Global structural rearrangement of the cell penetrating ribonuclease colicin E3 on interaction with phospholipid membranes

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Abstract

Nuclease type colicins and related bacteriocins possess the unprecedented ability to translocate an enzymatic polypeptide chain across the Gram-negative cell envelope. Here we use the rRNase domain of the cytotoxic ribonuclease colicin E3 to examine the structural changes on its interaction with the membrane. Using phospholipid vesicles as model membranes we show that anionic membranes destabilize the nuclease domain of the rRNase type colicin E3. Intrinsic tryptophan fluorescence and circular dichroism show that vesicles consisting of pure DOPA act as a powerful protein denaturant toward the rRNase domain, although this interaction can be entirely prevented by the addition of salt. Binding of E3 rRNase to DOPA vesicles is an endothermic process ($\Delta H = 24 \text{ kcal mol}^{-1}$), reflecting unfolding of the protein. Consistent with this, binding of a highly destabilized mutant of the E3 rRNase to DOPA vesicles is exothermic. With mixed vesicles containing anionic and neutral phospholipids at a ratio of 1:3, set to mimic the charge of the *Escherichia coli* inner membrane, destabilization of E3 rRNase is lessened, although the melting temperature of the protein at pH 7.0 is greatly reduced from 50°C to 30°C. The interaction of E3 rRNase with 1:3 DOPA:DOPC vesicles is also highly dependent on both ionic strength and temperature. We discuss these results in terms of the likely interaction of the E3 rRNase and the related E9 DNase domains with the *E. coli* inner membrane and their subsequent translocation to the cell cytoplasm.

Keywords: conformational changes; enzymes; membrane-associated proteins; circular dichroism; fluorescence; thermodynamics; hydrodynamics; calorimetry

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Abbreviations: DOPA, 1,2-dioleoyl-sn-glycero-3-phosphate (monosodium salt); DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; E3 rRNase, the isolated 12-kDa rRNase domain of colicin E3; Im3, the colicin E3 immunity protein; E9 DNase, the isolated 15-kDa endonuclease domain of colicin E9; KPi, potassium phosphate; R_{L-P} , lipid:protein molar ratio; CD, circular dichroism; ITC, isothermal titration calorimetry.

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The ability of some proteins and peptides to penetrate cells has opened up a potential route through which therapeutic biomolecules can be effectively delivered to the cell cytoplasm (Fawell et al. 1994; Schwarze et al. 1999). The colicins provide an excellent model system to study membrane penetration since they possess a conserved modular organization that permits delivery of a range of cytotoxic activities to different targets within a bacterial cell (James et al. 2002).

With respect to their mechanism of translocation, the colicins are grouped into types A or B, with the import of group A colicins dependent on the Tol protein complex and the B group on the Ton system (Lazdunski et al. 1998). However, despite identification of the proteins required for colicin translocation, the exact role of the Tol and Ton complexes in colicin translocation is not fully understood. Both A and B type colicins include toxins that kill cells through depolarization of the inner membrane, the pore-forming colicins, and nuclease type colicins. In the case of the pore-forming colicins (A, B, E1, Ia, K, N), the cytotoxic domain need only be transported to the periplasmic side of the inner membrane, where it spontaneously inserts into the inner membrane, forming voltage dependent ion channels that depolarize the cell (Zakharov and Cramer 2002). The mechanism of ion channel formation involves the translocation of significant portions of charged polypeptide chain across the hydrophobic core of the membrane (Lakey and Slatin 2001). The pore-forming colicins share similar helical structures and show conservation of key residues involved in pore formation (Cramer et al. 1995).

In contrast to the pore-forming colicins, the nuclease-type colicins form a diverse group, including both RNA and DNA hydrolyzing enzymes. This family of toxins can be divided by their enzymatic activities into the DNases (E2, E7, E8, and E9) that kill cells through cleavage of chromosomal DNA (Pommer et al. 2001), the rRNases (E3, E4, and E6) that specifically cleave 16S RNA (Bowman et al. 1971; Walker et al. 2004), and the tRNases (E5 and D) that cleave some tRNAs (Ogawa et al. 1999; Tomita et al. 2000). Within each of the DNase and rRNase groups the toxins share a high degree of sequence identity, whereas the tRNases E5 and D share no sequence identity. Structures of the cytotoxic domains of colicins E9, E3, and D emphasize that there is no structural similarity between the three types of nuclease domain (Kleanthous et al. 1999; Carr et al. 2000; Graille et al. 2004), yet each is transferred to the cytosol by the same translocation machinery.

