Interaction of the Eukaryotic Pore-forming Cytolysin Equinatoxin II with Model Membranes: $^{19}$F NMR Studies

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Sea anemones produce a family of 18–20 kDa proteins, the actinoporins, which lyse cells by forming pores in cell membranes. Sphingomyelin plays an important role in their lytic activity, with membranes lacking this lipid being largely refractory to these toxins. As a means of characterising membrane binding by the actinoporin equinatoxin II (EqTII), we have used $^{19}$F NMR to probe the environment of Trp residues in the presence of micelles and bicelles. Trp was chosen as previous data from mutational studies and truncated analogues had identified the N-terminal helix of EqTII and the surface aromatic cluster including tryptophan residues 112 and 116 as being important for membrane interactions. The five tryptophan residues were replaced with 5-fluorotryptophan and assigned by site-directed mutagenesis. The $^{19}$F resonance of W112 was most affected in the presence of phospholipid micelles or bicelles, followed by W116, with further change induced by the addition of sphingomyelin. Although binding to phosphorylcholine is not sufficient to enable pore formation in bilayer membranes, this interaction had a greater effect on the tryptophan residues in our studies than the subsequent interaction with sphingomyelin. Furthermore, sphingomyelin had a direct effect on EqTII in both model membranes, so its role in EqTII pore formation involves more than simply an indirect effect mediated via bulk lipid properties. The lack of change in chemical shift for W149 even in the presence of sphingomyelin indicates that, at least in the model membranes studied here, interaction with sphingomyelin was not sufficient to trigger dissociation of the N-terminal helix from the β-sandwich, which forms the bulk of the protein.

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Introduction

The actinoporins are a family of sea anemone toxins that function by forming pores in cell membranes.¹–³ These highly basic proteins, of mass 18–20 kDa, display permeabilising activity in model lipid and cell membranes that is markedly enhanced by the presence of sphingomyelin (SM).

Abbreviations used: DHPC, dihexanoyl-phosphatidylcholine; DMPC, dimyristoyl-phosphatidylcholine; DPC, dodecylphosphocholine-²H₃₀; EqTII, equinatoxin II; F-Trp-EqTII, equinatoxin II with all five Trp replaced with 5-F-Trp; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PC, phosphatidylcholine; POC, phosphocholine; SM, sphingomyelin; SUV, small unilamellar vesicles.

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The actinoporins differ from the various classes of bacterial pore-forming toxins⁴–⁷ in several respects: they are more potent, the pore they form does not have a stable structure and has not yet been visualized directly, and they are smaller and extremely stable towards proteolytic degradation. Indeed, the potency and properties of these cytoly-sins have prompted their evaluation as the toxic component of chimeric proteins targeted at tumour cells⁸,⁹ and human parasites.¹⁰ The nature of their interaction with lipids in bilayer membranes and the specific role of sphingomyelin in pore formation are not understood at the molecular level.

In common with many pore-forming toxins, the actinoporins are highly water-soluble, stable proteins, and yet their only known activity is the formation of oligomeric pores in membranes, consisting of three or, more likely, four monomers.¹¹–¹⁵ Moreover, tetramers of the
Actinoporin sticholysin II have been observed in solution and when crystallized on lipid multilayers. The resulting pores have a radius of about 1 nm and are permeable to small molecules and solutes, with the resulting osmotic imbalance promoting cell lysis.

Sphingomyeloycin plays a key role in the lytic activity of the actinoporins. The haemolytic activity of a cytolysin from Stoechactis (now Stichodactyla) helianthus was inhibited by pre-incubation with SM, and treatment of erythrocyte membranes with sphingomyelinase rendered them resistant to the activity of the actinoporins. The haemolytic activity of the actinoporin equninoxin II (EqTII) was also inhibited by pre-incubation with SM. More recent studies with sticholysins I and II on model membranes confirmed that SM enhances lytic activity and suggested that cholesterol may have a minor role. The interaction of EqTII with large unilamellar vesicles containing phosphatidylcholine (PC) was reversible and did not involve major conformational changes, but the presence of SM enabled irreversible insertion and pore formation, which were associated with major conformational changes. Some EqTII-induced leakage was observed from large unilamellar vesicles containing only PC and cholesterol, again suggesting a contribution of this sterol lipid to actinoporin cytolytic activity, albeit a much less significant one than that of SM. Fluorescence studies of EqTII binding to lipid vesicles showed that association was markedly enhanced by the presence of SM.

Equinatoxin II, the subject of this work, is a 179 residue, 19.8 kDa cytolysin isolated from the Mediterranean anemone Actinia equina. It is essentially identical with tenebrosin-C, a cytolysin isolated from the Australian red waratah anemone Actinia tenebrosa, and the first of this class of toxins to be sequenced in full; tenebrosin-C has a variant S177T and EqTII has a variant P81D. Its structure, solved in solution and by X-ray crystallography, consists of two short helices packed against opposite faces of a β-sandwich structure formed by two five-stranded β-sheets.

