Review

Disparate proteins use similar architectures to damage membranes

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Membrane disruption can efficiently alter cellular function; indeed, pore-forming toxins (PFTs) are well known as important bacterial virulence factors. However, recent data have revealed that structures similar to those found in PFTs are found in membrane active proteins across disparate phyla. Many similarities can be identified only at the 3D-structural level. Of note, domains found in membrane-attack complex proteins of complement and perforin (MACPF) resemble cholesterol-dependent cytolysins from Gram-positive bacteria, and the Bcl family of apoptosis regulators share similar architectures with *Escherichia coli* pore-forming colicins. These and other correlations provide considerable help in understanding the structural requirements for membrane binding and pore formation.

Common roles – common folds

The aim of this review is to highlight the analogous groups of pore-forming and membrane-active proteins, which have been discovered through 3D-structural comparisons [1–9]. Comparative anatomy, which was developed by Edward Tyson (1650–1708), was an important philosophical advance of the 17th century, and similar studies in comparative biology can contribute greatly to the understanding of membrane–protein interactions. We will compare the structure and mode of membrane penetration of pore-forming toxins (PFTs; see Glossary) with similar proteins, which perform a range of functions. PFTs are one of the most-studied and best-understood groups of membrane-binding proteins (Box 1) because they are mostly bacterial in origin, easy to purify and easily assayed for function; moreover, they are implicated in disease. They use various mechanisms to kill target cells by forming holes in the membrane (Figure 1); this process might occur in combination with the translocation of a toxic protein, as in the case of anthrax or diphtheria toxins. PFTs are, classically, divided into two groups according to the secondary structural element of their transmembrane pores. Pores can be formed either by clusters of α-helices (α-PFTs) or by the formation of transmembrane β-barrels (β-PFTs) [10,11]. All PFTs possess the ability to convert from a water-soluble to a membrane-bound state [12]. In recent years, several eukaryotic proteins have been identified that undergo similar changes in localization from the cytosol to the membrane and, in some cases, back to the cytosol. Interestingly, these proteins also employ mechanisms that are used by PFTs for membrane binding and penetration. These similarities have been revealed recently, largely by comparisons of 3D structures. In addition, we will delineate the similarities and dissimilarities in the mechanism of action that is used by diverse PFTs as a way to guide the understanding of protein–lipid interactions (e.g. the fact that pores formed from α-helices might be partly lined by lipids, whereas those composed of β-barrels are not). This review describes several examples of conserved structures that are used in membrane interactions and provides the basis for cross-phyla comparisons of their actions.

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Glossary

Amphipathic: an amphipathic molecule is one that has spatially separated hydrophobic and hydrophilic regions, which determine its interactions with membranes and other related molecules.
Amphitropic protein: a protein that can switch between soluble and membrane-bound states, often in response to a physiological stimulus.
Architecture: this describes the overall shape of the domain structure as determined by the orientations of the secondary structures but ignores the connectivity between the secondary structures.
CATH: a database acronym that stands for class architecture topology homologous family, which are the top main classifications used. An alternative to SCOP.
CDC: cholesterol-dependent cytolysins, which require cholesterol in the target membrane to function because the lipid-binding domain specifically attaches to cholesterol.
Class: in CATH, this term means α, β or α/β SCOP divides into 11 classes.
Colicin: a multi-domain protein toxin that is exclusively produced by and toxic to *E. coli* and related cells. Its toxic domain can be a nuclease, a cell-wall synthesis inhibitor or a pore-forming toxin.
Family: a group of proteins with a clear evolutionary relationship that is based on sequence homology.
Fold: a group of proteins with major structural similarity but which might not have a common evolutionary origin. Such similarities can arise from the physics and chemistry of proteins favouring certain packing arrangements and chain topologies.
PA: protective antigen from *Bacillus anthracis*, the pore-forming component of anthrax toxin.
Peripheral membrane protein: a protein that, although bound to a membrane, does not insert deeply into the hydrophobic core nor traverse the bilayer. In the laboratory, this can often be extracted with salt solution alone without the need for detergents.
Pfam: a large collection of multiple sequence alignments and hidden Markov models that covers many common protein domains and families.
Planar lipid bilayers: freestanding model bilayers that separate two aqueous compartments across which electrical potentials are applied to measure ion currents through membrane pores.
Pore-forming toxin: a protein that relies upon pore formation in lipid membranes for its biological activity.
SCOP: a structural classification of proteins database, which classifies protein structures into 'class', 'fold', 'superfamily' and 'family'. An alternative to CATH.
Superfamily: a family of diverse proteins with a probable common evolutionary origin.
Topology: a description of the connectivity of secondary-structure elements in proteins; in some uses, when 3D information is included, topology is synonymous with fold

