DOI:
10.1002/cbic.201300799

Document Version
Peer reviewed version

Photobleaching reveals heterogeneous stoichiometry for Equinatoxin II oligomers

M. A. B. Baker,[a] N. Rojko,[b] B. Cronin,[a] G. Anderluh,[b,c] M. I. Wallace*[a]

[a] Associate Professor Mark I. Wallace, Dr Matthew A. B Baker,Dr Bríd Cronin
Chemistry Research Laboratory
University of Oxford
South Parks Road
OX1 3QZ Oxford, United Kingdom
E-mail: mark.wallace@chem.ox.ac.uk

[b] Professor Gregor Anderluh, Dr Nejc Rojko
Department of Biology
Biotechnical Faculty
University of Ljubljana
Večna pot 111
1000 Ljubljana, Slovenia

[c] Professor Gregor Anderluh
Laboratory for Molecular Biology and Nanobiotechnology
National Institute of Chemistry
Hajdrihova 19
1000 Ljubljana, Slovenia

* corresponding author

Keywords: equinatoxin, photobleaching, pore-forming toxins, pore stoichiometry, single molecule fluorescence

Abstract

Equinatoxin II (EqtII), a sea anemone cytolysin, is known to oligomerize to form pores that spontaneously insert into membranes. Crystallographic and cryo-EM studies of structurally similar cytolysins offer contradictory evidence for pore stoichiometry. Here we used single-molecule photobleaching of fluorescently labeled EqtII to determine the stoichiometry of EqtII oligomers in supported lipid bilayers. A frequency analysis of photobleaching steps revealed a log-normal distribution of stoichiometries with a mean of 3.4 ± 2.3 standard deviations. Comparison of our experimental data with simulations of fixed stoichiometries supports our observation of a heterogeneous distribution of EqtII oligomerization. These data are consistent with a model of EqtII stoichiometry where pores are on average tetrameric, but with large variation in the number of subunits in individual pores.

Introduction

Pore-forming toxins (PFTs) are water soluble proteins which bind to membranes and oligomerize to form transmembrane conduits that alter permeability and lead to cellular ionic imbalance [1–5]. Pores can form in a number of ways, including the insertion of both β-barrels [6] and α-helices [7,8] through the membrane. While structural information has provided significant insight into the function of PFTs, this work is hampered by the variable stoichiometries observed for many pores [1,9–11]. Key questions, including how the stoichiometry of an individual pore can vary, and the dynamics of how monomers assemble into an oligomer, remain unanswered [12,13].

Equinatoxin II (EqtII) is a eukaryotic actinoporin, found in the sea anemone Actinia equina [7]. EqtII consists of 179 amino acids and in its water soluble form EqtII is folded into a hydrophobic β-sandwich core, flanked on the opposite sides by two α-helices [14–17]. The pore forming activity of EqtII is enhanced by the presence of sphingomyelin in the target membrane [7,18], and pore insertion is thought to occur by binding of the toxin to sphingomyelin via a cluster of exposed residues [19–21], with movement of an N-terminal helix to lie parallel to the plane of the membrane at the lipid-water interface [21,22]. EqtII monomers then oligomerize and the N-terminal helix reorients to span the bilayer and form the pore [23]. Early investigations into EqtII assembly suggest that it forms a pore with 3-4 monomers arranged into a toroidal structure [24–27]. This data is supported by Cryo-electron microscopy (Cryo-EM) studies on a similar cytolysin, Sticholysin II (StnII), which also indicate a tetrameric structure [16,28]. Recently however, a third actinoporin, Fragaceatoxin C (FraC), with the same fold as EqtII and StnII but with structural variation in the N-terminal helix, was identified through Cryo-EM as a nonamer [17]. The true nature of the EqtII pore is thus uncertain, particularly as structural work is limited by factors including the need for high protein concentrations [17] and, in some cases, assembly on lipid monolayers at an air-water interface [16,28].

In the absence of high-resolution structures for these heterogeneous pores, we make use of step-wise photobleaching [29–31] to determine the mean stoichiometry present in oligomeric fluorescently labeled EqtII. By counting the number of photobleaching steps in individual labeled EqtII complexes we can determine the number of monomers present in each complex. We show that EqtII forms oligomeric complexes of varying stoichiometry, with a mean stoichiometry of 3.3 ± 2.2 standard deviations (s.d.). This suggests a nonamer is unlikely for EqtII. However more importantly, not all oligomers have the same number of monomer subunits, and there is a broad distribution of stoichiometries. To help us verify that the observed heterogeneity is a true reflection of the underlying biology we also compare our observations with simulations of homogeneous pores.

Results and Discussion

The distribution of stoichiometries for EqtII was broad with a mean of 3.3 ± 2.2 (s.d.) (Fig. 1). Controls with unlabeled toxin yielded few spots above the detection threshold, and these few were single bleaching events with high noise (Fig. 2A).

