mutants of the colicin A channel peptide, K39C (helix 2), T127C (between helices 6 and 7), and S16C (helix 1), gave useful derivatives. The conclusion reached by these investigators was that the amphipathic helices of the colicin A channel peptide open out on the surface of the lipid bilayer during the initial phase of membrane insertion in an "umbrella-like" fashion. More recently, Lakey et al. (1993) reported the application of FRET to the study of the membrane-bound topology of the hydrophobic helical hairpin of the colicin A channel peptide. The authors used a total of 5 single Cys mutants of the channel peptide and concluded that helices 1 and 2 open out onto the membrane surface while the hydrophobic hairpin structure remained closely packed against the rest of the structure. However, it appears unlikely that single-bilayer structures with a well-defined orientation of the COOH-terminal peptide were used. This creates a degree of uncertainty as to the correct interpretation of the topological data. Furthermore, the light scattering caused by protein–lipid complexes compromises considerably the accuracy of the FRET measurements, especially when using the quantum yield method for the determination of the efficiency of energy transfer.

Activation to the insertion-competent state results in the loosening of the colicin E1 channel peptide solution structure with significant changes in the measured FRET efficiencies (and estimated donor–acceptor distances) for the peptide's N-terminal Trp residues. Furthermore, the AEDANS fluorescence anisotropy measurements showed that Cys-505 becomes more mobile upon peptide activation (the anisotropy

values decreased ca. 20%). The extent of unfolding of the channel peptide upon activation was greatest in the N-terminal portion of the molecule (Figure 6), consistent with the idea that the N-terminus of the peptide was near the aqueous interface of the molecule with the COOH-terminal hydrophobic domain buried within the solution structure.

In the present study, activation of the colicin E1 channel peptide involves the unfolding/loosening of the N-terminal portion of the molecule, with generally smaller changes, upon peptide activation, occurring in the FRET efficiency reported by Trp residues located within the hydrophobic core (Trp residues 484, 495, and 507). These findings corroborate previous results obtained for the low pH-induced activation of the colicin E1 channel peptide as determined by acrylamide quenching of these single Trp peptides (Merrill et al., 1993). A model for the activation of the colicin E1 channel peptide, affectionately termed the "extension cord model", is proposed herein where low pH or the binding of small, amphipathic molecules affects the conformation of the soluble peptide at the periphery of the molecule (Figure 7). The colicin molecule may "unwrap" starting at its N-terminus, with diminishment in the extent of unwrapping, and thus the exposure to the aqueous medium, from the N-terminal to the COOH-terminal end of the molecule. Therefore, upon unwrapping, charged amino acid residues may become more exposed, including the ring of 8 positively charged residues proposed for colicin A by Parker et al. (1990), which is believed to facilitate the binding of the activated peptide to the negatively charged membrane surface. Upon binding to the bilayer surface, the hydrophobic helical hairpin (membrane anchor, helices 8 and 9 of colicin A) may penetrate the hydrocarbon portion of the bilayer to form the closed channel structure (Song et al., 1991; Zhang & Cramer, 1993).

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