Primary Events in the Colicin Translocon: FRET Analysis of Colicin Unfolding Initiated by Binding to BtuB and OmpF[†]

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ABSTRACT: Cellular import of colicin E3 is initiated by high affinity binding of the colicin receptor-binding (R) domain to the vitamin B_{12} (BtuB) receptor in the *Escherichia coli* outer membrane. The BtuB binding site, at the apex of its extended coiled-coil R-domain, is distant from the C-terminal nuclease domain that must be imported for expression of cytotoxicity. Based on genetic analysis and previously determined crystal structures of the R-domain bound to BtuB, and of an N-terminal disordered segment of the translocation (T) domain inserted into the OmpF porin, a translocon model for colicin import has been inferred. Implicit in the model is the requirement for unfolding of the colicin segments inserted into OmpF. FRET analysis was employed to study colicin unfolding upon interaction with BtuB and OmpF. A novel method of Cys-specific dual labeling of a native polypeptide, which allows precise placement of donor and acceptor fluorescent dyes on the same polypeptide chain, was developed. A decrease in FRET efficiency between the translocation and cytotoxic domains of the colicin E3 was observed upon colicin binding *in vitro* to BtuB or OmpF. The two events were independent and additive. The colicin interactions with BtuB and OmpF have a major electrostatic component. The R-domain Arg399 is responsible for electrostatic interaction with BtuB. It is concluded that free energy for colicin unfolding is provided by binding of the R- domain to BtuB and binding/insertion of the T-domain to/into OmpF.

The bactericidal colicin proteins are actively transported into *Escherichia coli* cells, parasitizing the import machinery for bacterial nutrients in the outer membrane. Colicin import is initiated by tight binding to the outer membrane β-barrel proteins, which for colicin E3 (colE3[1]) are BtuB ($K_d \leq 10^{-10}$ M^{-1}) and the OmpF or OmpC porins (*1*−*4*). These outer membrane proteins, together with the colicin, have been inferred to be components of an outer membrane translocon for the nuclease colicins (*4*−*6*).

The defined functions of the colicin E3 polypeptide are exerted by the N-terminal translocation (T), C-terminal endonuclease (C), and central elongate receptor-binding (R) domains (*7*). The T- and C-domains, responsible for cellular import and cytotoxicity, respectively, are held in close proximity through their interaction with the immunity protein at one end of the coiled-coil R-domain, which is ∼100 Å distant from the BtuB-receptor recognition site located on the opposite side of the coiled-coil (Figure 1).

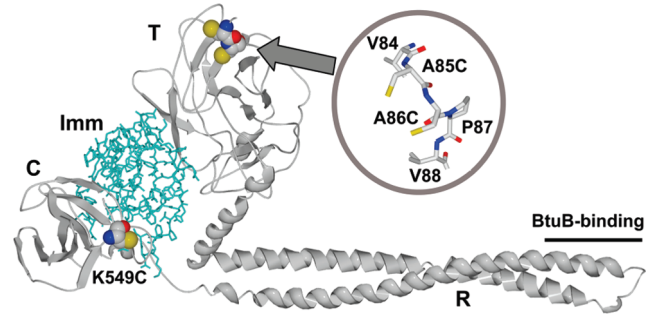

FIGURE 1: Ribbon diagram of colicin E3 with atoms of residues mutated to Cys shown as van der Waals spheres. Positions of Cys mutations K549C in the C-domain and A85C and A86C in the T-domain are shown. Inset: Cys side chains of the A85C/A86C mutant showing rotamers with a 3.5 Å distance between S atoms. Figure created using Swiss-PDB viewer v. 3.7 and POV-ray v. 3.6 programs. C, R, T, cytotoxic RNase, coiled-coil BtuB receptor binding, outer membrane translocation domains of colicin E3. Imm, immunity protein (blue).

Differential circular dichroism spectroscopy and the crystal structure of the 135-residue colicin E3 receptor-binding domain bound to the BtuB receptor revealed partial unfolding of coiled-coil termini far (>70 Å) from the BtuB-binding apex of the R-domain coiled-coil (*4*). This suggested an energy-transducing function of the colicin E3 coiled-coil, in which energy released upon colicin binding to the receptor is transduced to conformation changes in the distal part of the coiled-coil connected with the T- and C-domains, thus initiating the unfolding of T- and C-domains that is necessary for their insertion into the OmpF porin. The requirement for colicin import and cytotoxicity of unfolding of translocated

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[1] Abbreviations: Alx568, Alexa Fluor 568 C_5-maleimide; BSA, bovine serum albumin; colE3, colicin E3; C8E4, octyl tetraethylene glycol ether; CD, circular dichroism; DTT, dithiotreitol; FeCy, potassium ferricyanide; FRET, fluorescence resonance energy transfer; Imm, immunity protein; ITC, isothermal titration calorimetry; Org488, Oregon Green 488 iodoacetamide; MODIP, modeling of disulfide bonds in proteins; Omp, outer membrane protein; R_o, Förster distance; TCEP, tris-(2-carboxyethyl)phosphine.

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domains was shown by disulfide bond cross-linking in the distal part of the coiled-coil of the nuclease colicin E9 (*8, 9*). However, a calorimetric (ITC) study of the colicin E9 (colE9) interaction with BtuB did not detect the entropy increase that would be associated with BtuB-induced unfolding of colE9 (*5*).