The cytotoxic domains of the nuclease type colicins must completely traverse the inner membrane to reach their cytoplasmic targets. While this process is poorly understood, it has been shown that the DNase domain of colicin E9 is capable of forming channels in planar lipid bilayers and that the structural integrity of the domain is highly destabilized through its interaction with anionic phospholipids (Mosbahi et al. 2002, 2004). However, the lack of conserved sequence or structural features between the different colicin nucleases suggests either that their enzymatic domains traverse the inner membrane by different pathways or that they share a common pathway that is independent of tertiary structure.

In this work we expand our studies on the interaction of nuclease type colicins with phospholipid membranes.

We demonstrate that the rRNase domain of colicin E3 is destabilized by its interaction with artificial membranes in a manner similar to the DNase domain of colicin E9. Using fluorescence, circular dichroism, and isothermal titration calorimetry, we show that this destabilization is dependent on the anionic phospholipid content of the membrane, ionic strength, and temperature. Interaction of the E3 rRNase domain with anionic membranes is a strongly endothermic process, likely reflecting an unfolding process. Destabilization of the toxin also occurs with phospholipid membranes having a similar content of anionic and neutral phospholipids as the *Escherichia coli* inner membrane. We suggest that destabilization of the toxin is a prerequisite for translocation that likely occurs prior to traversal of the inner membrane.

Results

Anionic phospholipid membranes induce structural changes in the E3 rRNase

The transition from the water-soluble to membrane-bound state of cell penetrating polypeptides is likely to involve significant structural rearrangement. Colicin E9 is destabilized through its interaction with anionic phospholipid membranes, most likely as a prelude to membrane translocation (Mosbahi et al. 2004). The cytotoxic domain of colicin E3 (E3 rRNase) shares no sequence or structural homology with the E9 DNase but like the E9 DNase is very positively charged (pI = 9.5). Changes in λ_{max} occur with the E9 DNase on addition of anionic phospholipid but not with the E9 DNase-Im9 complex (Mosbahi et al. 2004). For the E9 DNase, changes in λ_{max} correspond to structural changes in the protein that can be observed by other techniques such as circular dichroism (CD). Changes in λ_{max} are widely used in protein lipid interactions and are interpreted similarly (Morillas et al. 1999; Sanghera and Pinheiro 2002).

In order to determine if E3 rRNase underwent similar interaction and destabilization on binding anionic membranes, which might suggest a general mechanism for inner membrane translocation by colicin nuclease domains, we initially used fluorescence to monitor structural changes in E3 rRNase on the addition of phospholipid vesicles. E3 rRNase contains two tryptophan residues at positions 43 and 54. In solution at pH 7.0, the enzyme displays a fluorescence emission maximum (λ_{max}) of 327 nm. On addition of 1,2-dioleoyl-sn-glycero-3-phosphate (monosodium salt) (DOPA) vesicles, the λ_{max} was red-shifted to 342 nm, with the fluorescence intensity showing an enhancement of ~70% at this wavelength (Fig. 1A). With vesicles composed of zwitterionic phospholipids (1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPC) no change in the λ_{max} of E3 rRNase was observed at pH 7.0

Figure 1. Changes in tryptophan fluorescence and far-UV CD spectra of the E3 rRNase induced by phospholipids. (A) Fluorescence emission spectra of the E3 rRNase in aqueous solution and in the presence of negatively charged phospholipid (DOPA) vesicles ($R_{\text{L-P}} = 150$) at pH 4.0 and 7.0 ($\lambda_{\text{ex}} = 280$ nm). The E3 rRNase concentration in each case was 1 μM . (B) Far-UV CD spectra of E3 rRNase (8.4 μM) in the absence and in the presence of DOPC and DOPA vesicles ($R_{\text{L-P}} = 150$). Spectra were recorded in 10 mM KPi (pH 7.0). (C) Dependence of the E3 rRNase fluorescence emission maxima on the lipid:protein ratio ($R_{\text{L-P}}$). Spectra were recorded at an E3 rRNase concentration of 0.2 μM in 10 mM KPi (pH 7.0). All experiments were performed at 25°C.

(data not shown). Thus, with anionic phospholipid vesicles, E3 rRNase undergoes significant change to its tertiary structure.