Asymmetric distributions are widespread in biological systems and arise due to multiplicative effects in measurement [41,42]. In the case of single molecule fluorescence, fundamental asymmetry arises from the

low numbers of photons. As a result, asymmetric distributions of stoichiometry are typical in photobleaching experiments [30,43,44]. Thus we sought to determine to what extent the log-normal distribution we observed reflected the underlying biological distribution and used simulations to probe how plausible alternate explanations were.

First, we generated simulation sets of homogeneous tetramer samples with identical signal to noise as our experiment. We then compared these simulations with the distributions observed for EqtII. Sets of simulated homogeneous tetramers had a far narrower distribution (s.d. = 0.65) compared to our observed distribution (s.d. = 1.89), therefore our data is unlikely to arise solely from a homogeneous population (Fig. 2B).

We also tested the ability of our algorithm to resolve tetrameric vs. larger, nonameric pores. Our experimental distribution suggests a mean stoichiometry consistent with tetrameric rather than nonameric oligomers. Hence we conducted simulations of varying ratios of tetramers to nonamers to enable us to test the detection threshold for a small subpopulation of nonameric pores within this distribution. Our simulations showed that when 5% of traces were nonamers, the population was significantly different ($p < 0.05$) to the 100% tetramer population, and a clear sub-population of nonamers could be observed in the stoichiometry distribution (Fig. S5). However, when nonamer traces constituted less than 5% of all traces, there was no significant difference in the mean stoichiometry from a set of 100% tetramers (SI Table 2). From this we conclude that, if present, our observed EqtII distribution contains, at most, 5% nonamers.

Another possibility for the observed heterogeneity is partial labeling, which would result in a binomial distribution of stoichiometry. Fitting a binomial model indicated that if the heterogeneity was due to partial labeling, the true oligomer stoichiometry would be heptameric (N = 7), with a labeling efficiency of only 0.34 (Fig. 2B). Our UV spectra measurements exclude this possibility, since they indicate that our labeling efficiency is 1.01 ± 0.04 (Fig. S1B).

Förster resonant energy transfer between multiple identical fluorophores (homo-FRET) would also affect observed stoichiometry, and fluorescence quenching would be expected when multiple fluorophores lie in close proximity [45]. If the level of homo-FRET reduces as photobleaching of fluorophores in a complex proceeds, then we would expect our analysis to underestimate the actual stoichiometry, since our calculated dominant decrement will be larger than quenched decrements at early times. To quantify these effects we calculated the bleaching decrement (i.e. the bleaching step size) in 1 s subintervals along each trace and surprisingly observed that the bleaching decrement was largest in the first segment, decreased in size over the first 5 s, and then was constant for the remaining segments (5 s to 50 s) (Fig. 2C). This observation suggests that in these experiments homo-FRET does not play a significant role.

Our analysis extracts the most common bleaching decrement value from a Fourier analysis of pairwise differences across all time-points. If, as Fig. 2C suggests, there were an underestimate of the decrement at early times compared to the overall decrement, then our overall estimate of stoichiometry would be, inversely, an overestimate. Conversely, if homo-FRET were significant, we would expect an underestimate of the calculated stoichiometry.

As a comparison, we fitted single step functions only to the best monomer traces, in place of using information from all steps in all traces (Fig. S6A). This provides a monomer bleaching decrement that is an underestimate of the overall bleaching decrement as it does not contain information from traces with multiple fluorophores. The distribution from this validation (Fig. S6B) was not significantly different to our distribution from the Fourier analysis (Fig1B) and it provided a qualitative means to judge our potential stoichiometry error as small, and on the order of 3%.

Following exclusion of these possibilities we concluded the breadth of the observed distribution was real, and a range of oligomer stoichiometries were present for EqtII. If the observed distribution were caused by an underlying biological heterogeneity, then EqtII assembly might be expected to follow a Poisson process where the true number of oligomers in a pore could vary. We simulated sets of traces with varying stoichiometries, following a Poisson distribution. The mean of this underlying Poisson distribution was optimized to minimize RMS error when compared with the observed EqtII distribution

($\mu = 2.68$, $E_{RMS} = 0.0286$). This good fit ($E_{RMS} < 0.05$) indicates that a Poisson process, where varying numbers of monomers come together to form a complex, could therefore satisfy our experimental observations (Fig. 2B). Although providing a good fit, it is worth noting that these two distributions do have a statistically significant difference in means ($p = 0.05$), and the Poisson distribution also fails to recreate some of the rare high stoichiometry events present in our observed distribution.

Having concluded that the log-normal distribution was a robust reflection of the underlying biology, we explored the dependence of EqtII stoichiometry on toxin concentration and on the presence of sphingomyelin and cholesterol (Fig. 3). Three separate L:T ratios (100:1, 200:1, 400:1) were prepared by adding Cy3B-labelled EqtII to a fixed concentration of lipid vesicles. Counting of photobleaching steps was then used to determine the stoichiometry of individual complexes.