Fluorescence resonance energy transfer (FRET) analysis can provide information about conformation and intramolecular distance changes in the structure of a protein. A wide variety of dyes that can be used for FRET covers a spectral range from the ultraviolet to the infrared region (*10*−*14*). Chemical conjugation of dyes under non-denaturing conditions is possible with amino, carboxyl, histidine, and thiol groups of amino acid side chains (*10, 11, 13*). The thiol groups are preferable due to the relatively small abundance of Cys in proteins and the high chemical reactivity of thiol groups under non-denaturing conditions. However, FRET application to the study of dynamics of an individual protein molecule is constrained by the placement of donor and acceptor fluorophores in defined positions on the same polypeptide chain.

In the present studies, a procedure based on conjugation of dyes with Cys residues specifically placed in the C- and T-domains has been developed for labeling a single colicin E3 molecule with two dyes for FRET measurements. Conjugation of donor and acceptor dyes was conducted under thiol-oxidized and thiol-reduced conditions. Application of the doubly labeled colicin in FRET studies of colicin interaction with its outer membrane BtuB receptor *in vitro* revealed a significant increase in distance between the T- and C-domains of the colicin E3 upon binding to the BtuB or OmpF receptor. Since the BtuB-binding site and imported domains of the colicin are located on opposite ends of the extended coiled-coil (Figure 1), it is inferred that free energy released upon the binding of colicin E3 to BtuB, in which the contribution of electrostatic interactions was found to be essential, is transmitted through the coiled-coil to disrupt the packing of the distal C-Imm-T-domains.

MATERIALS AND METHODS

Materials. Escherichia coli XL1 Blue strain was used as the host strain for cloning. All cloning was done in the pET41b vector with the colicin constructs containing a His8-tag at the C-terminus of the immunity protein. *E. coli* BL21 (DE3) was the host strain for the expression vector pET41b. In the pET41b vector, protein expression was under the control of a strong IPTG-inducible T7 RNA polymerase promoter. The fluorescent dyes, Alexa Fluor 568 C_5-maleimide and Oregon Green 488 iodoacetamide were obtained from the Molecular Probes Division (Eugene, OR) of Invitrogen, Inc.

Mutagenesis. Mutagenesis of colicin E3 and expression and purification of the resultant altered proteins were performed as previously described (*9, 15*), as were expression and purification of BtuB (*16*) and OmpF (*6*).

Cytotoxicity Assay. Cytotoxicity of colE3 was assayed by plating colicin-sensitive pT7-7 cells (Amp^r cells) on a LB Amp plate to which different concentrations of colE3 were applied as 20 μL drops. Lysis of the bacterial cells was detected through clear spots in a lawn of sensitive bacteria. The lowest inhibitory concentration ($I_{+/-}$) of colicin was defined as the smallest concentration that would generate a clear zone of inhibition or a decreased cell density compared to an unaffected lawn area.

Protein and dye concentrations. ColE3Imm, Alx568 and Org488 concentrations were determined, using extinction coefficients of 79.8, 92.0, and 68.0 mM^{-1} cm^{-1} measured at 280, 580 and 498 nm, respectively. Because of the dye absorbance at 280 nm, a correction is required if the protein concentration is measured spectrophotometrically. Bradford (*17*) or Lowry (*18*) assays were also used for estimation of protein concentrations.

Protein Thiol Content. Spectrophotometric assay of thiol content was performed using Ellman's reagent (DTNB) (*19*) as described previously (*20*), using an extinction coefficient of 14.2 mM^{-1} cm^{-1} at 412 nm.

Thermal Melting Analysis Using CD at 222 nm. Far-UV thermal scanning CD measurements were carried out with a J-810 spectropolarimeter (JASCO, Easton, MD) equipped with a Peltier type temperature control system. Thermal scanning was carried out at 222 nm in a 5 or 10 mm cell with magnetic stirring. Scanning rate, 0.5 °C/min. Step interval, 0.1°. The midpoint temperatures of the thermal transitions were determined, using the JASCO "Protein Denaturation" program, or as the first derivative peaks of the thermal scanning functions, after noise reduction using a fast Fourier transform program (FFT).

Absorbance and Fluorescence Measurements. Absorbance spectra were measured with a Cary-3 spectrophotometer (Varian, Inc.) in the spectral range 240−650 nm using quartz cuvettes with an optical path length of 1 or 10 mm.

Fluorescence excitation and emission spectra were measured with a photon counting spectrofluorimeter FluoroMax-3 (Horiba, Jobin-Yvon Inc.) at 20 °C. Excitation spectra of Alx568 fluorescence emission at 620 nm (half-band width, 2 nm) were measured in the spectral range 450−600 nm (half-band width, 5 nm). Excitation for the emission spectra in the spectral range 490−650 nm was at 470 nm.

FRET Analysis. The distance between the Org488 donor and the Alx568 acceptor (R) was calculated using the formula

$$R = R_o(E^{-1} - 1)^{1/6} \quad (1)$$

where R_o is the Förster radius, the distance at which the FRET efficiency (E) equals 50% (*12*). The value, $R_o \approx 62$ Å, which was used for the distance calculations, was determined previously (*10*) for the FRET pair Alx488, an analogue of Org488, and Alx568. The efficiency of energy transfer, E, was calculated using the formula

$$E = (F_{498} - F_{alx498})/(F_{580} - F'_{580}) \quad (2)$$

where F_{498} and F_{580} are the fluorescence intensities of doubly labeled colE3 at 620 nm, excited at 498 and 580 nm, respectively. F_{alx498} is a correction for the emission of Alx568 in response to excitation at 498 nm. F'_{580} is the correction for the contribution of extra-stoichiometric content of acceptor dye, arising from the labeling of colE3 with Alx568 at the disulfide site.