Far-UV CD spectra of E3 rRNase display a minimum at ~ 196 nm and a maximum at 226 nm (Fig. 1B). Estimates of E3 rRNase secondary structure from far-UV CD indicate 7% α -helix and 26% β -sheet, values close to those observed in the X-ray structure of the E3 rRNase in complex with its immunity protein Im3 (5% α -helix, 25% β -sheet) (Walker et al. 2004). Consistent with fluorescence data, in the presence of DOPC vesicles there is little change to the far-UV CD spectra of E3 rRNase. However, with DOPA and 2:1 DOPA:DOPC vesicles there is a shift in the minimum to 200 nm and a loss of negative ellipticity in the region of the spectra from 190 to 200 nm, indicating a change in secondary structure. In addition, the maximum at 226 nm is absent in the presence of anionic phospholipids. The loss of this feature is also observed in heat-denatured protein as well as mutant proteins (e.g., Y64A E3 rRNase) that have highly destabilized structures (Walker et al. 2004). Interaction with anionic vesicles therefore affects both the secondary and the tertiary structures of the E3 rRNase.

To determine the lipid:protein molar ratio ($R_{\text{L-P}}$) required for saturation of the change in protein fluorescence, we measured λ_{max} over a range of lipid:protein ratios. With phospholipid vesicles consisting of pure DOPA and 2:1 DOPA:DOPC, the λ_{max} increased up to a $R_{\text{L-P}}$ of ~ 50 , with the addition of further phospholipid vesicles, up to a $R_{\text{L-P}}$ of 500, having little effect (Fig. 1C). Thus, a $R_{\text{L-P}}$ of > 50 is required to ensure sufficient binding sites for E3 rRNase. In addition, these experiments indicate that this value is essentially independent of the DOPA:DOPC ratio, at least down to a value of 2:1. With pure DOPC vesicles no binding was observed at any $R_{\text{L-P}}$.

In solution at pH 4.0 E3 rRNase has a λ_{max} of 354 nm, indicating complete solvent exposure of its two tryptophan residues (Fig. 1A). On addition of DOPA vesicles at this pH we observed a blue shift of the λ_{max} to 340 nm, indicating that the tryptophan residues become less solvent-exposed in the presence of anionic phospholipid vesicles. The value of 340 nm observed at pH 4.0 is very similar to that observed at pH 7.0, suggesting that E3 rRNase tertiary structure is not required for the interaction with anionic phospholipid vesicles.

This was further tested using a destabilized mutant of E3 rRNase, in which Tyr64 is substituted for Ala. We have shown previously using fluorescence and far-UV CD that this mutant is largely unfolded in solution (Walker et al. 2004). At pH 7.0, Y64A E3 rRNase has a λ_{max} of 354 nm, identical to the wild-type protein at pH 4.0. On addition of DOPA phospholipid vesicles to E3 rRNase Y64A at pH 7.0 the λ_{max} is blue-shifted to

343 nm, similar to that observed for wild-type E3 rRNase at pH 4.0 (data not shown). Hence, although the E3 rRNase Y64A mutant lacks native structure, it is capable of forming a complex with phospholipid vesicles similar to that seen for the wild-type enzyme. This suggests that binding of wild-type E3 rRNase to anionic phospholipid vesicles must trigger a global rearrangement and loss of its native structure. If this is indeed the case, then significant differences in the thermodynamics of the binding of Y64A and wild-type E3 rRNase to anionic phospholipid vesicles should be evident.

Thermodynamics of the E3 rRNase lipid interaction reflect a loss of native protein structure

Fluorescence and circular dichroism experiments indicate that the interaction of E3 rRNase with phospholipid vesicles leads to a loss of native-like structure. To generate quantitative thermodynamic data on the E3 rRNase-lipid interaction we used isothermal titration calorimetry to monitor the binding process. Isothermal titration calorimetry has previously been used to measure the interaction between phospholipid vesicles and

polypeptides ranging from small cell-penetrating peptides such as HIV-1 TAT (Ziegler et al. 2003), to larger anti-microbial peptides such as the 34-residue nisin Z (Breukink et al. 2000), and large intact serum proteins (Dimitrova et al. 2000).

