Supplementary Discussion

Structure-function relationship in BinA and BinB carbohydrate binding modules: Resemblance of the β-trefoil domains to the lectin binding domain of the deadly toxin, ricin, suggests that they might chaperone entry of BinAB into the cell by binding carbohydrates on the host cell surface (Extended Data Fig. 3a). To the contrary, the canonical carbohydrate modules (α, β, and γ) of BinB are located on the opposite surface of the trefoil (*i.e.* the cap surface) from the mutations which disrupt receptor binding in *Culex quinquefasciatus* (*i.e.* the barrel surface) (Figs. 2b-d and 3b). Specifically, the receptor binding sensitive mutations are located near the barrel opening comprising Tyr42 and residue ranges Ile85-Arg86-Phe87 and Phe147-Gln148-Phe149 (Supplementary Table 2). Recognition of the receptor at this surface is probably through protein-protein interactions since this pocket does not correspond structurally to any known carbohydrate-binding motif. The analogous region in the BinA trefoil shares a similar backbone, implying a similar role. However, BinA binds only non-specifically to the midgut in *Culex*, and plays only a supporting role in *Anopheles gambiae* (Supplementary Table 1). Apparently, the difference in residues (Arg19, Ile56-Phe57-Phe58, Tyr106-Tyr107-Leu108) makes BinA ineffective in binding receptor in *Culex* species, and contributes to the distinct functional roles of BinA and BinB (Fig. 2d).

The three carbohydrate modules of BinA, and the two accessible modules of BinB could play a role in toxicity, if not in receptor binding. Numerous mutations in the carbohydrate binding modules have been identified as causing loss of toxicity, implying a role for carbohydrate binding in toxic function. In BinA, these include Asn52 [59], disulphide-linked Cys31 and Cys47 in the α−module [60], Leu93 in the β−module [61], and Arg97 [62] and Val99 in the non-canonical site III_A [10]. In BinB, these include Tyr150 in site III_A [47] and disulphide-linked Cys67 and Cys161 in the α−module and γ−module [63], respectively (Fig. 2b-d).

Three out of four of BinAB propeptides are visible in the complex structure: Activation of the 42 kDa pro-BinA and 51 kDa pro-BinB requires cleavage by mosquito-larvae gut proteases, resulting in 39 and 43 kDa fragments, respectively. Deletion analysis of BinA concluded that larval gut proteases remove 10 amino acids from the N-terminus, and 17 amino acids from the C-terminus [64] (Supplementary Tables 1 and 2). Similarly, for BinB, larval gut proteases remove 21 amino acids from the N-terminus and 53 amino acids from the C-terminus of BinB [65]. In both our pH 7 and pH 10 structures, three of the four propeptide segments are ordered. Removal of the propeptide coordinates from our pH 7 BinAB dimer model eliminates 1539 $Å^2$ combined interface surface area (propeptide surface plus opposing surface) from the original 3655 $Å^2$ (BinA surface plus opposing BinB surface), leaving only 2116 $Å^2$ buried surface area after cleavage, or 1058 $Å^2$ per monomer (Supplementary Table 10).

Propeptides also participate in three other crystal contacts, mediating an additional 1160 Å^2 of surface area (Supplementary Tables 5, 6 and 8). Hence, removal of the propeptides would irreversibly commit BinAB to advance from the crystalline state to solution.
The TM subdomain also plays a notable role in the BinA-BinB interface. Although it contributes less interface area than the trefoil, sheet, and propeptide regions, it contributes the largest area per residue (about 9 Å^2) (Extended Data Fig. 5c). This value is twice greater than observed in the trefoil domain. Hence, evolution appears to have emphasized the role of the TM domain in dimer packing, perhaps to restrain this domain from inserting into a membrane until after the BinAB dimer dissociates.