Protocol (i–iv) for Site-Specific Double Labeling. The starting material was colE3Imm containing a K549C mutation in the C-terminal cytotoxic domain and a double A85C/A86C mutation in the N-terminal translocation domain (Figure 1).

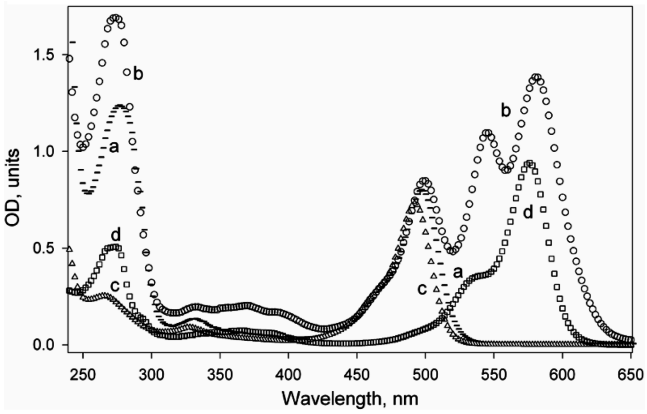

FIGURE 2: Spectral properties of colE3(A85C,A86C,K549C)Imm and its conjugates with Org488 and Alx568. Absorbance spectra: (a) colE3(549C-Org488)Imm (λ_{max}, 275 and 498 nm; horizontal marks), (b) colE3(549C-Org488;85C/86C-Alx568)Imm (λ_{max}, 273, 500, 545 and 580 nm; circles), and aqueous solutions of (c) Org488 (λ_{max}, 269 and 496 nm; triangles) and (d) Alx568 (λ_{max}, 274 and 576 nm; squares).

(i) Disulfide Bond Formation. To prevent labeling of the two Cys residues in the N-terminal T-domain while labeling C-domain Cys549, colE3 (0.05−0.1 mM) was treated with 0.4 mM ferricyanide (FeCy) for 5 min (*21*) followed by FeCy removal on the desalting column. This results in formation of a Cys85−Cys86 disulfide bond. Incubation of colE3 with a higher concentration of FeCy can result in intermolecular disulfide bonding. ColE3 protein dimers were removed by size-exclusion chromatography.

(ii) Labeling of Cys549 with Oregon Green 488 Iodoacetamide. The FeCy-oxidized colE3 (0.1 mM) with one available cysteine, Cys549 (Figure 1), was incubated with Org488 (0.5 mM) at room temperature for 1 h. Unbound dye was removed by gel filtration on a Sephadex G-25 (medium, 2 mL) column. Eluted fractions were analyzed using absorbance spectra, 240−550 nm, to estimate the efficiency of labeling.

One hour of protein incubation with Org488 at room temperature was usually sufficient for conjugation of >0.9 mol of Org488 with 1 mol of colE3, yielding the absorbance spectrum shown in Figure 2 (spectrum a). For proper double labeling, it is important to use only monomeric protein in the second conjugation step. Therefore, high-resolution size-exclusion chromatography (Superdex 200, 10/300) was used to remove protein dimers after FeCy oxidation, or after the first labeling and before disulfide bond reduction.

(iii) Disulfide Bond Reduction. ColE3(549C-Org488) was incubated with 20 mM DTT (30 min), which was removed by size exclusion chromatography on a Superdex 200 or desalting spin column.

(iv) Labeling of ColE3(549C-Oregon488) at Cys85/Cys86 with Alexa 568. The second labeling with Alx568 was started immediately after removal of DTT to prevent Cys oxidation by ambient oxygen. ColE3(549C-Org488) (20−100 μM) was incubated with Alexa Fluor 568 C_5-maleimide (0.2 mM) for 1 h at room temperature. Unbound dye was removed by gel filtration on a Sephadex G-25 (10 mL) column. Absorbance spectra of eluted fractions in the 240−650 nm range were used to estimate the concentration and extent of labeling of colE3(Cys85/Cys86-Alx568;Cys549-Org488). If necessary, the colE3 conjugates were concentrated using a Centricon 50 (Millipore).

Incubation with Alx568 for 1 h at room temperature was sufficient to achieve a sufficient extent of labeling. The use of TCEP (*10*) to keep thiol groups reduced during labeling significantly decreased the yield in reactions with both Org488 and Alx568 (data not shown). All steps of the labeling procedure were performed in dim light.

RESULTS

Colicin Labeling. Design of Colicin Cys Mutants. Colicin E3 does not contain any cysteine residues. However, its tightly bound inhibitor, the 9 kDa immunity protein (Imm), has one Cys residue at position 47 (*7*). A Cys47Ala mutant of Imm was used in the present study to avoid interference by Cys47 in the labeling procedure. Ala85Cys and Ala86Cys replacements were made in the solvent-accessible area of the translocation (T) domain that follows the intrinsically disordered N-terminal 83-residue segment (Figure 1). This provided additional flexibility in the backbone, increasing the probability of disulfide bond formation. A minimal distance of 3.5 Å between S atoms is predicted from the X-ray structure of colicin E3 (PDB accession, 1JCH (*7*)) with the Ala85Cys and Ala86Cys replacements (Figure 1 inset). Disulfide bond formation in the double Cys mutant colE3(A85C/A86C), oxidized with FeCy, was confirmed by quantitative assay of thiol groups using the Ellman reaction (*19*). The content of thiol groups in FeCy-oxidized and DTT-reduced colE3(A85C/A86C) was 0.2 ± 0.2 and 2.1 ± 0.3 mol/mol (n = 2), respectively.