On titration of E3 rRNase into a solution of DOPA vesicles (25 μM) at pH 7.0 we observed a strongly endothermic interaction (Fig. 2). This was found to be consistent over a range of lipid concentrations, although at higher lipid concentration (100 μM DOPA) it was apparent that aggregation occurred toward the end of the titration. However, titrating E3 rRNase into DOPA vesicles (25 μM) allowed us to obtain an entire binding isotherm (Fig. 2A). Fitting the data to a single site binding model yielded a value of 56 (± 5) for the lipid:protein molar ratio required for saturation binding, similar to the value of 50 estimated by fluorescence (Fig. 1C). These experiments yielded a value for $\Delta H = 23.9 (\pm 1.0) \text{ kcal mol}^{-1}$ of E3 rRNase. An apparent equilibrium dissociation constant (K_d) of 8.8 (± 2.6) nM was obtained, yielding a free energy change (ΔG) of $-11.0 (\pm 0.1) \text{ kcal mol}^{-1}$. Thus the overall process of E3 rRNase binding DOPA vesicles at 25°C and pH 7.0 is

Figure 2. Thermodynamics of the interaction of wild-type and Y64A E3 rRNase with anionic vesicles determined by isothermal titration calorimetry. Wild-type (A) and Y64A E3 rRNase (B) were titrated into a solution of DOPA vesicles in 10 mM KPi (pH 7.0) at 25°C. Data were fitted to a single site binding equation after subtraction of experimentally determined heat of dilution of protein into buffer.

entropically driven ($T\Delta S = 34.9 (\pm 1.0) \text{ kcal mol}^{-1}$), consistent with the loss of the highly ordered protein structure as suggested by fluorescence and CD.

To test this idea further we used the Y64A E3 rRNase mutant, which lacks wild-type structure and a cooperative unfolding transition in solution (Walker et al. 2004). Since CD and fluorescence indicate that the final protein–lipid complex for wild-type and Y64A E3 rRNase are similar, then formation of the protein–lipid complex should be significantly less endothermic if this mainly reflects unfolding of the wild-type protein. Indeed, titration of Y64A E3 rRNase into DOPA vesicles was in fact found to be exothermic with $\Delta H = -18.5 (\pm 3.7) \text{ kcal mol}^{-1}$ (Fig. 2B). Overall, the interaction of Y64A E3 rRNase with DOPA yields a free energy change (ΔG) of $-9.4 (\pm 0.4) \text{ kcal mol}^{-1}$. Therefore, in contrast to the interaction of wild-type E3 rRNase with DOPA vesicles, binding of Y64A E3 rRNase is entropically unfavorable ($T\Delta S = -9.1 (\pm 4.1) \text{ kcal mol}^{-1}$). Thus, although the protein–lipid complex can be formed from either the native form of the protein or from a highly destabilized state, in the former case a prerequisite for complex formation is loss of the native protein structure.

Binding of the E3 rRNase to anionic phospholipids is electrostatically driven and temperature-dependent

Using phospholipid vesicles consisting of purely anionic phospholipids provides a useful model system to investigate interactions between the positively charged colicin nuclease domains and the anionic cytoplasmic membrane. Indeed, such vesicles are also used to study the structural rearrangements of pore-forming colicin domains (Muga et al. 1993). However, the *E. coli* cytoplasmic membrane consists of $\sim 70\%$ – 80% zwitterionic and only 20% – 30% anionic phospholipids (Kadner 1996). Fluorescence and far-UV CD show that E3 rRNase does not interact with vesicles composed solely of neutral lipids (Fig. 1). We therefore investigated the interaction of E3 rRNase with phospholipid vesicles composed of both DOPA and DOPC at different ratios.

In solution at pH 7.0, E3 rRNase displays cooperative and reversible thermal unfolding, with a melting temperature (T_m) of $\sim 50^\circ\text{C}$ when monitored by the change in fluorescence λ_{max} (Fig. 3). In the presence of DOPA vesicles this cooperative transition is lost and replaced by a small and a gradual rise in the λ_{max} in the temperature range 15° – 80°C (Fig. 3). With 1:2 DOPA:DOPC vesicles the thermal denaturation profile is broadly similar to that observed with DOPA vesicles. However, with 1:2.5 or 1:3 DOPA:DOPC vesicles we observe a decrease in the λ_{max} at low temperature and the return of a cooperative unfolding transition. With 1:3 DOPA:DOPC vesicles an approximate T_m of 26°C could be determined from the