A number of studies have been carried out in an effort to elucidate the molecular mechanism of actinoporin pore formation. CD and FTIR studies both detected small increases in β-sheet and α-helical content at the expense of random structure in the presence of unilamellar vesicles. By site-directed mutagenesis, it was shown that at least two regions of EqTII became embedded in lipid membranes, the N-terminal region (residues 10–28) and the surface aromatic cluster including tryptophan residues 112 and 116. The current model of pore formation proposes that EqTII binds to the membrane initially via the aromatic-rich region, then the N-terminal region is transferred to the lipid membrane and, finally, across the bilayer to form a final functional pore. The structural details of the final oligomeric assembly are not known.

The perturbation by EqTII of model lipid membranes composed of PC and SM has been investigated using solid-state NMR and electron microscopy showed the formation of an isotropic lipid phase in SM-containing bilayers with EqTII. The toxin enhanced slow motions in the membrane lipids and destabilized membranes containing SM, suggesting a preferential interaction between the toxin and SM.

Our goal is to elucidate the structural basis for the interaction of EqTII with lipid membranes, and the role of SM in that interaction. Here, we describe 19F NMR studies of EqTII with all five Trp residues replaced with 5-fluoro-Trp. Previous studies of other membrane-binding proteins have shown that 19F NMR is a sensitive and informative probe of protein interactions with lipid membranes, and this proves to be the case for EqTII.

Results

Expression, isolation and characterization of F-Trp-EqTII

A Trp auxotroph strain was constructed that grew only when Trp was present in the medium, the lowest Trp concentration required for normal growth being 0.4 mM (Figure 1(a)). As 5-F-Trp proved to be inhibitory for growth (Figure 1(a)), bacteria were first grown in complex LB medium until they reached the middle of the exponential phase, then transferred to M9 medium containing 1% Casamino acids that lack any Trp. After one hour shaking, 5-F-Trp was added to the medium and after another half-hour expression of EqTII was induced by addition of 0.4 mM (final) IPTG.

EqTII with all five Trp replaced by 5-F-Trp (F-Trp-EqTII) was isolated as described. The isolated protein was >95% pure according to SDS-PAGE (Figure 1(b)) and RP-HPLC. The RP-HPLC retention times were virtually the same for EqTII and F-Trp-EqTII (data not shown). Importantly, the 19F-labelled protein was also of the expected size as determined by mass spectrometry (EqTII expected 19,833 Da (Thr179 variant), obtained 19,831.6 ± 2; F-Trp-EqTII, expected 19,923 Da; obtained 19,922.5 Da), indicating that 5-F-Trp was incorporated quantitatively at all five positions in the sequence. The same purification procedures were used for the four Trp→Phe mutants used in making resonance assignments.

Absorbance spectra of F-Trp-EqTII were qualitatively and quantitatively different from those of unlabelled EqTII (Figure 1(c)). There was a shoulder present at around 300 nm in F-Trp-EqTII that was not visible in spectra of EqTII. The A280 ratio between F-Trp-EqTII and unlabelled EqTII is 0.96 ± 0.01 (n = 2; ± SD). It appears that F-Trp-EqTII has an increased Trp fluorescence quantum yield, the fluorescence of F-Trp-EqTII being more than twice that of EqTII. Fluorescence was blue-shifted in both proteins relative to equimolar concentrations of tryptophan in solution, indicating
folding and burial of some of the Trp residues. The shift was from 356 nm for free Trp to 339 nm for EqTII, which is in accord with published data, and slightly less for F-Trp-EqTII, from 357 nm for free F-Trp to 343 nm for F-Trp-EqTII.

The activity of F-Trp-EqTII was compared with that of EqTII in two different assays. As shown in Figure 2, both the haemolytic activity and calcein release from small unilamellar vesicles (SUV) of F-Trp-EqTII were similar to that of unlabelled protein.

These data indicate that replacement of the five Trp residues in EqTII with 5-F-Trp did not affect the structure or activity of EqTII. This conclusion is further supported by analysis of one and two-dimensional $^1$H NMR spectra of F-Trp-EqTII and its four Phe-containing mutants recorded at 600 MHz. These spectra (not shown) differed slightly in the 9.5–11 ppm region where the indole NH resonances occur, but this was a direct consequence of the fluorine substitution on chemical shifts rather than an indicator of structural differences. Other regions
of the spectra that are sensitive monitors of native structure, such as 5–6 ppm and 0–0.7 ppm, were similar for EqTII and F-Trp-EqTII. Spectra of F-Trp-EqTII, F-Trp-EqTII-W112F and F-Trp-EqTII-W116F were very similar to one another. Differences were evident in F-Trp-EqTII-W45F and F-Trp-EqTII-W149F, suggesting that some local structural changes accompanied these Trp to Phe mutations; this is discussed below in relation to 19F NMR spectral changes in the mutants. All of these F-Trp-EqTII mutants displayed activity similar to that of F-Trp-EqTII itself. The key result, however, is that the structure and activities of EqTII and F-Trp-EqTII are essentially identical.