Comparison of pro-BinB and activated BinB Structures: Pro-BinB and activated BinB structures are similar to each other (369 aligned alpha carbons superimpose with RMSD 1.5 Å) with exceptions relating to residues involved in BinB's extensive interface with BinA, and inclusion of the pro-peptide, neither of which were present in the activated BinB structure [11]. The interface between BinA and BinB affects the location of a hairpin loop (residues 138-145) near a putative receptor binding-site on BinB. Specifically, Tyr140 near the tip of this hairpin moves over 10 Å away from its contacts with BinB Tyr180 in the activated BinB structure to contact BinA residues 262, 263, 290, and 292 in the BinAB complex. The movement exposes Tyr150, which has been shown from mutation studies to be essential for receptor binding [47] (Supplementary Tables 1 and 2). Tyr150 is located in a pocket, observed to bind carbohydrate in a structural homolog (PDB ID 3AH1). The proximity of this mobile hairpin to the putative receptor binding-site suggests a mechanism by which receptor binding might weaken the interactions between BinA and BinB, possibly resulting in a conformational change in the complex structure. There is also a hinge motion between the trefoil domain and the pore forming domain, resembling that observed in a structurally homologous toxin, lysenin [49].

Exposure of the receptor binding epitope of BinB: The putative receptor binding epitope of BinB is buried in the BinAB dimer interface. If BinAB is to remain associated as a dimer while bound to the receptor, a limited conformational change may be required to expose the epitope which is located on the trefoil domain of BinB. Intramolecular difference distance matrices (DDMs) calculated between the pH 7 and the pH 10 structures demonstrate that upon pH elevation, both trefoils undergo conformational changes, resulting in a more compact conformation of these (Extended Data Fig. 9c, d). In addition, the DDMs highlight drastic conformational changes in trefoil loops 110-120 of BinA (Extended Data Fig. 9c) and 177-184 of BinB (Extended Data Fig. 9d). Furthermore, the intermolecular DDM reveals that the two trefoils come closer to one another at elevated pH (Extended Data Fig 9a). The F_o-F_o map calculated between the pH 10 dataset and the pH 7 native dataset reveals that the hydrogen bond between BinB Gln448 terminal carboxyl and BinA Asp22 side chain nitrogen breaks upon pH elevation (Fig. 4d and Extended Data Fig. 8b). Increased dynamics of BinB C-terminal propeptide could release the trefoil to alter its

position with respect to the PFD, thereby exposing the receptor binding motif (Figs. 2b and 3b). To test this hypothesis, we engineered a pH-insensitive hydrogen bond between BinA and the C-terminal propeptide of BinB, by mutation of BinA Asp22 into asparagine. The resulting binary toxin, $BinA^{D22N}B$ showed decreased toxicity (main text and Supplementary Table 13).

Additional evidence of conformational flexibility comes from the comparison of BinB and pro-BinB structures. A hinge motion of the BinB trefoil domain with respect to the PFD conformational change was observed, as well as a conformational change in its 138-145 hairpin loop. This hairpin is in the vicinity of BinA loop 341-345, another loop which we observe as mobile in response to pH elevation. In addition, the pH 10 structure reveals a rearrangement of the H-bond network between BinA Glu14, Arg19, Glu98 and BinB Gln336, which results in the N-terminal propeptide of BinA transitioning from helix to β-strand. Hence, other regions of BinA and BinB that could become exposed during the toxic cycle could include the putative transmembrane domains (TMs; Extended Data Fig. 4c) . In the pH 7 structure, access to these is indeed reduced by the joint presence of BinA and BinB N-terminal propeptides. Proteolytic activation would thus expose the TMs to bulk solvent. The extended conformation of BinA N-terminal propeptide (L4-E14; BinA residues 1-3 are not visible in the *pH10* structure) observed in the pH 10 structure could effect in facilitating access to proteases (Fig. 4c and Extended Data Figs. 8a and 9a, c). In BinB, it was shown that TM residues Leu314 and Phe317 are determinant for the host range [11] (Supplementary Tables 1 and 2), but whether residues within BinA's TM domain are crucial for activity was not challenged by mutation as yet (Supplementary Tables 1 and 2). In fact, most of the residues we observe to be sensitive to pH have been left untouched in mutagenesis experiments.

Evidence that structural changes observed between pH 7 and pH 10 data sets are due to pH change and not radiation damage: BinA and BinB each feature a disulphide bond in their trefoil domains (BinA C31-C47 and BinB C67-C161). In comparing datasets collected at pH 7 and pH 10, we found that both disulphide bonds are perturbed, as revealed by strong positive and negative F_o-F_o peaks proximal to the bonds. These peaks have two conceivable origins: either pH-induced structural change, or radiation damage-induced disulphide rupture resulting from an unintended difference in radiation dose. Both data sets were collected using single XFEL pulses of close wavelength and duration. However, a difference in dose arose from changes in LCLS power and beam focal size between experimental sessions (Extended Data Table 1). As a result, we estimate that the pH 10 dataset was collected with a ~500 fold higher dose than the pH 7 dataset. Nevertheless, the bulk of evidence supports the hypothesis that the observed structural changes originate from differences in pH rather than radiation dose.