As we progressively diluted the toxin we observed a small but statistically significant ($p < 0.05$) difference in the mean stoichiometry (Tables S2&3). When cholesterol was present we again observed a small but statistically significant ($p < 0.05$) difference in mean stoichiometry at L:T 100:1 compared to 400:1 and 200:1, but no significant difference between 400:1 and 200:1. Lastly, when ESM was omitted from vesicle bilayers at a lipid:toxin ratio of 100:1, there was again a significant ($p < 0.05$) but small difference in the mean stoichiometry. However, for all these conditions, there were no gross changes in the distribution, and a linear fit to mean stoichiometry across all conditions was approximately constant (slope = 10^{-3} monomers / lipid:toxin ratio) (Fig. 4A, Table S1).

Our analysis shows a broad distribution of stoichiometries with a mean of 3.4 ± 2.3 s.d. in EqtII monomers per oligomer. We conclude that the breadth of this distribution reflects the biology and is not due to low signal to noise ratio, partial labeling, or homo-FRET.

Although we know that the EqtII-Cy3B used in these experiments is both active and capable of forming pores (Fig. S1) [34], in the photobleaching assay presented here we do not directly test pore functionality whilst simultaneously determining stoichiometry. We can however, look more closely at our data to determine whether we are photobleaching assembled complexes or merely co-localized monomers present on a single vesicle. We first examined the overall dependence of stoichiometry on the lipid to toxin ratio. If the observed stoichiometry were due to co-localized monomers, then we would expect to see a drop in the mean stoichiometry as the proportion of toxin was decreased, since these monomers would be more sparsely spatially located. However, within error, the mean stoichiometry was constant (Fig. 4A), which is in agreement with previous findings that the pore diameter of sticholysin does not change as the L:T ratio is increased [46].

Additionally, we examined the change in the mean stoichiometry while restricting the maximum centroid variance (MCV) across the excised 3 × 3 pixel patch used to generate each trace. We excluded traces whose position during photobleaching varied by more than a fixed distance. If the measured stoichiometry was due to multiple monomers localized to the region where a vesicle initially fused to the surface, we would expect there to be high variance in the centroid moment as each fluorophore was bleached, up to approximately the diameter of a vesicle (~100 nm). Alternately, if the traces with multiple bleaching steps were due to pore complexes, we would expect the centroid variation to be significantly less, around the inter-monomer distance of a pore (~6 nm) [15,28]. Thus, if our system were composed of co-localized monomers, we would expect to measure a higher mean stoichiometry as we increased the MCV. In fact, we observed a plateau regime between 10 - 50 nm MCV where the stoichiometry was constant, and a sharp drop in stoichiometry as the MCV was reduced below 10 nm (Fig. 4B). As we reduced the MCV below 10 nm, we began to exclude multimeric pore-complexes from our analysis, and the mean stoichiometry was sharply reduced. Since nearly all (95%) of the analyzed traces had fluorophores located no further than 10 nm apart, this uniform proximity indicates that the toxin monomers are present as complexes, not as coincidentally co-localized monomers.

Progressive dilution of the toxin did not yield gross changes in stoichiometry. Each population at lipid:toxin ratios of 100:1, 200:1 and 400:1 had significant, but small differences in means (Tables S2&3). However overall, the shape of these three distributions was very similar, and the variation in the means

was small (mean difference: 0.5) in comparison to the standard deviation (mean s.d: 2.3). The significant difference in these means may indicate that dilution of toxin places a small bias on the oligomerization reaction. However, there is no consistent trend, such as higher dilution biasing the formation of smaller complexes (Fig. 4A). Given the inherent breadth of our observed distributions, and with only small changes in stoichiometry occurring, it is not possible for us to quantify any kinetic effects on oligomerization arising from toxin dilution.

We only observe small, albeit significant, differences in stoichiometry in the presence of ESM and cholesterol, which, considering the breadth of our photobleaching distributions (mean s.d. = 2.4), indicates that both ESM and cholesterol also have no gross effect on EqtII stoichiometry. This supports a role for ESM and cholesterol prior to EqtII oligomerization.

Cholesterol, in large amounts, is known to aid toxin binding to lipid [47]. The lack of any change in stoichiometry with cholesterol is consistent with this observation; Cholesterol affects binding not oligomerization. Repeating our measurements across a range of lipids, may shed further light on how lipid composition can affect variable pore stoichiometry, but our findings here indicate that any stoichiometric shift due to a change in cholesterol content would be too subtle to observe using this single-molecule technique. Egg sphingomyelin also had only a small, but significant, effect on mean pore-complex stoichiometry. We propose that this is because again ESM primarily affects EqtII binding, not oligomerization. Binding is known to be poor in membranes devoid of ESM [48], thus we can consider the absolute number of complexes detected as a measure of effective insertion. For the DOPC:ESM (100:1) data there were 12.1 detected events above the signal to noise threshold per image (1030 events out of 85 image stacks), whereas for the DOPC data there were only 3.4 high-signal events per image (306 events out of 90 image stacks). If binding were deficient, then we would indeed expect lower insertion rates and fewer events in ESM-free membranes.