NMR spectrum in aqueous solution

The 19F NMR spectrum of F-Trp-EqTII in aqueous solution shows five well-resolved peaks, as shown in Figure 3(a). The peaks were assigned to individual Trp residues using a series of four single Trp/Phe mutants. The resolution of the five peaks in the spectrum of wild-type F-Trp-EqTII was sufficient to enable clear assignment of the missing peak in each Trp → Phe mutant, even though there were slight changes in the chemical shifts of remaining Trp resonances in some mutants. In particular, F-Trp-EqTII-W45F showed a downfield shift of the W149 peak and a smaller upfield shift of W117 (Figure 3(b)), suggesting some structural perturbation associated with this Trp→Phe substitution. In F-Trp-EqTII-W116F, the W149 peak moves downfield and W112 and W117 were slightly affected (Figure 3(d)), while in F-Trp-EqTII-W149F the W116 peak moves downfield (Figure 3(e)). Substitution of W112 did not perturb any of the other Trp resonances, consistent with its high degree of solvent exposure as part of a surface loop. Despite these small secondary shifts in some Trp→Phe mutants, the assignment of the missing peak in each case was unequivocal. Because of this, assignment of the fifth resonance, from Trp117, was made by elimination, without production of the W117F mutant.

The 5-F-Trp resonances in F-Trp-EqTII span a chemical shift range of about 3.3 ppm. The chemical shift of the central resonance, from W112, which is also the sharpest, corresponds to the chemical shift of acid-denatured F-Trp-EqTII. This is consistent with the observation that W112 is located on a highly solvent-accessible eight residue loop connecting strands β5 and β6, which includes four aromatic residues, Y108, Y110, W112 and Y113. However, there is no simple correlation between chemical shift and degree of solvent exposure, as the other two exposed tryptophan residues, 116 and 149, are well separated from the W112 resonance (Figure 3(a)). The closest resonance to W112 is from W45, which is buried. Both van der Waals and electrostatic interactions can have a significant effect on 19F chemical shifts in proteins, and it seems that both are important in F-Trp-EqTII.

NMR relaxation in aqueous solution

The 19F NMR relaxation times of F-Trp-EqTII are summarised in Table 1. The T2 values were remarkably uniform at both resonance frequencies and, therefore, did not correlate with degree of solvent exposure. By contrast, the T1 values showed a significant spread, with the most solvent-exposed residue, W112, having the longest T1 at both frequencies. Of the three solvent-exposed tryptophan residues, W112 and W116 had T1 values clearly longer than the others, but the third, W149, was the same as the buried residues at 376 MHz and only slightly longer at 470 MHz. Thus, there is a rough, but by no means perfect, correlation between T1 and solvent exposure.

Interaction with micelles

Significant changes in 19F chemical shifts of F-Trp-EqTII were observed in the presence of DPC micelles, as illustrated in Figure 4. The W112
resonance shifted upfield by 0.9 ppm, W116 shifted downfield by 0.4 ppm and W117 shifted downfield by 0.2 ppm (Figure 4(b)). As a result of its upfield shift, the W112 resonance was close to that of W149, but was distinguishable by its sharper linewidth. These assignments were confirmed by parallel experiments on the W149F mutant (spectra not shown).

When a small amount of SM was added the overall appearance of the spectrum was similar, but the intensity of the W112 peak decreased and there was a small broad peak around $\delta_{47.9}$ ppm (indicated in Figure 4(c)). In fact, a weak peak at this position was visible even in the absence of SM (Figure 4(b)), and was seen also in corresponding spectra at 376 MHz of both F-Trp-EqTII and F-Trp-EqTII-W149F (not shown). Importantly, the same pattern of shifts was observed when half the concentration of F-Trp-EqTII was used, indicating that the EqTII binding capacity of the DPC micelles was not saturated at the concentration used in Figure 4. As the ratio of SM to DPC increased, the sharp W112 peak disappeared and the broad downfield peak grew (Figure 4(d) and (e)). The W116 resonance continued to move downfield and became broader, while W117 was largely unaffected by SM. When the ratio of SM to DPC reached 1:1, all peaks in the spectrum (not shown) became quite broad but the distribution of peak intensities was similar to that in Figure 4(e) except that the W112 linewidth had become more similar to the others.

A similar pattern of changes was seen at 376 MHz upon interaction of F-Trp-EqTII and F-Trp-EqTII-W149F, although the peak at $\delta -47.9$ ppm was sharper than at 470 MHz. One possible explanation for this is that there is some conformational averaging occurring on the millisecond timescale that produces more broadening at the higher observation frequency. In addition, chemical shift anisotropy contributions to relaxation$^{41,44}$ will be more pronounced at the higher field. For the purpose of this study we have not attempted to distinguish between these possible sources of broadening at 470 MHz. The spectra of F-Trp-EqTII in DPC, with and without SM, showed no long-term time-dependence, being reproducible over a period of weeks to months.

The effect of SM on the spectrum of F-Trp-EqTII in these experiments is likely to be mediated entirely by SM bound to micelles. SM is very insoluble in aqueous solution, and when EqTII was

### Table 1. $^{19}$F NMR relaxation data for Trp residues of EqTII

<table>
<thead>
<tr>
<th>Trp</th>
<th>376 MHz</th>
<th>470 MHz</th>
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<tr>
<td></td>
<td>$T_1$ (s)</td>
<td>$T_2$ (ms)</td>
</tr>
<tr>
<td>W45</td>
<td>0.28</td>
<td>19</td>
</tr>
<tr>
<td>W112</td>
<td>0.74</td>
<td>23</td>
</tr>
<tr>
<td>W116</td>
<td>0.43</td>
<td>22</td>
</tr>
<tr>
<td>W117</td>
<td>0.32</td>
<td>24</td>
</tr>
<tr>
<td>W149</td>
<td>0.31</td>
<td>20</td>
</tr>
</tbody>
</table>