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Box 1. How peripheral proteins attach to lipid membranes

Peripheral or amphitropic proteins are water-soluble proteins that are reversibly associated with cellular membranes under certain physiological conditions. This group of proteins includes enzymes, transporters, small domains of signalling proteins, polypeptide toxins, antibacterial peptides, etc. The peripheral proteins attach to the lipid membranes through various mechanisms (Figure I). The amino acids that participate in protein–lipid interactions are arranged in various structural contexts (i.e. amphipathic helices, hydrophobic hairpins or long surface-exposed loops). Peripheral proteins can be classified according to the depth of their membrane insertion and the nature of their protein–lipid interactions; S-type (those localized at the membrane surface), I-type (those that penetrate to the water–lipid interface region of the lipid membrane) and H-type (those that penetrate to the hydrocarbon core region of the membrane) [21]. Various methodological approaches are used to determine the nature of interactions, depth of the membrane insertion or thermodynamics aspects of protein–membrane binding. These include fluorescence spectroscopy, electron paramagnetic resonance, reflectivity analysis and surface plasmon resonance [42,70–72]. Many membrane-binding domains show structural similarity at extremely low sequence conservation and, therefore, traditional sequence-based searches do not work well in identifying novel peripheral proteins from genomic data; hence, only few such studies exist [73]. A recent large-scale computational approach accurately predicted the position of many peripheral proteins within the lipid membrane. In particular, this approach indicated that most peripheral proteins penetrate through the interfacial region and reach the membrane hydrocarbon core, which is consistent with published data [74]. Readers can also consult the Orientation of Proteins in Membranes (OPM) database (http://opm.phar.umich.edu/) and the Membrane Targeting Domains Resource (MeTaDor) (http://proteomics.bioengr.uic.edu/metador/) databases for further information that is specific to peripheral proteins.

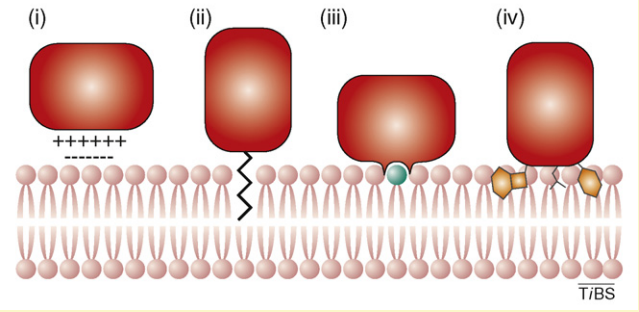

Figure I. Peripheral membrane proteins. Proteins attach to membranes by various mechanisms including electrostatic attraction of positively charged patches on protein surfaces to negatively charged lipid membranes (e.g. colicin) (i) or covalent protein modifications with fatty acid acyl chains (ii) (e.g. *E. coli* α haemolysin and eukaryotic transducin, GPI and Ras proteins). A particular membrane lipid can be specifically recognized, thereby promoting binding (e.g. actinoporins and CDC) (iii). Non-specific hydrophobic interactions between surface-exposed hydrophobic or aromatic amino acid residues with the interfacial and hydrocarbon regions of the membrane lipids also occur (e.g. melittin, actinoporins, CDC) (iv).

Simple rules for membrane-binding peptides

Several databases, including CATH (class architecture topology homologous family) and SCOP (structural classification of proteins), define domains by structural similarity and not by sequence homology. The most recent CATH release (3.1.0) defines 305 pure α, 191 pure β and 496 α/β topologies or folds (Box 2). SCOP lists 1086 folds in seven types including α (259), β (165) and α/β (475), plus 50 membrane or cell-surface proteins. Thus, structural information is necessary both to define domains and to define interdomain similarities. It follows that searches using sequence information alone can rarely predict these similarities. Among the membrane-binding domains that can be predicted from sequence information alone are structures that result from general physico-chemical responses to the membrane surface environment. The simplest