Conclusion

We conclude that pores form from a heterogeneous number of monomers, with a mean of 3.4 ± 2.3. A maximum of 5% of complexes could be present as nonamers. Our work suggests EqtII pore stoichiometry is heterogeneous, consistent with planar lipid bilayer data [24,49]. Our data, obtained at physiological protein concentrations, thus corroborates the structural data for StnII of Mancheño et. al. [16] and functional data obtained for EqtII and other actinoporins [24,46], and differs from the structural data for FraC of Mechaly et. al. [17]. This may be due to real differences between EqtII and FraC pores, or the result of lattice effects in crystal structures exerting stabilizing forces on oligomeric complexes that are not present either in supported lipid bilayers, or *in vivo* [50,51]. Future fluorescence work should seek to correlate stoichiometry with a direct measurement of pore formation.

Experimental Section

Materials

All materials were from Sigma-Aldrich unless otherwise stated. Buffers were filtered prior to use (0.2 μm cellulose acetate, Nalgene). 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), Sphingomyelin (ESM, egg, chicken), and cholesterol (Chol, ovine wool) were obtained from Avanti Polar Lipids.

Purification, labeling and characterization of EqtII

An EqtII A179C mutant was expressed recombinantly and purified in E. coli (DE3) as described previously [32]. Dithiotreitol (DTT, 2 mM) was present throughout the purification protocol to prevent thiol oxidation. The C-terminal position of our cysteine labeling site is known to be unimportant for membrane binding [15,16,28], or formation of the final functional pore [20], and does not have an effect on the permeabilization of liposomes by EqtII [33]. We expect A179C to be exposed to solvent in both the soluble monomer and the membrane-bound pore.

EqtII A179C was concentrated (Amicon ultra centrifugal filter, Milipore) and washed with degased buffer (20 mM PO_4^{2-}, pH 7.2) to remove DTT. Cy3B maleimide (5 mg mL^{-1} in DMSO, GE Healthcare) was then

mixed with EqtII A179C to a final molar dye to protein ratio of 4:1. Following a 2 hr reaction at 20°C unreacted dye was removed by ion exchange chromatography (HiPrep, GE Healthcare). Finally, EqtII-Cy3B was concentrated (3 kDa centricon ultrafiltration membrane, Millipore) to a final concentration of 1.5 mg mL^{-1} and stored at -20°C.

To confirm successful labeling the protein was analyzed using SDS-PAGE fluorescence followed by Coomassie staining (Fig. S1A). A labeling efficiency of Cy3B to protein of 1.01 ± 0.04 (n = 3) was measured using UV-vis absorption spectroscopy (Fig. S1B). To compare EqtII-Cy3B activity with the wild type protein, hemolysis of bovine red blood cells was measured (Fig S1D) using a microplate reader (Dynex Technologies, Germany), as described previously [18]. Briefly, proteins were 2-fold serially diluted in a 96-well microplate. A suspension of red blood cells in erythrocyte buffer (10 mM Tris-HCl, 140 mM NaCl, pH 7.4) was added and the time course of hemolysis followed at 630 nm for 20 min at room temperature. Cy3B labeling of the protein did not reduce the protein hemolytic activity. Single-channel recording of Cy3B-labeled EqtII (Fig. S1C) in Droplet Interface Bilayers [34] also exhibited unitary changes in conductance (264 ± 70 pS) corresponding to channel insertion similar to those reported previously [24].

Vesicle preparation

Three different compositions of lipid vesicles were prepared: DOPC:ESM 1:1 (%mol), DOPC:ESM:Chol 1:1:1 (%mol), and DOPC alone. Lipids were mixed in a minimum amount of chloroform (for DOPC only preparations) or chloroform:methanol 9:1 (to aid dissolution of ESM and Chol in all other preparations) in a glass vial and dried under a gentle nitrogen stream before placing under vacuum for 1 hr to remove residual solvent. The resulting lipid film was dissolved in phosphate buffered saline (0.02 mM PO_4^{2-}, 150 mM NaCl, pH 7.2). Multilamellar vesicles were extruded 31 times through a 100 nm polycarbonate filter to produce vesicles of approximately 100 nm in diameter [35]. The final lipid concentration was measured using a phospholipid assay (LabAssay Phospholipid, Wako Chemicals).

Supported lipid bilayer preparation

Supported lipid bilayers (SLBs) were formed by vesicle fusion onto a plasma cleaned glass surface following published protocols [36,37]. Labeled protein was added to a reaction tube with 30 μM lipid vesicles to achieve the desired lipid to toxin ratio (L:T). Protein and lipid vesicles were incubated for 10 min at room temperature with vortexing every 3 minutes. After 10 min the required reaction mixture volume was added to a second tube containing fresh vesicles at 100 μM concentration. The solution was diluted to 100 mM NaCl by adding 0.02 mM phosphate buffer, to encourage vesicle swelling. This mixture was immediately transferred to a plasma-cleaned glass coverslip on a tunnel slide, and incubated for 30 min to allow vesicle fusion (Fig. S2). The coverslip was then washed with fresh buffer to remove excess unfused vesicles.