In the C-domain, the surface-exposed Lys549, which is not involved in the C-domain interactions with Imm, was replaced with a Cys residue (Figure 1). From the colicin E3 structure, the distances between S atoms for the 549C−85C and 549C−86C pairs (Figure 1) were found to be 51 and 47 Å, respectively. This is appropriate for FRET analysis using Org488 and Alx568 as the FRET donor and acceptor, for which the measured Förster distance, R_o = 62 Å (*10*).

Spectral Properties of Labeled ColE3. The concentrations of Org488 and Alx568 were determined using absorbance maxima in conjugates (Figure 2) and the extinction coefficients of 68,000 and 92,000 reported by the manufacturer (*10*). These maxima for protein-bound dyes are red-shifted to 497 ± 1 and 580 ± 1 nm (Figure 2a,b), relative to the those of the free dyes in aqueous solution, 493 ± 1 and 577 ± 1 nm, respectively (Figure 2c,d).

For the triple Cys mutant of colE3Imm, A85C/A86C/K549C, the extent of labeling of oxidized colE3Imm with Org488 was 0.9−1.0 Org488/colE3. The extent of labeling in the second stage with Alx568 was typically between 0.8 and 1.5, implying that the binding of dye to one Cys (Cys85 or Cys86) decreased labeling of the second Cys, probably due to steric hindrance.

The singly labeled colE3(549C-Org488)Imm has an emission maximum at 515 nm (Figure 3A, □) and negligible fluorescence at 620 nm, the emission spectral band used for spectral FRET measurements. The doubly labeled protein has two maxima, seen in the emission (516 and 598 nm) and excitation (497 and 576 nm) spectra (Figure 3A,B, ○). Excitation of Org488 in the doubly labeled protein at 470 nm results in significant emission of Alx568 centered at 598 nm (Figure 3B, ○), implying efficient FRET from Org488 to Alx568.

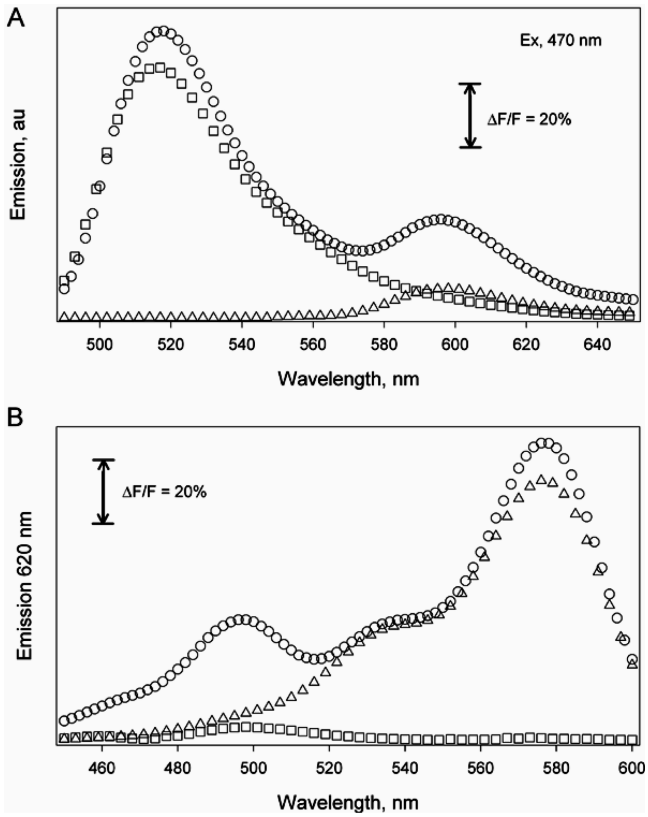

FIGURE 3: Emission and excitation spectra of colE3Imm conjugates. A. Emission spectra, with excitation at 470 nm. B. Excitation spectra were measured with the emission centered at 620 nm. The spectra of colE3(Org488)Imm (□), colE3(Alx568)-Imm (Δ), and colE3(Alx568,Org488)Imm (○) are shown. Buffer, 20 mM HEPES, pH 7.2, 0.1 M NaCl.

Table 1: Effect of Side Chain Replacements and Dye Conjugation on ColE3 Cytotoxicity

colE3Imm[a]	DTT[b] (mM)	$I_{+/-}$, nM
wild type	0	0.125 ± 0.05
wild type	10	0.125 ± 0.05
85C/86C/549C	0	0.25 ± 0.1
85C/86C/549C	10	0.125 ± 0.05
85C/86C;549C-Org488	0	1 ± 0.3
85C/86C;549C-Org488	10	1 ± 0.3
85C/86C-Org488	0	8 ± 3
85C/86C-Alx488	0	inactive (≤16 nM)
85C/86C-Alx568;549C-Org488	0	inactive (≤32 nM)

[a] In addition to the colE3 modifications, side chain replacement and dye conjugation, colE3Imm has C47A replacement in Imm. [b] ColE3 was dissolved in 20 mM HEPES, pH 7.2, 0.1 M NaCl. DTT, 10 mM, was present where shown; colicin E3 susceptible *E. coli* cells were grown on agar plates. Twenty μL aliquots of colE3Imm, 0.03−32 nM, with 2-fold dilution steps were applied to cell lawn 20 min after start of cell growth on the agar surface. ColE3 cytotoxicity was assessed by appearance of clear spots where colicin with a given concentration was applied; number of trials, $n = 2-3$.