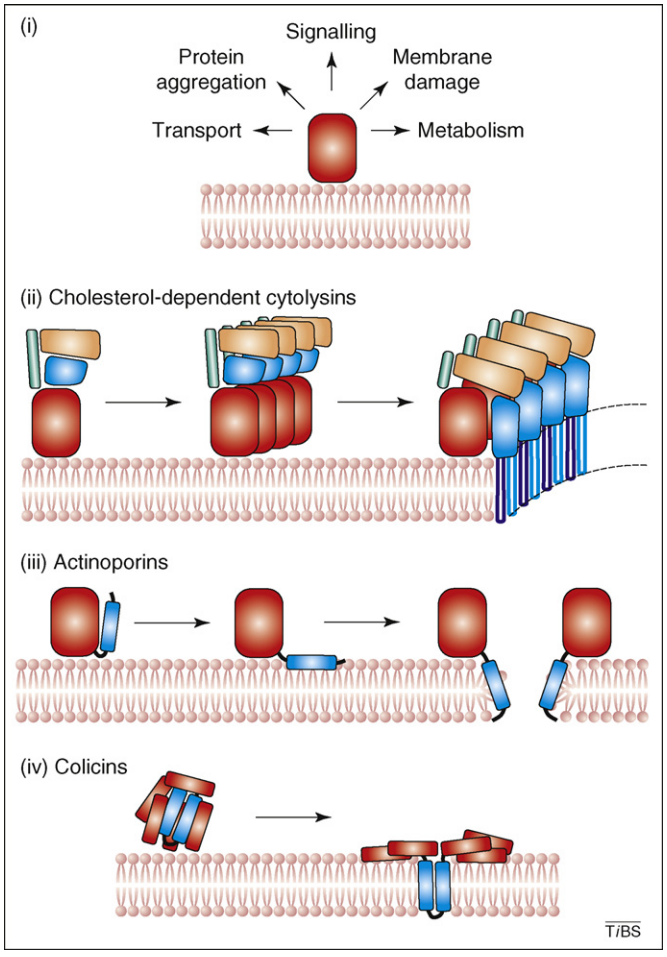

Figure 1. Mechanisms used by PFTs to disrupt cell membrane integrity. Membrane damage is only one of the biological functions that are elicited by the binding of a protein to the lipid membrane (i). Other described functions include signalling, metabolism of membrane components, the transport of compounds between different types of cell membranes and the aggregation of proteins induced by a binding event. PFTs comprise a diverse group of proteins that are usually composed of multiple domains. There are many ways to create a transmembrane pore, as highlighted here for three PFT families by using a colour scheme that is common to all figures in this article. Lipid membrane binding (membrane lipids are presented in pink) is achieved via a defined domain (red). This event can be followed by aggregation in the plane of the membrane before the insertion of part of the polypeptide chain (blue) across the lipid bilayer to form the final pore. The ordered arrangement of the pore for cholesterol-dependent cytolysins (ii) (and the heptameric barrels shown in Figure 2) is ensured by the need for lateral hydrogen bonding between the peptide bonds. Spanning a membrane with β-strands is more efficient than with helices, and only seven to nine residues are needed in each strand of the hairpin. β-strands also only need to be hydrophobic on one side, which means that because only 50% of the residues are hydrophobic, the transmembrane region can feature alternate runs of as few as four hydrophobic amino acids. Such a structure can be easily formed from small regions of the soluble protein, and the transmembrane regions are often insignificant in the structure of the soluble monomer. Helical proteins generally insert preformed helices into the membrane. These can comprise just a short region, as in the actinoporins (iii). Alternatively, the proteins are entirely helical, as in the pore-forming colicins (iv). Helices contain more residues and are more hydrophobic than β-strands. The lack of a fixed hydrogen-bonding network between helices means that they can be dynamic and their structures can be more difficult to define. Furthermore, it has been suggested for actinoporins [63], pore-forming colicins [64] and Bcl-family members (e.g. Bax) [50] that helices might adopt a tilted orientation in the bilayer [65] with lipids rearranged to form a toroidal pore (iii).