Single-molecule fluorescence

Fluorescence was measured using an inverted microscope (Nikon, Ti-E). For single molecule photobleaching a 2 mW 532 nm DPSS Nd:YAG laser (Compass 215M, Coherent Inc) provided illumination. The excitation light was focused at the back aperture of an oil immersion objective lens (100x Plan Apo N.A. 1.4, Nikon) so that it was totally internally reflected at the coverslip surface, with a power density in the image plane of 1 $\mu W\ \mu m^{-2}$. Emitted fluorescence was collected through the same objective, transmitted through suitable dichroic (C67195, Nikon) and emission filters (BrightLine 582/75, Semrock). Image stacks of 1000 frames with exposure time of 50 ms per frame were recorded using an electron-multiplying camera (iXon+ DV860E, Andor Technology).

Photobleaching analysis

Images were imported into MATLAB (Mathworks) and analyzed to determine the size of individual photobleaching steps. The mean pixel intensity was calculated across the first 50 frames, and this mean image was scanned for bright spots corresponding to protein complexes, with an overall average of 6.8 spots detected per image. 3 × 3 pixel regions of interest (a patch) of the image were excised at these spots,

in order of brightness. The signal was defined as the mean of the last 50 frames (the tail) subtracted from the first 50 frames (the head) and the noise was defined as the standard deviation of the tail. Bright spots were selected until the signal to noise multiple fell below 2, that is, when the range of the trace was less than approximately double the noise in the tail. The maximum pixel value at each time point was taken across each 3 × 3 section to give a single intensity versus time trace for subsequent frequency analysis. Traces where a fluorophore appeared midway through a recording were excluded by discarding traces with maxima outside the initial 50 frames.

Individual photobleaching traces were analyzed using a time-independent analysis of the distribution of intensities for each trace. The decrement in intensity corresponding to the photobleaching event of a single fluorophore was determined by calculating the Empirical Characteristic Function (ECF) of the pairwise difference density function (PDDF) [38]. The PDDF corresponds to the pairwise differences between every point in a single trace [39]. Both photobleaching and photoblinking events can be used in the analysis of pairwise differences, to calculate a final overall step size. The ECF was calculated directly over each wave vector of the inverse of possible decrements in intensity using a discrete Fourier transform:

$$P(f_j) = \frac{1}{T}\sum_{t=1}^{T} \exp\left(i f_j m_t\right)$$

where T is the total number of traces, f_j are the frequencies chosen for the analysis, based on desired decrement resolution, and m_t is the set of pairwise differences for an entire trace [38]. Direct evaluation of the ECF was preferred as this avoided artifacts introduced by arbitrary binning of the PDDF histogram which could affect the resolution of the Fourier transform. The power spectrum, $|P(f_j)|^2$, was calculated from the ECF. The largest peak in the power spectrum was selected as the dominant frequency in the trace (Fig. S3). Peaks in the power spectrum were determined by detecting zero crossings of the first derivative of the ECF, with peak properties (height, width) calculated by fitting a parabola to a 7-point window around each peak. The slope of the first derivative was used as a threshold to exclude very narrow peaks (slope threshold: 0.005), since narrow peaks at small decrement arise from high frequency noise and not photobleaching. Similarly, due to the harmonic nature of frequency analysis, the largest peak sometimes corresponded to a multiple of the actual decrement size. To avoid this, the lowest frequency peak was selected that was no smaller in height than a proportion (peak threshold: 0.8) of the largest peak. These thresholds were calibrated using simulation data of known stoichiometries at signal:noise levels matching our experiment. Following optimization (Fig. S4), the selected peak in the ECF was chosen as the decrement size (D) corresponding to a single photobleaching step. For each individual trace the maximum of the trace, M, and the mean of the last 50 frames in the tail of the trace (T) were found. By calculating $(M\text{-}T)/D$, the stoichiometry (i.e. the number of fluorophores observed in each diffraction limited spot) was determined. Stoichiometry histograms were then plotted (Fig. 3). The final stoichiometry is slightly inflated since the range of the trace ($M\text{-}T$) is overestimated, due to the influence of noise on the reported maximum of a trace. In practice, under conditions reflecting our experimental signal:noise, stoichiometries were inflated by approximately 0.45 units (e.g. the calculated stoichiometry of the simulated tetramer was 4.45 ± 0.53 units).