Values measured at 376 MHz are the mean of three sets of data, all recorded at 25 °C with a recycle time of 1.5 seconds; ranges were ±0.02–0.03 seconds for $T_1$ and ±2 ms for $T_2$. Values at 470 MHz were measured at 21 °C with a recycle time of 3.5 seconds. A second set of data at 470 MHz was recorded with a more dilute sample (ca one-sixth the concentration of the first sample); the $T_2$ values were similar to those shown in this table, as were the $T_1$ values for W45, W117 and W149. The $T_1$ values for W112 and W116 were longer (1.37 seconds and 0.76 seconds, respectively).
mixed with a suspension of SM in phosphate buffer it gave no detectable $^1$H NMR signal at 600 MHz. Furthermore, previous experiments have shown that EqTII bound to SM in aqueous solutions is not available to cause haemolysis.$^{22}$ Thus, essentially all of the SM added to DPC micelles is likely to be associated with the micelles. Any free SM will be insoluble and any F-Trp-EqTII associated with it would not be observable in our NMR experiments, as confirmed by $^1$H NMR (data not shown). It is also reasonable to assume that most of the toxin is associated with micelles under our experimental conditions (lipid to toxin molar ratio 500 at the highest SM concentration). At even lower lipid to toxin ratios, the majority of the toxin was associated with the membranes in liposomes composed of 1:1 PC/SM, with approximately 80% of EqTII$^{36}$ and sticholysins I and II$^{33}$ bound to small and large unilamellar vesicles, respectively.

To determine whether the order of addition had any influence, we mixed SM with DPC before adding F-Trp-EqTII. At an SM to DPC ratio similar to that shown in Figure 4(d) the W112 peak had not moved as far downfield as when SM was added to F-Trp-EqTII already mixed with DPC. This suggests that the distribution of F-Trp-EqTII and SM across the micelle population is affected slightly by the order of addition, although the pattern of chemical shift changes is the same. The toxin may induce a change in lipid micelle structure that enables easier partition of SM into the micelle or easier access of SM to F-Trp-EqTII, or F-Trp-EqTII may preferentially attract SM to micelles in which it is embedded.

An impurity peak at $-42.3$ ppm was evident in most, but not all, spectra in DPC, with or without SM (Figure 4) and in bicelles with SM (see below). Its sharper linewidth suggests that it arose from an impurity in the lipids, rather than from native F-Trp-EqTII. This was confirmed by the appearance of this peak in spectra of DPC micelles and bicelles in the absence of SM or F-Trp-EqTII. Its $T_1$ at 470 MHz was similar to that of W45 in F-Trp-EqTII in DPC with 16 mM SM (sample as in Figure 4(d)), i.e. about 0.4 second.

### Interaction with bicelles

Binary bilayered mixed micelles, known as bicelles, represent a model membrane system more similar to natural biomembranes than DPC micelles. They are considered to be discoidal bilayers typically composed of long-chain dimyristoylphosphatidylcholine (DMPC) localized in the planar section, and short-chain dihexanoylphosphatidylcholine (DHPC) stabilizing the torus of the discs.$^{43-49}$ Because of the diamagnetic susceptibility of their phospholipid components, bicelles can spontaneously align in the magnetic field with the bilayer normal perpendicular to the direction of the field, but this alignment depends on the temperature, concentration, and long-chain to short-chain lipid ratio ($q$). In this study, we have used bicelles with a small $q$ ratio (0.25), which are expected to be cylindrical in shape, with a height around 45 Å and a radius of about 25 Å.$^{46-47}$ Although they are larger than micelles they undergo sufficiently rapid tumbling in solution to allow studies of bicelle-associated peptides and proteins by solution-state NMR.$^{48,49}$

The changes in $^{19}$F chemical shift in the presence of DMPC/DHPC bicelles were qualitatively similar to those in the presence of DPC micelles, but there were some quantitative differences. Comparison of 376 MHz spectra of F-Trp-EqTII in DPC micelles (Figure 5(a)) and DMPC/DHPC bicelles (Figure 5(b)) showed that the chemical shift changes for W112 and W116 followed the same pattern, but were larger. Thus, W112 moved slightly further upfield in bicelles such as Figure 5.

#### Figure 5.

$^{19}$F NMR spectra of F-Trp-EqTII and F-Trp-EqTII-W149F in DMPC/DHPC bicelles with or without SM. Spectra were recorded at 376 MHz and 30 °C except for the micelle spectrum in (a), which was run at 25 °C. (a) F-Trp-EqTII in DPC micelles; (b) F-Trp-EqTII in DMPC/DHPC bicelles, 0.3 mM F-Trp-EqTII, 123 mM PC (23 mM, DMPC, 92 mM DHPC) in 50 mM phosphate buffer, pH 6.8; (c) F-Trp-EqTII in DMPC/DHPC bicelles, plus 15 mM SM (ratio of SM to PC 0.13); (d) F-Trp-EqTII-W149F in DMPC/DHPC bicelles; (e) F-Trp-EqTII-W149F in DMPC/DHPC bicelles, plus 15 mM SM (ratio of SM to PC 0.13). With time, the W112 peak narrowed and appeared to increase in intensity slightly, but did not change its chemical shift. All spectra in bicelles were run with 30–50K scans.
that it overlapped with W149, and W116 moved further downfield. Even W117, which moved only slightly in micelles, shifted further in bicelles. It appears that the interactions of F-Trp-EqTII with micelles and bicelles are very similar, but the association with lipids is somewhat tighter in bicelles. Interestingly, the small peak at $-47.9$ ppm, which was present in spectra of F-Trp-EqTII and F-Trp-EqTII-W149F in DPC micelles and became stronger as SM was added, was not observed in spectra of F-Trp-EqTII or F-Trp-EqTII-W149F in bicelles in the absence of SM (Figure 5(b) and (d)).