Box 2. Domain classification

Domains are best defined as independent folding units; however, there are many ways to analyze protein molecular taxonomy to recognize the regions that have similar folds in other proteins. The most common approach is to use the large amount of available sequence information to discover domains that share sequence homology; this approach forms the basis for Pfam (http://pfam.sanger.ac.uk/) and related approaches [75]. The structure-based SCOP database (http://scop.mrc-lmb.cam.ac.uk/scop/) uses the following generally accepted terms in order of increasing relatedness: 'fold', 'superfamily' and 'family'. In the CATH database (http://www.cathdb.info/), the term 'fold' is replaced by 'topology' and then divided into homologous superfamilies. Two toxins, anthrax protective antigen (PA) and the pore-forming colicin Ia, represent how folds or domains are assembled in toxins (as defined by the original authors for PA and according to CATH for Ia because some databases differ) [76,77] (Figure I). The receptor-binding domain 4 of PA has an immunoglobulin-type fold that is found almost universally in nature. It is, thus, a widespread, highly modified fold with many functions and little evidence remaining of any common evolutionary origins. Owing to its inherent stability and simplicity, this fold might have arisen independently multiple times. Such disparate structures reveal fundamental functional rules especially when, in spite of their unrelated sequences, the proteins perform similar functions. In particular, we have discussed the CDC or pore-forming colicins, which form homologous families. Yet it is the links between these families and unrelated folds in other membrane-binding domains (e.g. the MACPF domain and Bcl-xl, respectively) that provide the important structure function information that is unaffected by residual sequence homology. This information helps to define the essence of what enables each domain to function. It should also be remembered that multiple domains in single proteins might not act independently. Indeed, this is a crucial consideration and is covered in detail elsewhere [78].

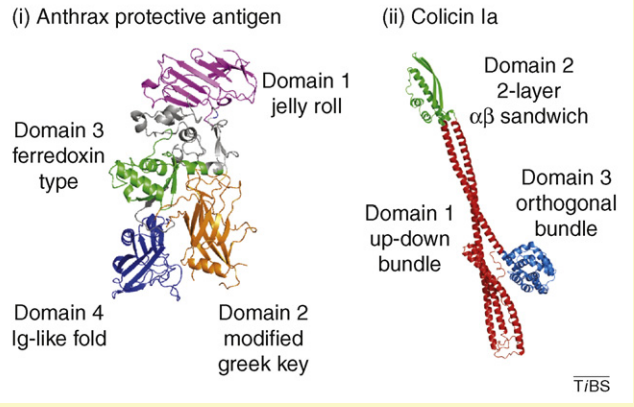

Figure I. Multidomain structure of PFTs. Anthrax protective antigen (PDB: 1acc) [77] (i),and colicin Ia (PDB:1cii) [76] (ii) are shown.

domains are the lytic peptides [13], which include bee-venom toxins such as melittin (a 26-residue amphipathic peptide), or antimicrobial peptides such as the magainins, cecropins, dermaseptin and delta-lysin. These peptides trigger the lysis of target cells via lipid bilayer permeabilization and act directly upon the membrane lipids via amphipathic helices (with a polar and a non-polar face), which are often recognizable from sequence analysis. The polar face usually contains lysine residues, and these cationic properties are prevalent because many biological membranes carry a net negative charge. The combination of these properties causes an initial surface localisation, possibly coupled with folding and helix formation, with the hydrophobic side of the helix contacting the non-polar core. Insertion into the membrane can follow, in response to a voltage gradient and/or peptide aggregation. Various models have been proposed to explain their lytic action, including their ability to act as detergents [13]. Although lytic peptides can lyse phosphatidylcholine containing (zwitterionic) membranes [14], they are often more active on those containing negatively charged lipids (e.g. phosphatidylglycerol or phosphatidylserine). Phosphatidylethanolamine, which normally forms a hexagonal phase, can be induced to form bilayers by peptide insertion [13]. The other general feature of such peptides is the presence of aromatic, and particularly tryptophan, residues: these residues can enhance the interfacial solubility of peptides [15] and are found in many lytic peptides. The replacement of three central tryptophan residues, by tyrosine or phenylalanine, in tritrpticin reduces the membrane interaction with zwitterionic membranes [16], whereas the removal of the single tryptophan in melittin abolishes its haemolytic activity [17]. Remarkably, even an anticoccidial peptide selected by phage display methods contains a commonly found Trp–Trp–Arg motif in addition to an amphipathic character [18]. Thus, these peptides represent protein structures that can differentially interact with a range of membrane phospholipids at the most fundamental physicochemical level by adhering to a few simple constraints that are detectable by primary structure analysis.