Three lines of evidence support the hypothesis that pH elevation, and not radiation dose, is the cause of the disulphide-proximal F_o^{pH10}-F_o^{pH7} peaks. (1) If dose has been

responsible for the appearance of these peaks, an analogous set of peaks would have been expected in comparison of pH 5 and pH 7 datasets, which also differ in dose by ~500 fold (Extended Data Table 1). However, the lack of peaks in the F_o^{pH5}-F_o^{pH7}, φ^{pH7} map (R_{iso}=0.26) around disulphides indicates that the corresponding peaks in the F_o^{pH10}-F_o^{pH7}, φ^{pH7} map (R_{iso}=0.28) are not due to radiation damage (Extended Data Fig. 8e, f). (2) An analogous set of disulphide-proximal peaks is reproduced in the F_o^{pH10}-F_o^{pH5}, map (R_{iso}=0.35) (Extended Data Fig. 8e, f), as would be expected for an additional pair of datasets which span the pH-induced transition occurring near pH 10. Moreover, these peaks cannot be attributed to radiation damage, since the pH 5 and pH 10 datasets were collected using the same dose, with the same experimental setup, on the same day. (3) If the disulphides had been reduced by radiation damage, or ruptured by some other process, such as oxidation upon pH elevation (e.g. Cys-S-S-Cys → Cys-S- + Cys-S-OH), we would have expected negative density to be centred between the two sulphur atoms of the disulphide, with positive peaks indicating the new position of sulphur atoms. Instead, the negative density is displaced about 1 Å away from the bond, with a positive peak, equidistant and in the opposite direction (Extended Data Fig. 8e, f). This observed pattern is more consistent with movement, rather than bond disruption. Indeed, the pattern of positive and negative peaks correlates with a larger pattern of negative-positive peak pairs throughout the trefoil domains arising from a pH-induced rigid body motion of the trefoils (Extended Data Figs. 8g, h). This motion of the trefoils toward their respective PFDs is further evidenced in Cα-Cα difference distance matrices (Extended Data Fig. 9c and d, lower left corners), and is notably absent from a comparison made between pH 5 and 7, two values far from pH 10 transition (Extended Data Fig. 9c and d, upper right corners). We hypothesize that the peaks originating from motion of the disulphide bonds stand out among all the trefoil motion-related peaks in F_o-F_o maps simply because the sulphur atoms are electron dense compared to carbon, nitrogen and oxygen. Hence, we conclude that radiation damage had no contribution to the structural changes near disulphides, or any of the other regions identified as pH-sensitive, in our comparisons between data collected at different pH values.

Features observed in the F_o^{pH10}-F_o^{pH7}, φ^{pH7} map: In both BinA and BinB, peaks are concentrated on the trefoil domains, suggesting that these are more affected by pH elevation than the PFDs (Fig. 4, Extended Data Fig. 8 and Supplementary Tables 11 and 12). We observe more F_o-F_o peaks on the trefoil of BinB than on that of BinA, consistent with the hypothesis that following crystal dissolution, the next step in the intoxication process is a conformational change in the trefoil of BinB, to expose the barrel subdomain featuring the epitope for the receptor. Inside BinA and BinB trefoils, most prominent are the pairs of positive and negative peaks on disulphide bridges (Cys31-Cys47 in BinA, and Cys67 to Cys161 in BinB). The positive and negative F_o-F_o peaks are at -3.5 and +5.5 σ in BinA, and at -4.4 and +5.8 σ in BinB, respectively. We propose that these indicate motion, rather than reduction, oxidation,

or damage (see paragraph above). Apart from peaks around disulphides, strong F_o-F_o peaks are seen at four specific locations in the crystal:

1 - The N-terminal extremity of BinA (Fig. 4c and Extended Data Fig. 8a). The highest peaks on BinA N-terminal propeptide (residues 1-10) are at -6.8 and -4.5 σ, on Gly15 and Ser9 carbonyl oxygens, respectively. Strong negative peaks are also observed Ile7, Ile11, Ly16, Tyr17 (-3.7, -4.4, -4.5 and -4.1 σ).