Addition of SM to bicelles caused changes qualitatively similar to those seen in micelles, but again there were quantitative differences. W112 did not move as far downfield in the presence of similar amounts of SM, and W116, which had already moved further downfield in bicelles than micelles, did not move much further. Addition of more SM shifted the W112 peak further downfield (not shown). The same trends were evident for F-Trp-EqTII-W149F (Figure 5(d) and (e)) but W112 moved further in F-Trp-EqTII-W149F (Figure 5(e)) than F-Trp-EqTII (Figure 5(c)) even though the amounts of PC lipid and the ratios of SM were the same. It is possible that the Trp149 → Phe mutation altered the interaction with SM, but the amount of protein was much lower in the case of F-Trp-EqTII-W149F (hence the much lower signal-to-noise ratio in the spectra), and perhaps the effect of SM depended not only on the SM to PC ratio but also the ratio of SM to EqTII (unlike the effect of PC, which was clearly saturating even at the highest F-Trp-EqTII concentrations added). An effect of SM that was dependent on the SM to F-Trp-EqTII ratio would be more consistent with a specific interaction with the protein than with a bulk effect on the lipid. In order to test this, we repeated the experiment using a concentration of F-Trp-EqTII even lower than that of F-Trp-EqTII-W149F in Figure 5. With much less toxin, the same ratio of SM to PC caused a larger shift in W112, very similar to that in Figure 5(e) (Figure S2 in Supplementary Material). By contrast, in DPC micelles the 376 MHz spectra of F-Trp-EqTII and F-Trp-EqTII-W149F had similar chemical shifts in the presence of SM, although the W112 resonance was considerably sharper for the more dilute mutant.

It appears that EqTII associated more tightly with bicelles than micelles and, perhaps because of this, SM was less effective at causing subsequent changes in the EqTII–lipid interaction, even though the changes were qualitatively similar. This is consistent with previous reports of actinoporin preference for binding to and permeabilisation of SM-containing large unilamellar vesicles, which have a lower curvature than SUV. Some broadening was seen in the $^{31}$P spectra of 1:1 DPC to SM micelles, but even here the lineshape was consistent with small, isotropically tumbling “micelles”.

### 19F NMR relaxation in lipid micelles

$^{19}$F NMR spin-lattice relaxation times were measured at 376 MHz for F-Trp-EqTII in DPC micelles in the presence of SM at a ratio of 0.13, similar to that shown in Figure 4(d) but with a sharper W112 peak because of the lower field strength. The $T_1$ values were: W45 0.41 second, W112 0.74 second, W116 0.74 second, W117 0.53 second, W149 0.62 second. The least solvent-exposed Trp in the native structure, W45, was still the most rapidly relaxing, but W112 was now much more similar to the other three than for the aqueous protein (Table 1), where it was almost twice as long as the others. These data suggest that W112 is no longer as highly solvent-exposed, presumably reflecting its interaction with micellar lipids.

### Comparison with $^{13}$C NMR

The sensitivity of the 5-F-Trp resonances of F-Trp-EqTII to its interaction with model membranes prompted a comparison with $^{13}$C NMR studies of $^{13}$C-labelled Trp in the same protein. The aromatic resonances of Trp are very sensitive to their environment within native proteins, and partial assignments for the Trp aromatic $^1$H and $^{13}$C resonances are available. $^{29}$ $[2-^{13}$C$]$Trp was incorporated quantitatively using the same expression system as for 5-F-Trp, and the labelled EqTII was fully active (Supplementary Material). A 2D
The 13C(1H)-HSQC spectrum (Figure 6) showed that the five resonances were well dispersed in both the 13C and 1H dimensions. The sharpest resonance in the 1H dimension is that of W112, reflecting its high degree of solvent exposure. When this sample was mixed with DPC micelles in the absence of SM, good-quality spectra could still be obtained but only with significantly longer spectral acquisition times. However, few changes in chemical shift were observed, the only significant shift being that of the 13C resonance of W116, from 129.7 ppm to 130.1 ppm (Table S3, Supplementary Material). When SM was added to a molar ratio of 0.14, the spectral quality decreased to the point where only the two sharpest resonances (W112 and W149) were observable in the HSQC at 25 °C; W112 showed a significant change in chemical shift with this addition of SM, moving from 127.2 ppm to 127.6 ppm. At 35 °C all five sites were observable and there was significant broadening of some resonances, but the spectral quality was poor even after 24 hours spectral acquisition on a cryoprobe-equipped spectrometer. When the SM ratio was increased to 0.27, only the W112 resonance was visible even after 40 hours of spectral acquisition. Thus, whatever spectral changes may have occurred upon lipid binding, they were considerably more difficult to observe than the corresponding 19F NMR spectral changes.