Simulated fluorescence data

Simulated traces at 50 ms time resolution matching our experimental data were generated using the following procedure: (1) Gaussian noise (mean 0, standard deviation 10) was added to a constant baseline (1000 a.u.) estimated from the background noise across the last 50 frames of all measured traces; (2) photobleaching steps were added sequentially, with the frame in which the photobleaching event appears determined by sampling at random from an exponential distribution (τ = 3.7 s). This time constant was set to the mean photobleaching decay of all EqtII L:T 100:1 traces. Steps were added with a fixed decrement size of 156 a.u. equal to the mean EqtII L:T 100:1 decrement size, and each step was simulated to include a Gaussian noise per fluorophore (mean 0, standard deviation 24) resulting in larger noise when larger numbers of fluorophores are present. This noise per fluorophore was estimated using a Kalafut-Visscher

(KV) step-fitting algorithm to both our simulated and observed data [40]. We adjusted the noise per fluorophore in our simulation traces until the root-mean-square (RMS) error of a KV piecewise constant fit to our simulation matched the RMS error of a KV fit to our observed data.

To compile a set of tetrameric traces we simulated 200 traces of length 40 s, all with 4 bleaching steps over the trace. To compile the Poisson simulation sets we determined the number of steps for each trace by sampling a Poisson distribution of fixed mean, iterating over different values of this mean to determine the best fit to our observed data ($\mu = 2.68$). To compare how similar these simulated distributions were to the observed data we measured the difference between density functions over each unitary bin (from 1 to 40) and then calculated the total RMS error, where a fit was judged as good when this error was less than 0.05.

Acknowledgements

G. A. and N. R. would like to thank Slovenian Research Agency and Ad Futura, Slovene Human Resources Development and Scholarship Fund, for support. M.I.W. is funded by the ERC and BBSRC. B.C. is an EPSRC LSI postdoctoral fellow.

References

[1] [1] R. J. C. Gilbert, *Cell. Mol. Life Sci. CMLS* **2002**, *59*, 832–844.

[2] G. Anderluh, J. H. Lakey, *Trends Biochem. Sci.* **2008**, *33*, 482–490.

[3] M. Gonzalez, M. Bischofberger, L. Pernot, F. van der Goot, B. Frêche, *Cell. Mol. Life Sci.* **2008**, *65*, 493–507.

[4] R. Aroian, F. G. van der Goot, *Curr. Opin. Microbiol.* **2007**, *10*, 57–61.

[5] M. W. Parker, S. C. Feil, *Prog. Biophys. Mol. Biol.* **2005**, *88*, 91–142.

[6] S. J. Tilley, H. R. Saibil, *Curr. Opin. Struct. Biol.* **2006**, *16*, 230–236.

[7] G. Anderluh, P. Macek, *Toxicon* **2002**, *40*, 111–124.

[8] M. Mueller, U. Grauschopf, T. Maier, R. Glockshuber, N. Ban, *Nature* **2009**, *459*, 726–730.

[9] R. K. Tweten, *Infect. Immun.* **2005**, *73*, 6199–6209.

[10] J. C. Sharpe, E. London, *J. Membr. Biol.* **1999**, *171*, 209–221.

[11] M. Palmer, R. Harris, C. Freytag, M. Kehoe, J. Tranum-Jensen, S. Bhakdi, *EMBO J.* **1998**, *17*, 1598–1605.

[12] I. Iacovache, M. Bischofberger, F. G. van der Goot, *Curr. Opin. Struct. Biol.* **2010**, *20*, 241–246.

[13] M. A. Dunstone, R. K. Tweten, *Curr. Opin. Struct. Biol.* **2012**, *22*, 342–349.

[14] A. Athanasiadis, G. Anderluh, P. Maček, D. Turk, *Struct. Lond. Engl. 1993* **2001**, *9*, 341–346.

[15] M. G. Hinds, W. Zhang, G. Anderluh, P. E. Hansen, R. S. Norton, *J. Mol. Biol.* **2002**, *315*, 1219–1229.

[16] J. M. Mancheño, J. Martín-Benito, M. Martínez-Ripoll, J. G. Gavilanes, J. A. Hermoso, *Struct. Lond. Engl. 1993* **2003**, *11*, 1319–1328.

[17] A. E. Mechaly, A. Bellomio, D. Gil-Cartón, K. Morante, M. Valle, J. M. González-Mañas, D. M. A. Guérin, *Struct. Lond. Engl. 1993* **2011**, *19*, 181–191.

[18] B. Bakrač, I. Gutiérrez-Aguirre, Z. Podlesek, A. F.-P. Sonnen, R. J. C. Gilbert, P. Maček, J. H. Lakey, G. Anderluh, *J Biol Chem* **2008**, *283*, 18665–18677.

[19] G. Anderluh, A. Barlic, Z. Podlesek, P. Maček, J. Pungercar, F. Gubensek, M. L. Zecchini, M. D. Serra, G. Menestrina, *Eur. J. Biochem. FEBS* **1999**, *263*, 128–136.

[20] P. Malovrh, A. Barlic, Z. Podlesek, P. Maček, G. Menestrina, G. Anderluh, *Biochem. J.* **2000**, *346 Pt 1*, 223–232.

[21] Q. Hong, I. Gutierrez-Aguirre, A. Barlic, P. Malovrh, K. Kristan, Z. Podlesek, P. Maček, D. Turk, J. M. Gonzalez-Manas, J. H. Lakey, et al., *J. Biol. Chem.* **2002**, *277*, 41916–41924.