Figure 3. Effect of anionic phospholipid vesicles on thermal stability of the E3 rRNase. Change in tryptophan fluorescence λ_{max} was used to monitor unfolding of E3 rRNase in the presence of DOPA, 1:2, 1:2.5, and 1:3 DOPA:DOPC vesicles and in solution at pH 7.0 ($\lambda_{\text{ex}} = 280 \text{ nm}$). Experiments were performed at a protein concentration of $0.2 \mu\text{M}$ and a phospholipid concentration of $30 \mu\text{M}$ ($R_{\text{L:P}} = 150$) in 10 mM KPi (pH 7.0).

unfolding transition for the E3 rRNase. Therefore, using vesicles composed of anionic and neutral phospholipids at a ratio reflecting the charge composition of the *E. coli* inner membrane, we still observe significant destabilization of the E3 rRNase domain. This suggests that the interaction of E3 rRNase with the *E. coli* membrane may be enough to destabilize the protein sufficiently for it to be transported across the lipid bilayer.

This dependence of the E3 rRNase binding on the DOPA content of the vesicles suggests that electrostatic interactions are important to the E3 rRNase–phospholipid interaction. To test this we determined the effect of salt on the binding interaction of E3 rRNase with phospholipid vesicles of differing DOPA content. Measuring the fluorescence λ_{max} of E3 rRNase at NaCl concentrations from 0 to 500 mM in the presence of phospholipid vesicles composed of differing ratios of DOPA:DOPC showed that the ionic strength dependence of the protein–lipid interaction is strongly dependent on the anionic lipid content of the vesicles (Fig. 4). The concentrations of salt that yield half-maximal protein–lipid binding in the presence of DOPA, 2:1 DOPA:DOPC, and 1:3 DOPA:DOPC vesicles were $\sim 345 \text{ mM}$, $\sim 220 \text{ mM}$, and $< 25 \text{ mM}$, respectively. Thus, electrostatic forces are a key determinant of the ability of E3 rRNase to form a protein–lipid complex.

With 1:3 DOPA:DOPC phospholipid vesicles at higher temperatures the concentration of salt required for half-maximal protein–lipid binding is increased from $< 25 \text{ mM}$ at 25°C to $\sim 60 \text{ mM}$ at 35°C and $\sim 80 \text{ mM}$ at 40°C (data not shown), presumably due to destabilization of the protein structure with increasing temperature. To investigate this further we determined the ionic strength

Figure 4. Effect of ionic strength on the E3 rRNase–lipid interaction. Change in fluorescence $\lambda_{\max}$ was used to monitor binding of E3 rRNase to phospholipid vesicles containing DOPA, 2:1 DOPA:DOPC, and 1:3 DOPA:DOPC vesicles. Experiments were performed at a protein concentration of 0.2 μM and a phospholipid concentration of 30 μM ($R_{L:P} = 150$) at 25°C in 10 mM KPi (pH 7.0) with sodium chloride added from 0 to 500 mM.

dependence of the destabilized Y64A E3 rRNase–lipid interaction. With this destabilized mutant a salt concentration of ~ 120 mM was determined for half-maximal protein–lipid binding at 25°C, compared to < 25 mM NaCl with wild-type E3 rRNase (data not shown).

Discussion

The mechanism of protein translocation across membranes remains a complex problem. In this work we have studied the interaction of E3 rRNase with phospholipid vesicles as a model of how E3 rRNase might be able to interact with phospholipid membranes prior to translocation across the *E. coli* inner membrane. We have shown previously that another colicin nuclease domain, the DNase domain of colicin E9, is destabilized in the presence of anionic phospholipid vesicles, undergoing rearrangement of the secondary and tertiary structure of the protein (Mosbahi et al. 2004). Here the interaction of the structurally unrelated rRNase domain of colicin E3 with phospholipid vesicles has been investigated, showing that this basic enzymatic domain ($pI = 9.5$) undergoes a similar destabilizing interaction on adsorption to the membrane surface.