Discussion

By creating an E. coli strain auxotrophic for tryptophan we have been able to produce an analogue of EqTII with all five Trp residues replaced by 5-F-Trp. This enabled 19F NMR studies of the binding of EqTII to lipid membranes, which offer the advantages of high NMR detection sensitivity (83% that of 1H), good peak resolution and no natural background. Trp residues were labelled because previous studies of EqTII interactions with membranes by means of fluorescence and mutagenesis had suggested that two regions of EqTII were particularly important, namely the N-terminal helical region and a surface aromatic cluster involving Y108, W112, Y113, W116, Y133, Y137, and Y138.41,35,37 In addition, Trp residues play an important role in orienting peptides and proteins in the interfacial region of lipid membranes.53 The 5 position of the Trp ring was chosen as 5-F-Trp resonances exhibit greater dispersion than 4 or 6-F-Trp (7 ppm versus 6 ppm or 3 ppm, respectively)40 and show less broadening at higher magnetic fields due to chemical shift anisotropy.41 A significant advantage of 19F NMR, which becomes important in interpreting our data, is its ability to observe all five Trp residues, rather than a subset that, for example, gives rise to fluorescence spectra.24,37 The labelled protein, F-Trp-EqTII, was fully active in haemolytic and calcein release assays and had 1H NMR spectra similar to those of native EqTII, although some differences were evident in its UV absorbance and fluorescence spectra. The 19F resonances of F-Trp-EqTII were well resolved, and were readily assigned by a series of single-site Trp→Phe mutations.

Compared with 19F labelling, 13C labelling offers the potential advantages that 13C is likely to be more readily incorporated without the risk of cell toxicity and to cause less perturbation of the local structure. Moreover, if resonance assignments have been made in the course of other NMR studies of the target protein, there should be no need to resort to mutational studies for resonance assignments. On the other hand, 13C-labelled amino acid residues are considerably more expensive than their 19F-labelled counterparts. In the current case, 19F labelling proved to be far more informative, with larger spectral perturbations observed upon interaction with lipids and better-quality spectra obtained in the presence of model membranes. The additional effort required to assign the resonances via single-site mutants was minimal and, in any case, the mutants proved to be valuable in their own right for probing lipid interactions.

In the presence of DPC micelles, W112 showed the largest shift (upfield), while W116 moved downfield and W117 moved slightly downfield. The downfield shift of W116 suggests that it has moved to a less polar environment upon membrane binding40,43 whereas the upfield shift of W112 may reflect its proximity to the zwitterionic headgroups of the lipids. To test this possibility, we added phosphocholine (POC) to F-Trp-EqTII. By analogy with sticholysin II, POC should bind in the vicinity of W112. At a molar ratio of 1:1, POC caused a slight (0.04 ppm) upfield shift of the W112 resonance while not affecting other resonances, but at a ratio of 10:1 aggregation occurred and spectra could not be obtained. These preliminary observations suggest that interaction with the phosphatidylcholine head group may contribute to the upfield shift of W112 in micelles and bicelles, but further work is required to establish the exact magnitude of this contribution.

The addition of SM shifted W116 even further downfield, but caused a shift reversal of W112, which now moved downfield. This shows that SM either interacts directly with W112 in the membrane-bound state of EqTII or modifies the environment of W112 by changing the interaction of EqTII with the membrane or the conformation of membrane-bound EqTII. This is the first demonstration that SM directly affects EqTII, as opposed to simply stabilising the membrane-bound form of the toxin. Recently, Barlič et al.54 concluded that, although specific interactions between actinoporins and SM could not be excluded,13,14 it was the coexistence of lipid phases that seemed to modulate the interaction of the toxin with the membrane. Our results show that SM does have a direct effect on EqTII in two different model membranes, so its role in EqTII pore formation involves more than whatever effects it has on bulk lipid bilayer properties. Evidence of an interaction between Trp residues...
and SM has been reported recently for the unrelated haemolytic toxin lysenin.\(^\text{55}\)

The fact that significant shifts in the resonances from W112 and W116 are observed upon binding of F-Trp-EqTII to DPC micelles, and even more so, DMPC/DHPC bicelles, shows that EqTII makes significant interactions with PC even in the absence of SM. Although binding to PC is not sufficient to enable pore formation,\(^\text{13,14,23,54}\) our results show that the interaction has as much effect on the tryptophan residues of EqTII as the subsequent interaction with SM. They also show that EqTII does not undergo a major conformational change upon binding to membranes, and thus rule out the possibility that the pore could be formed by \(\beta\)-barrel structures of the type seen for bacterial cytolytins.\(^\text{56}\)