The presence of FRET is also seen in the excitation spectrum for Alx568 emission at 620 nm (Figure 3B, ○). In addition to the excitation maximum at 578 nm caused by the direct excitation of Alx568, a maximum at 498 nm is observed, caused by FRET from Org488 to Alx568.

Dual Labeling Inhibits ColE3 Cytotoxicity, but Does Not Affect Binding to BtuB. Wild type colE3 is cytotoxic toward sensitive *E. coli* cells at picomolar concentrations in a survival spot-test assay ($I_{+/-}$, 30−100 pM, Table 1). Approximately the same toxicity was observed with the unlabeled triple-Cys mutant of colE3, A85C/A86C/K549C, when it was applied in the presence of the disulfide bond reducing agent, DTT. However, doubly labeled triple-Cys colE3 was not cytotoxic (Table 1). Colicins labeled at the double-Cys site, A85C/A86C, with either Alx568 or Org488, were also inactive. The triple-Cys mutant, A85C/A86C/K549C, carrying only a single Alx568 dye at 549C, was 10 times less efficient than unlabeled colicin in its expression of cytotoxicity (cytotoxic at 1 nM compared to 0.1 nM for unlabeled colE3) (Table 1). The absence of cytotoxicity of labeled colE3 is believed to be a consequence of the necessity for cytotoxic colicin to cross the outer membrane through the OmpF pore. Dye attachment at C- and T-domains would hinder this translocation.

Table 2: Double Competition Assay of Doubly Labeled ColE3Imm Binding Affinity to BtuB

wt colE3[a]	BtuB	colE3-Org488-Alx568	cell growth
A. 0.5 nM	−	−	−[b]
0.5	1 nM	−	+
0.5	1	1 nM	+/−
0.5	1	2	−
0.5	4	4	−
B. 0.1 nM	−	−	−
0.1	0.1 nM	−	+/−
0.1	0.2	−	+
0.1	0.4	−	+

[a] Spot-survival assay similar to that as in Table 1 with the exception that wild type colE3 before application on a plate bacterial lawn was preincubated with BtuB alone or its mixture with doubly labeled colE3Imm. 0.7% octyl-POE was added to buffer solution. [b] Fully active (−), inactive (+) or intermediate toxicity (+/−) of colE3 was detected by appearance of clear spots, spots with cell density close to untreated lawn or of intermediate cell density, respectively.

Taking into account the location of the labeling sites (Figure 1), it is expected that the affinity of doubly labeled colE3 for BtuB should not be affected by conjugation with colicin of Org488 and Alx568. Using a double competition assay, the cytotoxicity of the fully active wild type colE3, added at a concentration of 500 pM, was prevented by prior incubation *in vitro* with 1 nM BtuB in detergent solution (Table 2A). It is known that preincubation of colicin E3 with BtuB in detergent solution can neutralize colicin cytotoxicity and that the R135 receptor-binding domain can neutralize the binding of the colicin to BtuB (*4*). Similarly, incubation of doubly labeled colE3 with BtuB at equimolar concentrations neutralized BtuB so that it was not able to bind to, and abolish the cytotoxicity of, wild type colE3 (Table 2A). Thus, the doubly labeled colE3 is not impaired in its ability to compete with wild type colE3 for high affinity binding to BtuB.

Cytotoxicity Assay of Colicin E3 Affinity for BtuB. The colicin neutralization assay by BtuB can be used to estimate colicin affinity for BtuB, and shows that the K_d of ∼1 nM previously measured for ColE3 using surface plasmon resonance (*4*), and for colE9 using ITC (*5*), defined an upper limit for this parameter. If the K_d for dissociation of colE3 from BtuB is 10^{-9} M^{-1}, then an equilibrium incubation of 1 nM BtuB with 0.5 nM colE3 would leave ∼0.25 nM colE3 free in a cytotoxic state. However, unlabeled colE3 was completely neutralized under these conditions (Table 2A, lane 2), implying that $K_d \ll 10^{-9}$ M for colE3 binding to BtuB. Thus, the efficient neutralization by isolated BtuB of colE3 cytotoxicity at 0.1 nM concentration (Table 2B) implies a colicin affinity ≤0.1 nM. In addition, colicin affinity for BtuB

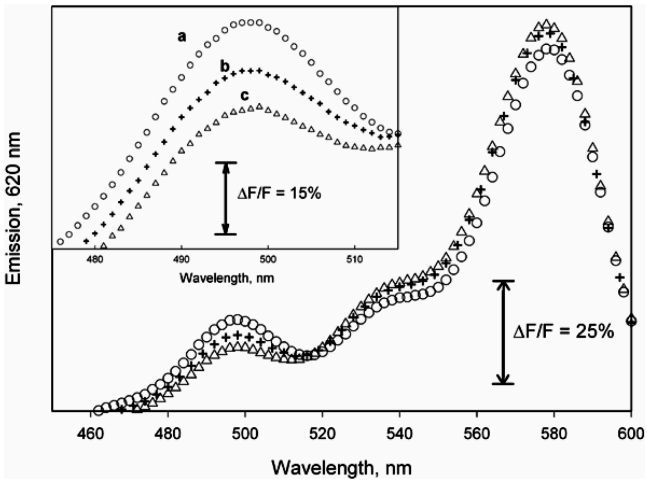

FIGURE 4: Conformation changes in colE3Imm upon binding to BtuB. Conformation changes were detected by FRET from Org488 to Alx568 attached to the colicin C- and R-domains, respectively. Excitation spectra of the colE3(549C-Org488;85C/86C-Alx568)Imm, 20 nM, alone (○), and after addition of BtuB, 0.2 μM, (+), and then of OmpF, 2 μM (△). Emission of Alx568 was measured at 620 nm. Buffer, 0.6% C8E4, 20 mM Tris, pH 8.0.