[22] K. Kristan, Z. Podlesek, V. Hojnik, I. Gutiérrez-Aguirre, G. Gunčar, D. Turk, J. M. González-Mañas, J. H. Lakey, P. Maček, G. Anderluh, *J. Biol. Chem.* **2004**, *279*, 46509–46517.

[23] K. Kristan, G. Viero, P. Macek, M. Dalla Serra, G. Anderluh, *FEBS J.* **2007**, *274*, 539–550.

[24] G. Belmonte, C. Pederzolli, P. Maček, G. Menestrina, *J. Membr. Biol.* **1993**, *131*, 11–22.

[25] M. Tejuca, M. Dalla Serra, M. Ferreras, M. E. Lanio, G. Menestrina, *Biochemistry (Mosc.)* **1996**, *35*, 14947–14957.

[26] C. A. Valcarcel, M. Dalla Serra, C. Potrich, I. Bernhart, M. Tejuca, D. Martinez, F. Pazos, M. E. Lanio, G. Menestrina, *Biophys. J.* **2001**, *80*, 2761–2774.

[27] G. Anderluh, M. D. Serra, G. Viero, G. Guella, P. Maček, G. Menestrina, *J. Biol. Chem.* **2003**, *278*, 45216–45223.

[28] J. Martín-Benito, F. Gavilanes, V. de los Ríos, J. M. Mancheño, J. J. Fernández, J. G. Gavilanes, *Biophys. J.* **2000**, *78*, 3186–3194.

[29] M. H. Ulbrich, E. Y. Isacoff, *Nat. Methods* **2007**, *4*, 319–321.

[30] S. K. Das, M. Darshi, S. Cheley, M. I. Wallace, H. Bayley, *ChemBioChem* **2007**, *8*, 994–999.

[31] H. McGuire, M. R. P. Aurousseau, D. Bowie, R. Blunck, *J. Biol. Chem.* **2012**, *287*, 35912–35921.

[32] E. Limpert, W. A. Stahel, M. Abbt, *BioScience* **2001**, *51*, 341–352.

[33] E. Limpert, W. A. Stahel, *PLoS ONE* **2011**, *6*, e21403.

[34] M. C. Leake, N. P. Greene, R. M. Godun, T. Granjon, G. Buchanan, S. Chen, R. M. Berry, T. Palmer, B. C. Berks, *Proc. Natl. Acad. Sci.* **2008**, *105*, 15376–15381.

[35] N. Groulx, H. McGuire, R. Laprade, J.-L. Schwartz, R. Blunck, *J. Biol. Chem.* **2011**, *286*, 42274–42282.

[36] L. W. Runnels, S. F. Scarlata, *Biophys. J.* **1995**, *69*, 1569–1583.

[37] N. Rojko, B. Cronin, J. S. H. Danial, M. A. B. Baker, G. Anderluh, M. I. Wallace, *Accept. Biophys J* **2014**.

[38] M. Tejuca, M. Dalla Serra, C. Potrich, C. Alvarez, G. Menestrina, *J. Membr. Biol.* **2001**, *183*, 125–135.

[39] D. Martínez, A. Otero, C. Alvarez, F. Pazos, M. Tejuca, M. E. Lanio, I. Gutiérrez-Aguirre, A. Barlic, I. Iloro, J. L. Arrondo, et al., *Toxicon Off. J. Int. Soc. Toxinology* **2007**, *49*, 68–81.

[40] B. B. Bonev, Y. H. Lam, G. Anderluh, A. Watts, R. S. Norton, F. Separovic, *Biophys. J.* **2003**, *84*, 2382–2392.

[41] K. Črnigoj Kristan, G. Viero, M. Dalla Serra, P. Maček, G. Anderluh, *Toxicon* **2009**, *54*, 1125–1134.

[42] A. McPherson, *Methods San Diego Calif* **2004**, *34*, 254–265.

[43] D. L. Minor Jr, *Neuron* **2007**, *54*, 511–533.

[44] G. Anderluh, A. Barlič, I. Križaj, G. Menestrina, F. Gubenšek, P. Maček, *Biochem. Biophys. Res. Commun.* **1998**, *242*, 187–190.

[45] N. Rojko, K. Č. Kristan, G. Viero, E. Žerovnik, P. Maček, M. D. Serra, G. Anderluh, *J. Biol. Chem.* **2013**, *288*, 23704–23715.

[46] D. G. Hunter, B. J. Frisken, *Biophys. J.* **1998**, *74*, 2996–3002.

[47] P. S. Cremer, S. G. Boxer, *J. Phys. Chem. B* **1999**, *103*, 2554–2559.

[48] R. P. Richter, R. Bérat, A. R. Brisson, *Langmuir* **2006**, *22*, 3497–3505.

[49] M. A. Little, N. S. Jones, *Proc. R. Soc. Math. Phys. Eng. Sci.* **2011**, *467*, 3115–3140.