2 - The C-terminal extremity of BinB (Fig. 4d and Extended Data Fig. 8b). Strong negative peaks are observed on BinB Gln448 (main chain; -4.1 σ) and BinA Asp22 (side chain; -4.3 σ) carboxyls, which are H-bonded in the pH 7 structure. A positive peak is also seen, at +6.1 σ, whose position matches that refined for Asp22 side chain in the pH 10 structure.

3 - The crystal contact zone 5 (Fig. 4e, Extended Data Figs. 6 and 8c, and Supplementary Table 7), which displays the smallest buried area as compared to other interfaces in the crystal, is the only to be clearly affected by the pH change in the F_o-F_o map. Notably, this is the only crystal interface that involves the trefoil domains of both BinA and BinB. The interface is principally contributed by the H-bonds between BinB Glu59 (OE1, OE2) and BinA His 125 (ND1) and Tyr134 (OH). In the F_o-F_o map, we observe pairs of positive and negative peaks on BinB Glu59 (-4.1 σ; +4.7 σ), Phe60 (-4.3 σ; +4.2 σ) and Pro61 (-4.3 σ; +4.8 σ), suggesting a large conformational change in this region of BinB (Supplementary Tables 11 and 12). Negative peaks are also seen on BinA His125, Ser126, His127 and Leu129 (-4.1, -4.3, -5.0 and -4.3 σ). No peaks are seen, however on BinA Tyr134.

4 - The 341-345 loop in BinA (Fig. 4f and Extended Data Fig. 8d). This loop is at the junction between the sandwich, sheet, and TM subdomains of BinA and precedes the penultimate strand of the sandwich subdomain of BinA PFD. The largest negative peak of the F_o-F_o map is found on Asp342 (-6.0 σ, 70 voxels) (Supplementary Table 11), which H-bonds to BinA Glu240 from the second stand of the sandwich subdomain, in the pH 7 structure. A positive peak is also seen in the vicinity of Asp342 (5.0 σ), matching the new conformation observed in the pH 10 structure. In addition, strong peaks are seen on main and side chain atoms of residues Asn341 (-3.9; +4.2 σ), Asn343 (-4.5; +4.1) and Tyr344 (-4.1 σ), suggesting increased structural dynamics across the whole loop. Interestingly, no peak is seen on Glu240 or on Tyr299, in the close vicinity. The B-factors of the latter however increase by 70% upon elevation, to compare to 21 and 11 % increase for Glu240 and the full BinA molecule.

Conformational changes observed upon elevation of pH from 7 to 10: The pH 7 and pH 10 models superimpose closely, with an rmsd of 0.48 Å RMSD over 775 aligned alpha carbons. The pH 10 structure is characterized by hinge motions of BinA and BinB trefoil domains over their respective PFD (Extended Data Fig. 9), resulting in an overall more compact structure (volume occupied by BinAB in the pH 7 and pH

10 structures: 8.39 x 10^4 and 8.29 x 10^4 $Å^3$). Interestingly, pH elevation leaves the complex untouched in terms of buried surface area, with a loss of -56 $Å^2$ at the dimer interface and a gain of +38 $Å^2$ at crystal contacts. The Complex Formation Significance Score (CSS) of the dimer interface nevertheless drops from 1.0 to 0.89 upon pH elevation (calculated with PDBePISA[66], http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html). Comparison of the two structures allows pinpointing regions most sensitive to pH elevation in BinAB. Below, we recapitulate our findings on a monomer basis. Residues which have been challenged by mutagenesis studies are highlighted by a (*) (Supplementary Table 1 and 2).