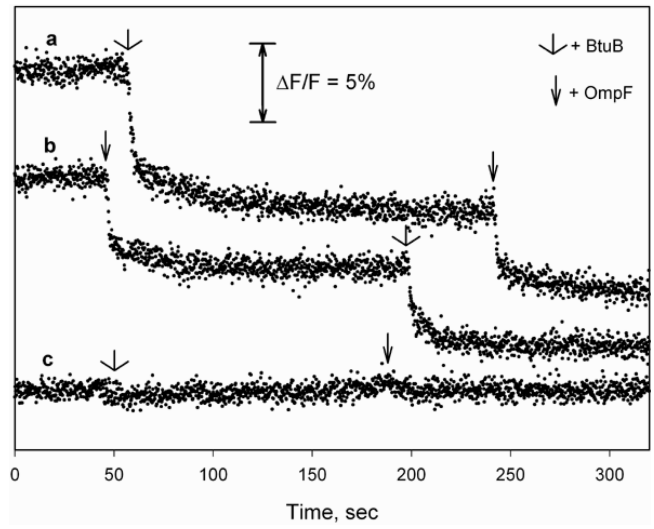

FIGURE 5: Kinetics of FRET decrease upon colE3 interaction with outer membrane receptors. The time course of Alx568 emission at 600 nm was measured upon excitation at 497 nm with continuous magnetic stirring. Time resolution, 0.2 s. ColE3(549C-Org488;85C/86C-Alx568)Imm, 20 nM. Additions of BtuB, OmpF to concentrations of 0.2 and 2 μM, respectively, are marked by arrows. Buffer, consisting of 0.6% C8E4, 20 mM Tris, pH 8.0. 0.15 M NaCl, was added in trace c only. Three traces (a, b and c) were recorded separately and are shown with shifted positions for better presentation.

was found to depend on ionic strength. The neutralization by isolated BtuB of colE3 cytotoxicity required a higher BtuB concentration in the presence of 0.3 M ambient ionic strength and was abolished at 1.0 M (data not shown), whereas under low salt conditions (~20 mM), colE3 is cytotoxic at a concentration of 10−30 pM (not shown).

Colicin E3 Affinity for OmpF. Preincubation of OmpF with wild type colicin E3 in detergent solution did not completely neutralize colicin cytotoxicity. However at an OmpF concentration ≥10 μM, cell growth was diminished, while it was not abolished entirely (not shown). This implies (i) a relatively low binding affinity of the colicin E3 to OmpF and/or (ii) the necessity of prior interaction of the colicin with BtuB that would unfold the colicin and expose the OmpF-binding site located in the colicin N-terminal glycine-rich segment (*16*). While this segment is disordered, its interaction with OmpF could be prevented by involvement in interactions with other parts of the colicin, as in the case of colicin Ia where an extended N-terminal segment resolved in the crystal structure (residues 23−46; residues 1−22 are not resolved) is embedded between the long helices of the T- and C-domains (*22*).

ColE3 Unfolding Initiated by in Vitro Binding to BtuB and OmpF. Spectral Measurements. The amplitude of the maximum at 498 nm in the excitation spectrum colE3(85C/86C-Alx568;549C-Org488)Imm, which is associated with FRET from Org488 to Alx568, was not affected by the presence of detergent, 0.6% C8E4, or BSA (not shown), but under low ionic strength conditions rapidly decreased after addition of BtuB, 0.2 μM (Figure 4, +). This implies that BtuB binding triggers a change in the conformation of the colE3Imm coiled-coil R-domain that results in an increase of the distance between the T- and C-domains. Random orientation of donor and acceptor dyes is assumed because of surface location and flexibility of the dye-conjugated cysteine residues used in this study. Mixing of the other component of the colicin E3 outer membrane translocon, OmpF (2 μM), also caused a decrease in FRET efficiency (Figure 4, △). It was necessary to use a concentration of OmpF larger than that employed with BtuB, because of the lower binding affinity of colicin E3 for OmpF compared to BtuB.

After correction of Alx568 emission for the presence of 1.5 mol of this dye per 1 mol of colE3, a decrease in FRET efficiency from 50% to 41% and 36%, respectively, resulted from addition of BtuB and OmpF. Using formulas 1 and 2 (Materials and Methods) and a value of R_o = 62 Å, this corresponds to an increase between the donor (C-domain) and acceptor (T-domain) dyes of approximately 4.4 ± 0.5 Å and 3.5 ± 0.5 Å. Thus, the total increase in donor−acceptor distance that can result from colicin interactions with BtuB and OmpF is 7−8 Å. Such a distance change does not necessarily imply an extensive colE3 unfolding. However, they are in agreement with crystallographic data that show an extended helix displacement upon the coiled-coil binding with BtuB (*4*). Such a displacement could be translated to the partial separation of the T- and C-domains.

The presence of the isolated 135 residue R-domain, R135 (1 μM), which efficiently competes with intact colicin E3 for binding with BtuB (*4*), prevented FRET changes upon addition of BtuB to the doubly labeled colE3 (data not shown). However, R135 with an R399A mutation that has a decreased affinity for BtuB (see data presented in Figure 6 and in ref *4*) was unable to prevent FRET changes even at a 2-fold excess of the R399A mutant.