[50] M. A. Little, B. C. Steel, F. Bai, Y. Sowa, T. Bilyard, D. M. Mueller, R. M. Berry, N. S. Jones, *Biophys. J.* **2011**, *101*, 477–485.

[51] B. Kalafut, K. Visscher, *Comput. Phys. Commun.* **2008**, *179*, 716–723.

Figure Captions

Figure 1. **Photobleaching of EqtII.** *(A) Example individual photobleaching traces for monomer through decamer detected for EqtII. Although it is straightforward to determine a photobleaching decrement manually for low stoichiometries, this task becomes more difficult as the number of fluorophores, and thus the noise, increases. (B) Distribution of EqtII stoichiometry with lipid composition DOPC:ESM 1:1 and lipid:toxin 100:1. Stoichiometry was calculated by dividing the total intensity decrease for a trace by its bleaching decrement determined by frequency analysis. This distribution of stoichiometry has a mean of 3.3 ± 2.2 s.d.*

Figure 2. ***Controls & simulations.*** *(A) Stoichiometry analysis of background noise. Supported vesicle bilayers of unlabeled EqtII in DOPC:ESM (1:1) yielded significantly fewer spots than in the labeled data (~2 per image here, vs. ~16 per image in Fig. 1). Representative traces are shown in the inset. (B, top) A binomial simulation (blue line -●-, N= 7, p = 0.34, E_{RMS} = 0.0153) fit to the observed distribution in Fig. 1 (red bars). Partial labeling could only explain the breadth of the observed distribution if labeling efficiency was low. The stoichiometry distribution from a simulated Poisson distribution (black line -◆-, μ = 2.68) fits the observed EqtII data (E_{RMS} = 0.0286), but has a significantly different mean (p = 0.05) and fails to recreate some of the rare high stoichiometry events in the observed distribution. (B, bottom) Stoichiometry distribution from simulated tetrameric traces corresponding to experimental conditions (red bars, σ = 0.66) shows a much narrower distribution than the experimental data. (Bottom) (C) Analysis of mean decrement across all traces, calculated over consecutive 1 s intervals (black circles -●-, error bars: standard deviation). The decrement decreases over the first 5 intervals, and is then constant over the remainder of the trace.*

Figure 3. ***Stoichiometry distributions of labeled EqtII in varying conditions.*** *Lipid:toxin ratio varies from left to right: (left) 100:1, (middle) 200:1, (right) 400:1. Supported vesicle bilayer composition changes from top to bottom: (top) DOPC:ESM 1:1, (middle) DOPC:ESM:Ch 1:1:1, (bottom) DOPC only. The x-axis in all subplots is the calculated stoichiometry, and the y-axis is the number of detected events. (Top row) As the lipid:toxin ratio is increased the mean stoichiometry is reduced from 3.3 ± 2.2 s.d. to 2.8 ± 1.7 s.d. Student's* t*-test analysis along this row shows all means are significantly different (p < 0.05). Plots comprise of 1031 (100:1), 962 (200:1), and 579 (400:1) total traces respectively. (Middle row) As the lipid:toxin ratio is increased the mean stoichiometry shifts from 2.8 ± 1.9 to 3.9 ± 3.0. For this row, only lipid:toxin 100:1 is significantly different (p < 0.05). Plots comprise of 576 (100:1), 402 (200:1) and 375 (400:1) traces respectively. (Bottom row) Distribution of stoichiometry in absence of sphingomyelin and cholesterol (lipid:toxin 100:1, N = 306). Absence of sphingomyelin does not prevent complex formation, and the mean stoichiometry of the measured traces is 2.9 ± 1.6. The mean of this distribution is not significantly different to DOPC:ESM 1:1 or DOPC:ESM:Ch 1:1:1 at identical lipid:toxin ratio (p > 0.05). P values for significance testing are presented in Table S1.*Figure Caption.))

Figure 4. ***Controls for co-localized monomers.*** *(A) Mean stoichiometry against lipid:toxin ratio (--□-- DOPC:ESM 1:1, ···●··· DOPC, -○- DOPC:ESM:Ch 1:1:1, red line: linear fit). As EqtII is diluted, the mean stoichiometry remains constant (slope of fit ~ 10^{-3}). (B) Mean stoichiometry calculated for DOPC:ESM (1:1) lipid:toxin (100:1) with increasingly stringent restrictions on centroid variance. When high centroid variance is tolerated (10 nm – 50 nm), the mean stoichiometry is constant, but as the*

centroid variance restriction was extended below 10 nm, the mean stoichiometry decreased as multimeric pores were excluded from the analysis. Error bars indicate standard deviation throughout.

Entry for the Table of Contents (Please choose one layout)

Layout 1:

Equinatoxin II (EqtII), a sea anemone cytolysin, is known to oligomerize to form pores that spontaneously insert into membranes. We used single-molecule photobleaching of fluorescently labeled EqtII to determine the stoichiometry of EqtII oligomers in supported lipid bilayers. These data are consistent with a model of EqtII stoichiometry where there is a large variation in the number of subunits in individual pores.