BinA:

1 - As suggested the F_o-F_o map (Fig. 4, Extended Data Fig. 8, and Supplementary Tables 11 and 12) and by the DDMs (Extended Data Fig. 9), a conformational change occurs in the N-terminal propeptide of BinA, which transitions from helix to β-strand as a result of the electrostatic repulsion of BinA Asp8(OD1) and Gly15(O) by respectively BinB Glu267(OE1) (distance increases from 3.5 to 8.3 Å) and BinA Tyr213(OH) (distance from 2.8 to 7.5 Å). The twisted β-strand structure of the N-terminal propeptide of BinA is stabilized by hydrophobic interactions as well as by new hydrogen bonds between BinA Ser9(OH) and BinB Glu267(OE1), BinA Ile7(N) and BinB Leu210(O), and Asp5(O) and Asn212(ND2) (distances: 2.9, 2.9 and 3.0 Å). However, the electron density around residues 1-3 is only poorly defined in the 2Fo-Fc map contoured at 1.0 σ. These residues were thus not included in the pH10 structure.

2 - At the N-terminus of the trefoil (after the propeptide), and as a results of BinA Glu98* forming a salt bridge with BinA Arg19 (distance between their OE1 and NH1 atoms decreases from 3.8 to 2.9 Å), BinA Glu14, whose side chain is H-Bonded to Arg19 in the pH7 structure, undergoes a conformational change (~180° rotation around the CB-CG angle) that results in the formation of another H-bond with BinB Glu336 side chain nitrogen (distance their OE2 and HE2 atoms: 2.8 Å) (Fig. 4c and Extended Data Figs. 8 and 9a, c).

3 - The formation of a salt bridge between BinA Arg120 and Glu119 results in a large conformational change in the trefoil loop Ile110-Arg120. The change results in this loop drawing closer to the rest of the structure (Extended Data Fig. 9b).

4 - Glu162, which is H-bonded to the main chain nitrogen of BinB Thr28 in the pH7 structure, adopts a new conformation that pushes the latter away (distance between OE2 and N increases from 2.9 to 4.3 Å). Thus, Glu162 is very likely protonated in the pH 10 Å structure.

5 - Tyr166 adopts a new conformation at pH 10 (~75° rotation of the aromatic cycle) that opens a route for new water, Wat530, in between the side chains of BinA Trp222, Tyr66, L318 and Tyr344. Of note, Tyr166 is neither involved in hydrogen-bonding interactions in pH 10 structure nor in the pH7 structure.

6 - A salt bridge forms between Lys241 and Glu296 (distance between their NH1 and OE2 atoms decreases from 4.7 to 3.0 Å).

7 - The electrostatic repulsion between Glu240 and Asp342 carboxyls, which are H-bonded in the pH 7 structure (Supplementary Table 3), results in a complete reorientation of the latter's side chain (~90° rotation) (Fig. 4f and Extended Data Figs. 8d and 9a, c). Of note, Asp342 is the residue that features the largest negative peak in the F_o-F_o map (-6.0 σ, 70 voxels) (Supplementary Tables 11 and 12). A positive F_o-F_o peak is also seen on the new conformation (+5.0 σ). Due to this change in Asp342, residues Asn341 to Thr346 are forced to also change their conformations. Accordingly, F_o-F_o peaks are seen on Asn343 side chain (-4.1 σ) and main chain atoms (-4.2; +4.3 σ). The pH 10 conformer of Asp342 is stabilized by a H-bond to BinA Ser242 hydroxyl (distance between OE2 and OH: 2.8 Å). The conformational change in BinA Asn341-Tyr344 loop participates to the increase of compactness observed, upon pH elevation, in the BinAB structure (Extended Data Fig. 9a, c). Following the conformational change in Asp342 side chain, Arg301 also changes conformation and establishes a salt bridge with Glu240 (distance between NH1 and OE1: 2.8 Å). Thus, our data suggests that Asp342, and possibly also Glu240, are deprotonated at pH 10. However, no F_o-F_o peak is seen on Glu240 nor on nearby Tyr299, whose hydroxyl lies within 2.8 Å of an obligate hydrogen-bond acceptor, BinB-Ile141(O). Deprotonation of Tyr299 hydroxyl would necessarily have disrupted the interaction. Thus, the pH 10 structure offers a demonstration that the pK_a of Tyr299 hydroxyl is higher than 10, possibly due to its reduced accessibility. Nevertheless, examination of B-factors suggests that Tyr299 side chain gains in dynamics, in the pH 10 structure (+70% increase in B-factor, to compare to 21 and 11% for Glu240 and all BinA, respectively). We note an additional contact between a tyrosine hydroxyl and an obligate hydrogen bond acceptor, BinB-Tyr395(OH):BinB-Ile201(O), which is expected to break upon deprotonation. Yet again, we did not observe F_o-F_o difference density nor a conformational change in these residues in the pH 10 structure, suggesting that the pK_a of BinB-Tyr395 hydroxyl is also higher than 10.