Finding membrane-binding domains among the crowd

The simple rules that apply to peptides do not enable us to identify which of the many structures with complex folds and topologies also bind membranes. For example, when the protein structure of the colicin pore-forming domain was first revealed by X-ray crystallography [19], the similarity with the well-known globin topology was made clear [20]. This topology is a subset of the orthogonal helical bundles (216 topologies and 393 homologous families) in which two layers of helices sit almost at 90 degrees to each other. In the CATH classification, the 'globin-like' topology contains five families: globins, phycocyanins, TipA-like, diphtheria toxin and pore-forming colicins. Of these, only the last two form ion channels in membranes. Furthermore, elsewhere within the orthogonal bundle architecture group can be found the Bcl-X family (named for B-cell CLL/lymphoma), plus the uteroglobins, amoebapores, granulysins and NK-lysins. These proteins all have membrane-binding functions that are more or less similar to those of pore-forming colicins. Thus, one architecture (e.g. orthogonal bundle) with mostly non-membrane-binding members might nevertheless contain several types of membrane-binding domains with different topologies.

The β-sandwich architecture is represented in CATH by 34 different topologies, of which two have members that are used in membrane binding. Domain 4 of anthrax protective antigen and cholesterol-dependent cytolysins (CDCs), and the C2 domains used in cell signalling [21] are immunoglobulin-like domains (Box 2), whereas the outwardly similar membrane-binding region of actinoporins [22] has a different β-sandwich topology that is similar to fungal lectins [2]. These functionally related architectures share no clear homology and probably are the result of convergent evolution.

In addition to revealing why certain domains are favoured in membrane interactions, understanding ways in which the various domains are recombined provides yet another route to dissect the membrane disruption process. For example, both CDCs and actinoporins attach by a β-sandwich, but whereas CDC pore formation occurs via a domain that is similar to membrane-attack complex proteins of complement and perforin (MACPF) [7,8,23], actinoporin pore formation employs an amphipathic α-helix [24–26]. *Vibrio cholerae* membrane attachment is further aided by a lectin domain, but pore formation occurs in a manner similar to that of *Staphylococcus aureus* α-toxin [5].

Similarities in membrane binding

The initial step in the formation of transmembrane pores is binding to the lipid membrane. In particular, the β-sandwich seems to be one of the most prevalent domains used for this purpose: it is found in many peripheral proteins and forms some of the most common membrane-targeting domains, such as C2 or PH (Pleckstrin homology) domains [21]. Membrane attachment is usually shallow and is mediated by exposed amino acids from one side of the domain (Box 1). These residues might participate in the specific recognition of membrane lipids; for example, the eukaryotic lipids, cholesterol or sphingomyelin, are often used by bacterial PFTs to target eukaryotic cells. The fourth domain of CDC is composed of a β-sandwich with long protruding loops at one side [27] (Figures 1 and 2), which enable the specific recognition of membrane cholesterol, although the mechanism for recognition remains unclear [28]. Yet, in a manner similar to the action of lytic peptides, shallow binding is enabled by exposed tryptophans, which cluster in this domain. Fluorescence spectroscopy and cryo-electron microscopy experiments indicate that this fourth domain does not insert deeply in the lipid bilayer during any pore-formation step [29,30]. This mode of membrane targeting is also found in other families of PFTs; for example, actinoporins show some structural resemblance to the fourth domain of CDC [22] and use residues from long protruding loops at the bottom of the β-sandwich to specifically recognize sphingomyelin in the lipid membranes [26]. Staphylococcal α-toxin also uses its rim domain, which contains exposed aromatic residues to bind phospholipid headgroups [31]. The subsequent steps of pore formation in these families are, however, completely different. Whereas α-toxin forms defined heptameric pores [32], CDCs form huge β-barrels [23], and actinoporins use their amphipathic N-terminal helix for pore formation [24,25]. This helix shows some sequence resemblance to melittin; hence, actinoporins could be regarded as molecular chimeras that use a β-sandwich fold for the specific attachment to the membrane and an amphipathic helix for pore formation (Figures 1 and 3). Notably, the peptides that correspond to the actinoporin N-terminal region do not exhibit the same haemolytic or permeabilizing activity as the intact molecule; they also lack the selectivity for sphingomyelin-containing membranes [33,34]. The actinoporin β-sandwich is, thus, crucial, not only for the lipid specificity and membrane targeting but also in the formation of the final transmembrane pore, where it probably helps to stabilize slightly tilted helices and lipids in a toroidal pore arrangement [35].