Time Course of the FRET Decrease. In Figure 5, the time course of FRET changes is shown with a 0.2 s time resolution. The measurements were performed using a conventional fluorimeter cuvette with magnetic stirring. Therefore, the rate of a fast phase in FRET change upon addition of BtuB and OmpF (1 ± 0.3 s^{-1}; Figure 5a,b) is probably limited by the mixing rate. The sequence of additions of BtuB and OmpF (OmpF followed by BtuB and *vice versa* in traces a and b) did not affect the rates and amplitudes of FRET changes, implying that the effects of

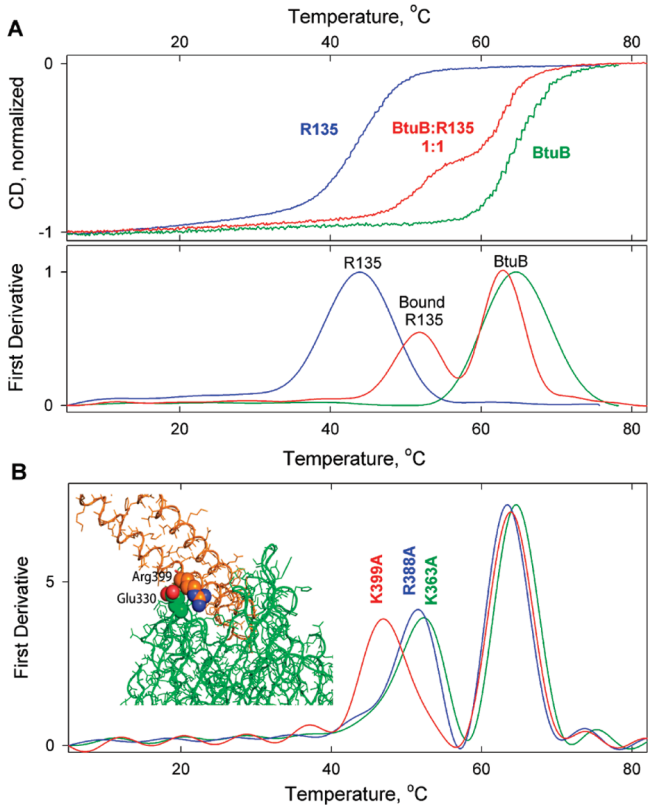

FIGURE 6: Changes in thermal stability of R135 and R135 mutants upon binding to BtuB. A. Thermal melting profiles (upper panel) and first derivatives of profile functions (lower panel) of R135 and BtuB, measured individually and in complex. BtuB (green); BtuB:R135, 1:1 mol/mol (red); R135 (blue). B. First derivative functions of CD thermal melting profiles of mutationally altered R135 complexed to BtuB. K363A (green); R388A (blue); R399A (red); concentration of peptides and BtuB, 1 μM. Optical path length, 5 mm. Inset: Ribbon diagram of R135 showing position of K363, R388, and R399 mutants, shown in ball-and-sick format. The peptide T_m values were determined using the maxima of first derivative curves after noise reduction. Buffer, 10 mM Tris, pH 8.0, 0.1 M NaCl, 0.1% LDAO.

BtuB and OmpF are independent and additive. The effects of both outer membrane proteins were significantly diminished in the presence of 0.15 M NaCl (Figure 5c), implying a major role of electrostatic interactions under these *in vitro* binding conditions. For comparison, BSA at a concentration (15 μM) larger than that used for BtuB and OmpF caused an insignificant decrease in FRET efficiency, comparable with the rate of photobleaching of Alx568 emission (rate constant $\leq$0.001 s^{-1}; not shown).

Electrostatic Interactions of Colicin E3 with BtuB. The high α-helical content (92%) of the colE3 R-domain is preserved in the 135-residue fragment of colE3 (R135; residues Thr313−Glu447). The coiled-coil structure of R135 melts cooperatively at 43.9 ± 0.4 °C (Figure 6A, blue), with complete restoration of the coiled-coil structure after cooling (not shown). The BtuB β-barrel structure melts at 64.6 ± 0.5 °C irreversibly (Figure 6A, green). The 1:1 complex of R135 and BtuB displays two cooperative unfolding transitions at 51.8 ± 0.7 and 63 °C, respectively (Figure 6A, red), implying that the binding to BtuB substantially increases the thermal stability of R135, which is attributed to a significant negative enthalpy of binding.

A requirement of low ionic strength conditions for BtuB to induce partial separation of C- and T-domains of colE3 underscores the importance of electrostatic interactions between the R-domain and BtuB. Several single point mutations in the R135 apex surface contacting BtuB were tested for their effect on the increase in R135 thermal stability. R388A and K363A mutations did not affect stability of R135 bound to BtuB (T_m, 51.5 and 52.3 °C, respectively; Figure 6B, blue and green), whereas the increase in its thermal stability caused by a K399A mutation in R135 obtained upon binding to BtuB (T_m, 46.9 °C; Figure 6B, red) was significantly smaller. In the crystal structure of R135/BtuB obtained under high salt conditions (*4*), the amino group of the BtuB Lys399 is 3.7 Å from the carboxyl group of Glu330. The strength of this salt bridge would significantly increase under conditions of low ionic strength.

DISCUSSION

Cys-Specific Dual Labeling of Proteins. Cys-specific dual labeling with fluorescent dyes has been used for FRET analysis of protein folding and unfolding (*14, 23*−*25*). For small proteins, the donor and acceptor dyes have been attached to cysteines introduced at the amino and carboxy termini of the proteins (*23, 24*). Protein labeled with one dye was separated from unlabeled protein and protein with two bound dye molecules by ion exchange chromatography, and subsequently was conjugated with the second dye. This method depends on separation and analysis of conjugation products after each step. The difference in reaction rates (*25*) and Cys accessibility (*14*) was also used for dual labeling of native proteins. In the present study, we used another feature of thiol groups, the capability for reversible formation of disulfide bonds, which makes cysteine residues unavailable for labeling. An alternative approach for targeted dual fluorescent labeling of proteins used poly histidine sequences, "His-tags" (*26*). In this case, a single Cys must be introduced, which would be conjugated with one fluorescent dye, and another dye can be attached to a His-tag that is often introduced for purification purposes at the amino or carboxy terminus.