BinB:

1 – The electron density around BinB residues 28-29 is only poorly defined in the 2Fo-Fc map contoured at 1.0 σ. These residues were thus not included in the pH10 structure.

2 - Glu65 displays a complete change of conformation, most likely as a result of that occurring in Glu65-Pro68.

3 - A large conformational change is observed in loop Lys175-Ser184, which results in the latter drawing away from the structure (Extended Data Fig. 9d).

4 - The electrostatic repulsion between BinA Asp22 side chain and BinB Asn448 main carboxyl-oxygens results in the latter drawing away from the former (distance

between Asp22 OD1 and Glu448 OXT increases from 3.1 to 5.2 Å) (Fig. 4d and Extended Data Fig. 8b).

Conformational changes observed upon decrease of pH from 7 to 5:

The pH 7 and pH 5 models are very similar to each other, displaying an RMSD of 0.31 Å over 780 aligned α-carbons. Cα-Cα difference distance matrices indicate that the BinAB complex is somewhat more compact at pH 5 than at pH 7 (volume occupied by BinAB in the pH 7 and pH 5 structures: 8.39 x 10^4 and 8.34 x 10^4 $Å^3$). Neither BinA nor BinB demonstrate hinge motions of the trefoil domain towards the PFD, contrary to what was observed in either comparison involving pH 10. Large amplitude conformational changes involve surface loops, and are mostly confined to the trefoil of BinA (Extended Data Fig. 9). Most notable of these, a large conformational change in the BinA trefoil loop Ile110-Arg120 draws it closer to the rest of the structure. The pH 5 conformation of this loop differs from that observed in the pH 10 and pH 7 structures, suggesting that this loop is sensitive to pH. The same can be said of the BinB trefoil loop Lys175-Ser184 which displays three different conformations in the three structures (Extended Data Fig. 9). Consistent with our interpretation of difference distance matrices, the F_o^{pH5}-F_o^{pH7}, φ^{pH7} map reveals that conformational changes are mostly localized to the trefoil domain of BinA. Some peaks are also notable in PFD regions that contact this trefoil domain (Extended Data Figs. 8 and 9).

The map also highlights a number of small conformational changes that occur in the propeptide of BinB, and which for the most correspond to side chain reorientations (Extended Data Figs. 8). Indeed, the pH 5 structure is characterized by multiple side chain reorientations in charged residues at the surface of BinAB. In BinA, noteworthy conformational changes include: Asp5, Asp8, Glu27, Arg49, Asp86, Arg97*, Glu128, Arg132, Glu140 (alternate conformation), Glu162, Lys168, Asp178, Glu184, Lys235, Lys241, Asp254, Arg267*, Glu296, Arg301*, Glu356 and Lys363. Tyr24, Met103, Tyr166 and Met225 also adopt new conformation. BinA residues highlighted by a (*) have been shown to play a role in intoxication; Supplementary Table 1). In BinB, new rotamers are observed for Lys47 (alternate conformation), Arg50, Glu59, Glu65, Glu71, Asp80 (new H-bond (2.6 Å) between OD1 and K57 (NZ)), His123 (alternate conformation), Arg129 (detachment from Arg122), Glu144*, Arg156, Arg162, residues Lys175 and Tyr180 in loop Lys175-Ser184, His245, Lys266 (concerted conformational change with Glu264, Thr370 and K372 in the sheet domain), Lys 284 (concerted conformational change with Glu347, Gln286 and Lys408), Arg318, Glu343 (new H-bond (3.1 Å) between OE2 and Arg288 (NH1)), Asp355 (new H-bond (3.1 Å) from (OD2) to symmetry related BinB Gln187 (NE2)), Lys380 (detachment from Asp302), Gln393* (new H-bond (2.9 Å) between (OE1) and Asn397 (ND2)), Arg413, Lys430 (concerted conformational change with Glu433 results in a new salt bridge between (NZ) and Glu433 OE2), Lys445 and Gln448. BinB residues highlighted by a (*) have been shown to play a role in receptor binding;

Supplementary Table 2). Altogether, these conformational changes increase the buried surface area at crystal contacts, in the pH 5 structure (+101 Å^2). The buried surface area at the dimer interface is impressively conserved (+0.4 Å^2), and its Complex Formation Significance Score (CSS) [66] accordingly remains equal to 1.0. Thus, BinAB crystals could to display increased resilience at pH 5, as compared to pH 7.