β-sandwiches are common robust modules, and similarities between the various protein families can be revealed only at the structural level. A striking example is the structural similarity between actinoporins and a novel family of fungal lectins that exhibit antiproliferative activity on a variety of epithelial cells [2]. Although the β-sandwich structure is extremely similar for both groups, the sequence identity is below 15% (Figure 3); moreover, fungal lectins bind sugars rather than sphingomyelin. Their primary ligand is the Galβ1–3GalNAc disaccharide (Thomsen-Friedenreich antigen) that is present on glycoproteins of malignant cells [36,37]. The residues used for binding are located at equivalent sites to the residues that actinoporins use to bind the sphingomyelin phosphocholine headgroup [26,35] (Figure 3). Strikingly, the fungal lectins lack the N-terminal amphipathic helix that is present in actinoporins. Thus, the archetypical actinoporin β-sandwich fold is not simply a lipid-recognition motif; it is also used to specifically bind cell-surface ligands [38]. Similarly, domain 4 of anthrax protective antigen (Box 1) retains many features of a lipid-binding β-sandwich but now binds a specific cell-surface receptor protein [39].

Helical bundles mediate membrane interactions

The colicin A pore-forming domain structure revealed the fold of all pore-forming colicin C-terminal domains [19] (Figure 4). The mechanism of insertion into the membrane, which is indicated by the presence of the hydrophobic helical hairpin, has been largely validated by experimental approaches [40–42]. Because hydrophobic helices are independent folding units, which are capable of insertion into the membrane in a dynamic manner, their exact structural resolution has been difficult to achieve. The central helical hairpin can easily penetrate the bilayer, but it has no charged tip to maintain a transbilayer conformation (Figure 1). Because it is shorter than transmembrane helices in intrinsic membrane proteins, the actual conformation of the hairpin might not be fixed. Furthermore, high-resolution structures of colicin-mediated pores have not been solved because pore formation requires a membrane potential. The topology has been mapped in planar lipid bilayers by using biotin labels that get trapped by streptavidin upon crossing the membrane. This method has revealed a flexible structure that can readily adapt to insertions and deletions [43]. Thus, colicins both bound to the surface and in the pore form are dynamic structures that are made possible by the ability of helices to move independently. The related diphtheria toxin and δ-endotoxin domains were solved soon after the colicin domain, and their similarity in burying the hydrophobic helices has been discussed previously [12].

The Bcl-xl protein, an apoptosis inhibitor, is surprisingly similar to pore-forming colicins [1] (Figure 4). Although the physiological relevance of its pore-forming activity remains unclear, Bcl-xl undergoes colicin-A-like structural rearrangements [40,41,44–46] (Figure 1). Colicins are delivered to the membrane by their translocation mechanism and might bind to the membrane electrostatically (Box 1). Bcl-xl