The lack of effect on the W149 \(^{19}\text{F}\) resonance in micelles and bicelles, with or without SM, proves to be very valuable in defining how EqTII binds to membranes and the extent of any conformational changes accompanying that binding. F-Trp-EqTII cannot be lying on the surface of the bicelles or micelles in such a way that the hydrophobic surface that is normally protected from solvent by the N-terminal helix (i.e. strands \(\beta_1\) (residues 32–40) and \(\beta_3\) (residues 69–75), and to a lesser extent \(\beta_{10}\) (residues 170–177)) is associated with the lipid surface, because this would bring W149 into close proximity to the lipid and affect its chemical shift (Figure 7(a)). In the model of the tetrameric pore proposed by Mancheno et al.\(^\text{17}\) on the basis of EM images of two-dimensional crystals of sticholysin II, the \(\alpha_2\) helix (residues 129–134 in EqTII) is intimately associated with the membrane (Figure 7 of Mancheno et al.). It is possible to orient EqTII such that this helix, as well as W112 and W116, are close to the lipid (Figure 7(a)), and this may be a good model of the micelle-bound and bicelle-bound EqTII in our experiments. In this orientation W112 and W116 are intimately associated with the membrane, W117 is the next closest, and W45 and

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**Figure 7.** (a) Possible models of the orientation of EqTII at the membrane surface. The magnitudes of the 5-F-Trp chemical shift changes upon membrane binding are consistent with the left-hand and middle, but not the right-hand, orientations. The middle orientation provides the best fit of the three to the observed shifts. In each case the closest-to-average structure of EqTII in solution\(^7\) is shown, with the Trp side-chains coloured as follows: W112 red, W116 magenta, W117 gold, W149 green, W45 blue. (b) Close-up view showing the proximity of W149 (green) to F16 (violet). Given the sensitivity of \(^{19}\text{F}\) chemical shifts to their environment, the lack of change in the W149 chemical shift upon binding to micelles or bicelles indicates that the N-terminal helix has not dissociated from the \(\beta\)-sheet, even in the presence of SM.
W149 are far enough away not to be affected. The N-terminal helix is still associated with the protein as in native EqTII and would have to undergo a large rearrangement in order to intercalate into the lipid bilayer. However, the lack of effect of lipid binding on W149 suggests that such a change has not yet occurred in micelles or bicelles. W149 is in close proximity to the side-chain of Phe16 in the native structure (Figure 7(b); the distance between W149 and F16 is 5.4 Å in the closest-to-average solution structure30) and a change in this interaction would be expected to change its 19F chemical shift.

The observation that the N-terminal dissociation does not appear to occur even in the presence of SM implies that the interaction with SM per se is not sufficient to trigger this conformational change, at least in the model membranes investigated here. It is possible that a membrane bilayer is required for N-terminal dissociation to occur. Alternatively, some additional interaction with the membrane, and/or with other molecules of EqTII that will participate in formation of the tetrameric pore, may be required. In the model of the tetrameric pore proposed by Mancheño et al.,17 the hydrophobic surface normally protected from solvent by the N-terminal helix appears to be exposed, which would be energetically unfavourable. Possibly the hydrophobic surfaces exposed upon lipid binding cause these protein surfaces to aggregate and promote pore formation.

In conclusion, we have shown that EqTII undergoes significant interactions with model membranes in the absence of SM, and that the addition of SM causes further changes in the membrane-bound protein, particularly in the vicinity of W112. The significant 19F chemical shift changes observed for some Trp residues, coupled with the absence of such changes for others, place significant restrictions on putative models of membrane-bound EqTII. In particular, the absence of change for W149 indicates that the interaction with SM is not sufficient to trigger dissociation of the N-terminal helix from the β-sandwich, at least in the model membranes studied here. Our results emphasize the value of 19F NMR in studies of the interactions of proteins with membranes, with good-quality spectra attainable in relatively short acquisition times and chemical shifts that are exquisitely sensitive to the local environment. Moreover, this technique is applicable in solid-state NMR experiments on lipid bilayers,57 and further studies will now proceed in that direction with the aim of characterising the subsequent steps towards formation of a tetrameric pore in bilayer membranes.

Materials and Methods

Creation of Trp auxotroph strain

Standard molecular biology protocols58 were used to create a Trp auxotroph strain (E. coli BL21(DE3) trp::Tn10). It was constructed by transduction of E. coli BL21(DE3) with P1 phage prepared on E. coli strain Y1090 (trp::Tn10), which harbours transposon Tn10, bearing tetracycline resistance, inserted within the trp operon. The resulting strain, selected as tetracycline-resistant transductant, was not able to grow in minimal media, in contrast to the original E. coli BL21(DE3) strain (Figure 1(a)). It grew only when Trp was present in the medium, the lowest Trp concentration required for normal growth being 0.4 mM. Bacteria were grown initially in minimal medium with the addition of 0.4 mM 5-fluoro-αL-tryptophan (5-F-Trp; Sigma), but, as 5-F-Trp proved to be inhibitory for growth (Figure 1(a)), we developed a protocol to bypass this limitation. Bacteria were grown overnight in LB medium supplemented with 100 μg/ml of ampicillin and 15 μg/ml of tetracycline at 37°C with shaking. In the morning, 500 ml of LB with added M9 salts and antibiotics was inoculated with 20 ml of the overnight culture and cells were grown under the same conditions until they reached mid-exponential phase (A490 ca. 1.5), then pelleted by centrifugation for five minutes at 4400g at room temperature. After washing with M9 salts they were transferred to M9 medium with 2% (w/v) glucose, 1 μg/ml of biotin, 1 μg/ml of thiamine, and 1% Casamino acids that lack Trp. After one hour shaking, 5-F-Trp was added to the medium to a final concentration of 0.4 mM and, after another 30 minutes, expression of EqTII was induced by addition of 0.4 mM (final) IPTG. Cells were incubated for an additional four hours, centrifuged at 4400g and 4°C and then frozen.