Of the four regions highlighted by the F_o^{pH10}-F_o^{pH7}, φ^{pH7} map as highly sensitive to pH elevation, two are also affected by the decrease of pH. In crystal contact zone 5 (Fig. 4e, Extended Data Fig. 6 and Supplementary Table 7), which holds together the trefoil domains of BinA and BinB in crystals, a conformational change occurs in BinB Glu59 upon decrease of pH, resulting in the breaking of H-bonds to BinA His 125 (ND1) and Tyr134 (OH), and in their replacement by two H-bonds to Arg132 (distances between BinB Glu59 OE2 and Bin Arg132 NH1 and NH2: 3.1 and 2.9 Å, respectively) (Fig. 4e and Extended Data Fig. 8c). At the junction between the TM and sandwich subdomains of BinA, a conformational change in Arg301 results in further stabilization of the pH 7 conformation of Asp342. Specifically, the two H-bonds of Asp342 (OD2) to Arg301 (NH2) and (NE) (2.7 and 3.0 Å distance, respectively) are replaced by a salt bridge between Asp342 (OD2) and Arg301 (NH2) (2.1 Å distance). Furthermore, the H-bond to Glu240 (OE1) of Asp342 (OD1) shortens from 2.8 to 2.6 Å. Thus, in the two regions previously identified as sensitive to pH elevation, decrease of pH result in further stabilization of the pH 7 conformation, and not in increase dynamics.

Toxic function of BinAB: Crucial to the toxic function of both BinA and BinB are residues involved in the proper folding of the trefoil (e.g. Cys31, Cys47, Asn52, Gln53, Arg97 and Glu98 in BinA and Tyr32-Glu36, Cys67, Cys161 and Phe147-Tyr150 in BinB) and the PFD (e.g. Leu295-Ile298 in BinA and Tyr387-Gln393 in BinB), and residues in the interface between the trefoil domain and the PFD (Figs. 2 and 3, and Supplementary Tables 1 and 2). Most PFD residues involved in the interface are contributed by a loop – helix motif, corresponding to residues Pro194-Tyr213 and Pro240-Tyr260 in BinA and BinB respectively. Mutations in residues that support the folding of this motif result in decreased (e.g. Cys195 and Cys241 in BinA and BinB, respectively) or abolished (Arg326 in BinA) toxicity, and likewise for the trefoil residues that interact with it (e.g. Ile85 and Phe87 in BinB) or shield it from the bulk (e.g. Leu93 in BinA). Interestingly, the natural mutation of Met197 into an arginine observed in *L. sphaericus* strain 6 results in increased toxicity against *Culex* mosquitos. In light of the structure, the mutation likely effects by destabilizing the interaction between the PFD and the trefoil, through the introduction of a charged residue in the centre of an otherwise hydrophobic cavity, consisting of Ile66, Tyr88, Pro194, Cys195, Ile196, Gly274 and Ile275. In BinB, the residue equivalent to BinA Met197 is Ile243, and the hydrophobic cavity is conserved, lined by Ile95, Tyr126, Pro240, Cys241, Ile242, Glu321 and F322. In both BinA and BinB, strong peaks in