The key step in the application of the method used in the present study is the design of a disulfide bond. The high propensity for disulfide bond formation between Cys residues [group A thiols in the MODIP prediction algorithm (*27*)] does not allow enough time to keep thiols available for conjugation under aerobic conditions. This problem could be solved by removal of reducing agents under anaerobic conditions. In the present study, another solution for this problem was found, design of disulfide bonds between neighboring Cys residues in the flexible region of the folded protein (A85C and A86C). Therefore, if the protein has internally disordered domains, neighboring residues in these domains are candidates for disulfide bond design.

Receptor-Induced Conformation Changes in ColE3. The method of Cys-specific dual labeling of colicin E3, developed in this study, allowed detection of the ~8 Å increase in distance between the C- and T-domains upon colicin binding in detergent solution to the outer membrane proteins, BtuB and OmpF (Figures 4 and 5). Such an interdomain separation induced by receptor binding was predicted as an early step in the cellular import of the nuclease colicins following binding to BtuB receptor (*4, 28*). It is considered to be necessary for polypeptide unfolding and subsequent insertion

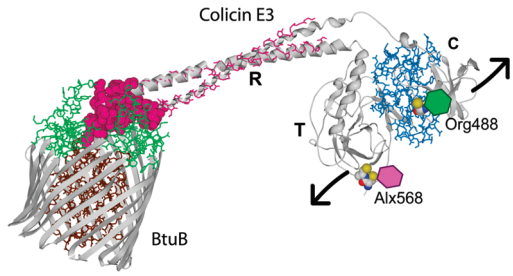

FIGURE 7: Energy transducing function of the colicin E3 coiled-coil domain. Extracellular loops of BtuB (green; backbone and side chain atoms are shown) are shown wrapping the apex of the coiled-coil R-domain shown in pink (space-filling presentation of the R135 residues 372−402). Only backbone atoms of the rest of R135 are shown (pink). Apex of R135 coiled-coil is superimposed with same atoms of entire colE3 molecule (T-, R- and C-domain; ribbon diagram in gray). Imm, blue. Mutated Cys residues in the T- (A85C, A86C) and C-domains (K549C) conjugated with Alx568 and Org488, respectively, are shown in space-filling presentations. FRET results (Figure 4) are interpreted as an increase in distance between the T- and C-domains upon colE3 binding to BtuB, arising predominantly from separation of the T-domain from the C-domain−Imm complex.

into the OmpF porin. However, taking into account the large distance of separation of the BtuB-binding site from the domain to be unfolded, with these sites connected only by a coiled-coil (Figure 7), it was not clear if the colicin interaction with BtuB alone would be sufficient to initiate concomitant conformation changes. This implies that deformation accumulated in the BtuB-binding apex of the coiled-coil, which was not seen at the 2.75 Å resolution of the structure of the R-domain−BtuB complex (*4*), is transmitted through the hydrogen bond network to the termini of the R-domain coiled-coil. This would result in separation of the T-domain from the Imm protein which, at this stage of colicin import, probably remains tightly bound [K_d, 10^{-13} M^{-1} (*29*)] to the C-domain (Figure 7).

A different view, that colicin binding to BtuB does not induce any conformation changes in the colicin, resulted from calorimetric analysis (ITC) of the binding of the DNAase colicin E9 with BtuB (*5*). The ITC measurements found a 1−2 nM K_d for dissociation to be associated with a large negative entropy change, which would not be expected if the binding was linked to a large scale unfolding event. The negative entropy changes were attributed to ordering of the BtuB extracellular loops. There is, however, a question in general about the accuracy of ITC measurements that yield a $K_d = 10^{-9}$ M when the concentration of substrate is in the μmolar range (*30, 31*). Thus, it is suggested that there may be some uncertainty in the ITC determination of the entropy change associated with the binding of colicin E9 to BtuB (*5*). A qualitative estimate of the K_d for dissociation of coliicn E3 from BtuB, using competition for BtuB in an assay of cytotoxicity (Table 2B), showed it to be $\leq 10^{-10}$ M, in approximate agreement with the ITC measurements (*5*).

An additional caveat regarding the ITC data (*5*) is associated with ionic strength conditions. The ITC analysis was carried out in 0.15 M NaCl, conditions under which the FRET changes described in the present study would not be detectable (Figure 5c).

The separation of the T- and C-domains detected in these FRET experiments could be a precursor to release of the immunity protein, which would require more free energy than is available from binding of the colicin E3 coiled-coil to BtuB (*4, 5*).

Thus, results of the present FRET experiments (Figures 4 and 5) imply an energy-transmission function of the colicin E3 coiled-coil. Structures have recently been obtained of the complexes of the receptor-binding coiled-coil of colicin E2 with BtuB (*32*) and of colicin Ia with its receptor, CirA (*33*). The oblique orientation of the R-domains relative to receptors in these structures is similar to that of the colE3 coiled-coil complex with BtuB. Thus, the mechanism proposed here for separation of the C- and T-domains is presumed to have general applicability to colicins with extended coiled-coiled R-domains.

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