Using tryptophan fluorescence and CD to monitor tertiary and secondary structure changes, we have shown that the E3 rRNase domain is destabilized in the presence of negatively charged phospholipid vesicles. Neutral phospholipid vesicles do not induce such structural alterations, establishing a clear dependence on electrostatic interactions in the interaction of the E3

rRNase with the membrane (Fig. 1). The loss of native-like structure on interaction with the membrane is reflected in the thermodynamics of protein–lipid complex formation. Direct calorimetric measurements of E3 rRNase interaction with DOPA vesicles show that this is a strongly endothermic and entropically favorable process, likely reflecting unfolding of the protein on adsorption to the vesicle surface. This is supported by the observation that for the highly destabilized Y64A E3 rRNase mutant the interaction is exothermic and entropically disfavored. Nevertheless, E3 rRNase–lipid complexes of both wild-type and Y64A E3 rRNase resulted in similar far-UV CD and fluorescence spectra, suggesting that the final protein–lipid complexes are the same (data not shown). Anionic phospholipids therefore act as a powerful denaturant of the E3 rRNase. Reducing the proportion of anionic phospholipids from 1:2 to 1:3 DOPA:DOPC leads to a significant reduction in the destabilizing effect of the protein–lipid interaction. With 1:3 DOPA:DOPC vesicles, a charge composition that reflects the *E. coli* inner membrane, a cooperative transition is observed but with a greatly reduced T_m , $\sim 26^\circ\text{C}$ (Fig. 3). We speculate that the apparent weakening of the interaction at this membrane composition may reflect a need for E3 rRNase to be destabilized but not bound tightly to the membrane for successful translocation across the bilayer. The importance of electrostatic interactions is also suggested by the ionic strength dependence of the protein–lipid interaction (Fig. 4).

The similarity between the interactions of colicin E3 rRNase and E9 DNase, two basic but structurally unrelated cytotoxic nuclease domains, suggests that this destabilizing interaction may play a role in translocation of these toxins into the cytoplasm. Membrane induced destabilization has been reported for a variety of proteins and is thought to be a key process in the action of the pore-forming colicins prior to their insertion into the membrane. For example, destabilization and loss of a cooperative unfolding transition, as monitored by far-UV CD and differential scanning calorimetry, has been reported for colicin A in the presence of anionic phospholipid vesicles (Muga et al. 1993). In this case, the helical secondary structure of the pore-forming domain is preserved while there is a loss of tertiary structure on binding to acidic membranes. Similarly, the membrane permeabilizing δ -endotoxin CytA is strongly destabilized on membrane binding, although there is no requirement for the presence of anionic phospholipids (Butko et al. 1997). In these cases the functional relevance of their interaction with phospholipids is clearly related to their cytotoxic action. This is not the case for the rRNase colicin E3, which kills cells through the cleavage of 16S rRNA, although membrane-induced destabilization seems to be a common requirement in either

translocation across or insertion into the membrane. It has also been suggested that membrane-induced destabilization is an important factor in conversion of the human prion protein (hPrP) to its pathogenic form, with anionic phospholipid interactions shown to have a destabilizing effect on the native protein that may be important in the conversion process (Morillas et al. 1999).

Although destabilization is likely to be important to translocation of colicin nuclease domains, it does not explain how the toxin is able to fully traverse the membrane and enter the cytoplasm as a folded, enzymatically active protein. Moreover, how this is achieved for the structurally dissimilar cytotoxic domains of the colicin E9 and E3 families in addition to the tRNase type colicin E5 and colicin D remains unknown. We note that in mitochondrial import it is also known that proteins must unfold to cross the inner mitochondrial membrane. In this case it has been demonstrated that this unfolding activity is not located at the mitochondrial surface, but for some proteins is driven by the membrane potential of the inner mitochondrial membrane (Huang et al. 2000, 2002). Possible mechanisms of colicin translocation are discussed below.

The pore-forming colicins (A, N, Ia, Ib, and K) are able to translocate a portion of their cytotoxic domain across the inner membrane to form a voltage-gated channel. Indeed, the cytotoxic domains of the pore-forming colicins are also capable of translocating a small functional protein across the lipid bilayer (Slatin et al. 2002). Could the enzymatic colicins mediate their own translocation across the inner membrane by an analogous mechanism? In this regard, the DNase domain of colicin E9 and the closely related E2, E7, and E8 DNase domains have all been shown to form channels in planar lipid bilayers (Mosbahi et al. 2002). However, models of channel formation by the pore-forming colicins indicate that the bulk of the protein remains anchored in the membrane, thereby preventing translocation of the whole cytotoxic domain (Lakey and Slatin 2001). Hence, it seems unlikely that channel formation by the DNase type colicins can wholly explain their translocation, although this may form an important part of the translocation mechanism. An alternative strategy is illustrated by ricin A chain, which uses the endoplasmic reticulum (ER) associated degradation (ERAD) pathway (Simpson et al. 1999). This protein, and related A-B toxins, initially enter eukaryotic target cells through receptor-mediated endocytosis and then enter the cell cytosol through the ERAD pathway. It is thought that although the majority of ricin A chain that enters the cytosol is degraded by the ERAD pathway, sufficient quantities of the toxin are able to enter the cytoplasm. It has been suggested that toxins such as ricin are, at least in part, able to avoid degradation by having relatively few lysine residues and so benefit from a