this region in the F_o^{pH10}-F_o^{pH7}, φ^{pH7} map. In the former, four of the residues lining the hydrophobic cavity features strong negative F_o-F_o peaks, *i.e.* Ile66 (-4.2 σ) Tyr88 (-4.7 σ), Cys195 (-3.9 σ), Ile196 (3.9 σ). In BinB, peaks are more numerous, and affect residues in (1) the hydrophobic cavity*, i.e.* Ile95-Ala97 (-4.1, -4.4 and -3.7 σ, respectively), Tyr124-Thr125 (+3.8 and -3.9 σ, respectively), Cys241 (-4.4 σ) and Gly321 (-4.9); (2) the Pro240-Tyr260 loop – helix motif, *i.e.* on His245 (4.1 σ), Asp246 (4.2 σ), Met255 (4.9 σ) and Tyr260 (-3.6 σ); and (3) in the receptor epitope of BinB, *i.e.* residues Ile85 (+4.2 σ), Phe87 (-4.6, +4 and +3.5 σ) and Phe147 (+3.9 σ) which contact the Pro240-Tyr260 loop – helix. The correlation between mutagenesis data (Supplementary Table 1 and 2) and peaks in the F_o^{pH10}-F_o^{pH7}, φ^{pH7} map (Supplementary Tables 11 and 12) supports a central role for the PFD/trefoil interface in triggering the biological activity of BinA and BinB. In both BinA and BinB, unplugging of the PFD loop – helix motif from the barrel subdomain of the trefoil would result in the disruption of PFD/trefoil interface and release of the two loosely associated domains. This could be the mechanism by which the otherwise unexposed receptor epitope of BinB becomes available for its target, at the cell surface. Release of BinA and BinB could also be required for the transformation of their PFD into a oligomeric pore. The increased toxicity displayed by the Met197Arg mutant of BinA supports this hypothesis. The exact role of residues such as BinA's Glu302 and Arg312, whose replacement results in complete loss of toxicity, is more difficult to establish. Given that these two residues are on both sides of BinA's PFD and separated by a 10-residues beta-hairpin loop, it could be that this tandem of charged residues is needed for BinA to exert its toxic function. In support to this hypothesis, the mutation of Arg312 into Lys (pK_a=10) does not preserve activity.

The structure does not explain, however, how the binary toxin converts to a membrane spanning, pore-forming oligomer. Experimental work has established that BinA and BinB remain associated throughout the whole toxic cycle. The observation that a 1058 $Å^2$ contact-surface area is preserved in the BinAB complex after proteolytic activation suggests that the 1:1 ratio between BinA and BinB is maintained in the toxic oligomeric complex. It is unclear, however, if both proteins are involved in the forming of the pore or if only BinA inserts into the membrane. Electrophysiology studies have shown that BinA is more efficient at forming pores than BinB [19,23,49], suggesting that BinA is sufficient to lend pore forming activity to the BinAB toxin. That the latter is at least ten fold more prone to form a pore than BinA suggests that BinB actively participate in the toxic function – either by stabilizing a pore-forming conformation of BinA or by forming a heteromeric pore with BinA.

Alternatively, BinB could act as an inhibitor of the conformational changes that allow BinA to form a pore. From our crystal structure, we know that the propeptides play a large role in the interface between BinA and BinB. We also know that association between BinA and BinB is important for chaperoning BinA into the cell; their large interface tethers BinA to the receptor-bound BinB. Our toxicity data show that dissociation of the C-terminal propeptide of BinB is a key player in triggering the

structural transition from crystal to mature protein (Supplementary Table 13). Cleavage of the BinB propeptide could be an effective trigger for dissociation of the BinA-BinB dimer and re-association into a ring-shaped oligomeric pore. If cleavage of the BinB propeptide were the trigger for the formation of the pore, then it would make sense that it occur after internalization, rather than on the cell surface. Logically, the BinB propeptide would be more functional if it remained intact outside the cell, able to assist with chaperoning BinA into the cell. Then, once inside the cell, cleavage of the BinB propeptide could trigger pore formation. Delaying cleavage until inside the cell is consistent with the idea that pore formation occurs inside the cell, rather than on the outer surface. However, a study from Cark and Baumann, 1990 [65] suggests cleavage occurs in the larval gut (before BinAB internalization). It could thus be that all propeptides (including the BinB propeptide) are cleaved outside the cell but that BinB propeptide remains in the dimer interface (doesn't dissociate from the interface) until the BinAB complex is internalized. After internalization, the BinB propeptide would dissociate, triggering the conformational change that leads to pore formation. The BinB propeptide interface with BinA and BinB is fairly large. It seems plausible that this would be the last propeptide segment of the four to dissociate. In support to this hypothesis, it has been shown that unactivated BinA and BinB associates, both in solution [67] and in the immobilized state [68]. Of note, another report suggests that association is lost upon trypsin activation [69] – meaning that proteolytic cleavage of propeptides would then have to occur in the pinocytotic vesicles of the target cells.