Isolation and characterization of F-Trp-labelled EqTII and mutants

F-Trp-EqTII and the mutants W45F, W112F, W116F and W149F were expressed in the Trp auxotroph E. coli BL21(DE3) strain as described above. The plasmids for the mutants were prepared as described.59 Recombinant proteins were isolated from the bacterial cytoplasm as described.42 Native EqTII was isolated from Actinia equina as described.25 The absorption coefficient at 280 nm for wild-type EqTII was taken as 2.21.50 The concentration of F-Trp labelled proteins was determined by BCA Protein Assay (Pierce), using bovine serum albumin (Merck) for the calibration curve.

Electrospray ionization MS analysis was performed using a high resolution magnetic-sector AutospecQ mass spectrometer (Micromass). The protein samples were introduced into an electrospray nebulizer at a flow rate of 10 μl/minute with a syringe pump. The spectra were obtained by scanning from m/z 2500 to 200 at 10 seconds/scan. Calibration was performed by sodium iodide cluster ions.

Haemolysis of bovine red blood cells was measured with a microplate reader on microtiter plates at room temperature (Dynex, Germany). The proteins were serially twofold diluted in 100 μl of erythrocyte buffer (130 mM NaCl, 20 mM Tris–HCl, pH 7.4); 10 μl of a suspension of washed bovine red blood cells (A490 0.5) was then added and haemolysis monitored at 630 nm for 20 minutes.

Calcine release was measured from SUV. SUV loaded with calcine were prepared by sonication, with excess calcine removed on a small Sephadex G50 column. Calcine fluorescence was excited at 495 nm and emission monitored at 520 nm at 25°C. SUV were in 140 mM NaCl, 20 mM Tris–HCl (pH 8.5), 1 mM EDTA. Toxin was then added to reach the desired lipid to toxin ratio (L/T) and...
Membrane Binding of Equinatoxin II

stirred. The permeabilisation (in %) was compared to the maximal, obtained at the end of the assay by the addition of (final) 2 mM Triton X-100.

Absorbance spectra were measured on a Shimadzu UV-2101PC spectrophotometer. Fluorescence spectra were measured with a Jasco FP-750 spectrofluorimeter at 25 °C. The excitation wavelength was set to 295 nm and emission was scanned from 300–400 nm. Excitation and emission slits were set to 5 nm.

NMR spectroscopy

Samples were prepared for NMR by dissolving solid protein in aqueous solution then adjusting the pH at room temperature; F-Trp-EqII and its Trp→Phe mutants were highly water-soluble. Micelle and bicelle solutions were prepared by dissolving the appropriate lipids in water. SM is essentially insoluble in aqueous solution, but dissolved in the micelle and bicelle solutions with gentle agitation at room temperature over a period of 0.5–1 hour. Protein concentrations were typically in the range 0.8–2 mM for aqueous solutions and 0.1–0.4 mM in micelles and bichelles. DMPC, DHPC, DPC and brain SM were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). POCl3 chloride calcium salt was purchased from Sigma-Aldrich (St. Louis, MO). All lipids were used without further purification.

19F NMR spectra were recorded at 470 MHz on a Bruker Avance500 spectrometer equipped with a dedicated 1H probe de-tuned to 19F, or at 376 MHz on a Varian Inova 400 (Palo Alto, CA) equipped with a triple-resonance gradient probe. Probe temperatures were 21 °C and 25 °C at 470 MHz and 376 MHz, respectively, for spectra of F-Trp-EqII in aqueous solution and micelles, and 30 °C for spectra in bichelles at 376 MHz. Recycle times (acquisition plus delay) were 1.5 seconds and 1.7 seconds, respectively. One and two-dimensional 1H NMR spectra were recorded at 25 °C on a Bruker DRX-600 equipped with a triple-resonance gradient probe. 31P spectra were obtained on a Varian Inova 400 operating at 162 MHz with proton decoupling; micelles were run at 25 °C and bichelles at 30 °C.

Spin-lattice relaxation times (1T) were measured using the inversion recovery (π-π/2) pulse sequence, with τ values in the ranges 1 ms to 1.5 seconds at 376 MHz and 10 ms to 2.0 seconds at 470 MHz. Spin-spin relaxation time (1T) measurements were made using a spin-echo experiment (π/2-π-τ) with τ values in the ranges 0.1–30 ms at 376 MHz and 2–100 ms at 470 MHz. Recycle times (acquisition plus delay) were 1.5 seconds and 3.5 seconds at 376 MHz and 470 MHz, respectively.

Spectra were processed with XWINNMR (version 3.5, Bruker) or VMR (version 6.1C, Varian). Unless stated otherwise, spectra were processed using an exponential window function with a line-broadening of 15 Hz. Structural Figures were created using MOLMOL. 36

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Supplementary Data


References

Membrane Binding of Equinatoxin II


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