reduced probability of ubiquitin modification and subsequent targeting to the proteasome (Deeks et al. 2002).

Like colicin E3 and colicin E9, ricin A chain is also destabilized by its interaction with negatively charged phospholipids (Day et al. 2002). Perhaps it is the case that the enzymatic colicins are translocated across the bacterial inner membrane through an analogous prokaryotic retrotranslocation process, although to our knowledge such a system has yet to be described in bacteria. This proposition is attractive since it would presumably be independent of sequence and structure and may be brought about by the colicins resembling misfolded or misincorporated membrane proteins. This is perhaps the state we observe in this work on the interaction of E3 rRNase with anionic membranes and that we have observed previously with the E9 DNase (Mosbahi et al. 2004).

Materials and methods

Protein purification and lipid vesicle preparation

E3 rRNase was purified and quantified as described previously (Walker et al. 2003). 1,2-dioleoyl-sn-glycero-3-phosphate (monosodium salt) (DOPA) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were supplied by Avanti Polar Lipids and used without further purification. Lipid vesicles were prepared using a film hydration method followed by extrusion through polycarbonate filters, as described previously (Mosbahi et al. 2004). The presence of large unilamellar vesicles with a diameter of ~ 100 nm was confirmed by electron microscopy after negative staining with ammonium molybdate (Olson et al. 1979).

Fluorescence measurements

Fluorescence emission spectra were recorded on a Spex-Fluor-oMax-3 spectrofluorimeter (Jobin Yvon) equipped with a Neslab RTE-111 circulating water bath. Spectra were recorded in 10 mM KPi (pH 7.0) at a protein concentration of 0.2 or 1 μ M using an excitation wavelength of 280 nm with excitation and emission slits set to 3 nm. All the spectra were an average of five scans and were corrected by subtraction of the blank (buffer or phospholipid vesicle-buffer) spectrum. Thermal denaturation experiment was obtained by measuring the λ_{max} in the temperature range of 15–80°C. For the measurements of binding of E3 rRNase to lipid membranes in the presence of NaCl, E3 rRNase was used at a protein concentration of 0.2 μ M in the presence of phospholipid vesicles with NaCl added as required. The protein–lipid vesicle mixture was allowed to incubate for ~ 30 min before each measurement.

Circular dichroism

Circular dichroism (CD) spectra of E3 rRNase were recorded on a Jasco J-810 spectropolarimeter equipped with a Jasco Peltier temperature controller (PFD-4255). Spectra were recorded in a 10-mm path-length quartz cuvette at a scan speed of 100 nm min⁻¹ with a response time of 1 sec and with the spectral bandwidth set to 1 nm. Measurements in the far-UV

(190–300 nm) were recorded in 10 mM KPi (pH 7.0) at 25°C and at a protein concentration of 0.1 mg mL⁻¹. Blanks (buffer in the absence or the presence of phospholipid vesicles) were recorded and subtracted from the original spectra. The spectra obtained were the average of 10 scans, with baseline subtraction.

Isothermal titration calorimetry

Isothermal titration calorimetry experiments were performed in 10 mM KPi (pH 7.0) using a Microcal VP-ITC calorimeter (Microcal). Data handling (baseline subtraction, peak integration, sample dilution) procedures were performed with MicroCal software. Binding isotherms were recorded by titration of protein, 10 µM for wild-type E3 rRNase and 20 µM for Y64A E3 rRNase, into a solution of lipid at 25 µM or 50 µM, respectively. Solutions were degassed prior to the ITC experiment and in both cases an initial injection of 2 µL was followed by multiple 10-µL injections. After baseline correction by subtraction of data from heat of dilution experiments, the data for protein–lipid complex formation were fitted to a single site binding equation using the MicroCal ORIGIN software.

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