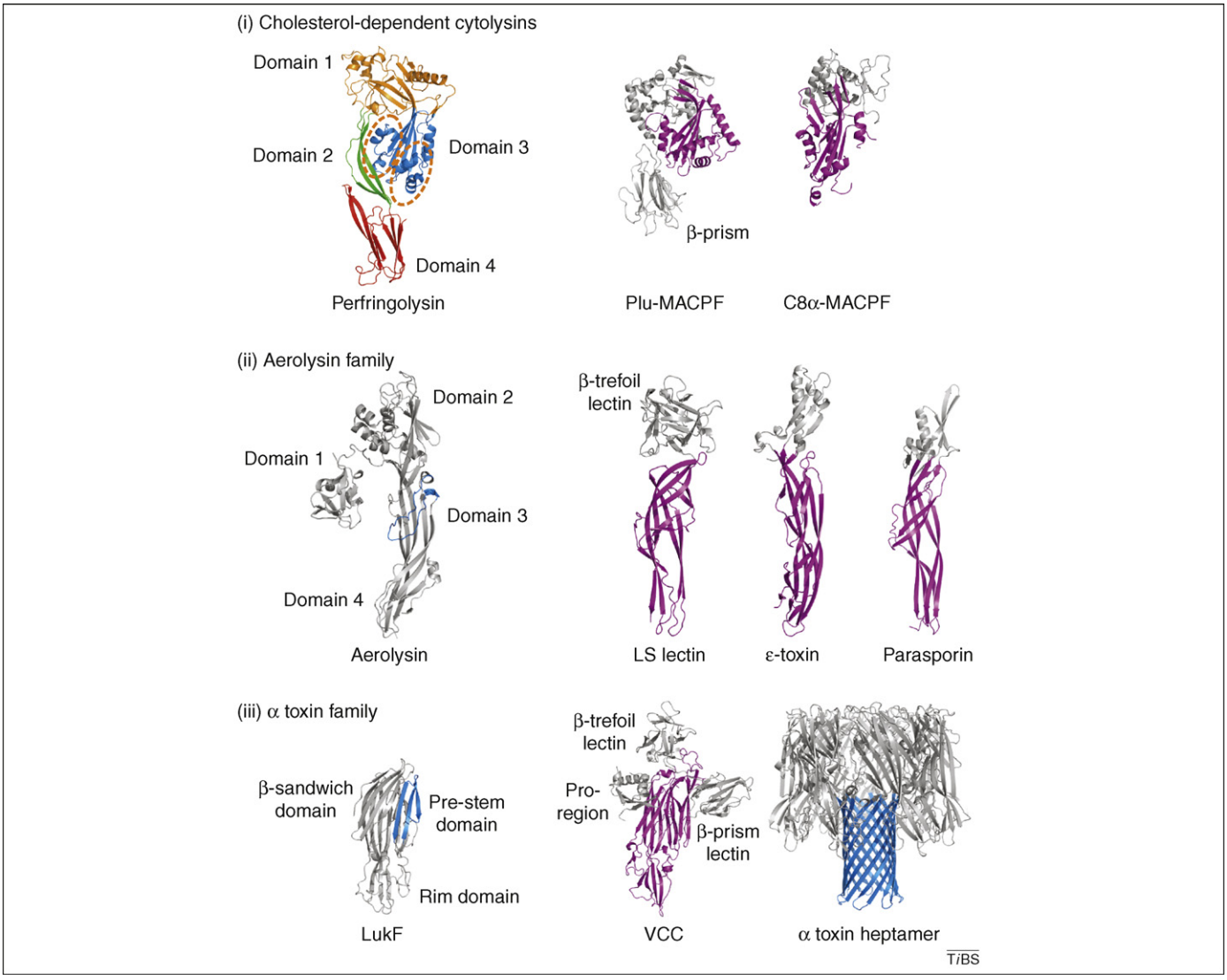

Figure 2. Examples of β-PFTs and structurally similar proteins. Three families of β-PFTs are shown for which the part of the polypeptide chain that forms the walls of the final pore was identified experimentally (shown in blue). The conserved parts in other proteins, which were identified only after the structures were solved, are shown in purple. In all cases, the portion of the polypeptide chain that contributes to the final β-barrel is shown. Other domains found in similar proteins are annotated as in the original publications quoted in this legend. Cholesterol-dependent cytolysins (PDB: 1pfo) [27] (i) domains are labelled as elsewhere in the article. They contain two clusters of helices in domain 3 that participate in the formation of the final transmembrane β-barrel (encircled) [23]. Similar organization of a kinked β-sheet decorated with helices was recently, and surprisingly, revealed in two proteins that possess MACPF domains: *Photorhabdus luminescens* Plu-MACPF (PDB: 2qp2) [8] and human complement component C8α (C8α-MACPF; PDB 2qqh) [7]. C8α domain structure was also determined in a complex with C8γ [9]. The aerolysin fold (ii) (PDB: 1pre) [66] was identified in other cytolytic proteins – ε-toxin (PDB: 1uyj) [3] and haemolytic lectin from the fungus *Laetiporus sulphurous* (LS lectin) (PDB: 1w3f) [4] – and also in non-toxic proteins, such as parasporin, a non-toxic spore protein from *Bacillus thuringiensis* (PDB: 2d42) [6]. *Vibrio cholerae* cytolysin (VCC) (PDB: 1xez) possess an α-toxin cytolysin core that is decorated with two lectin domains [5] but possess a central domain similar to members of the Staphylococcal α-toxin family (iii). The structure of a monomeric LukF (PDB: 1lkf) [31] is shown for comparison, as is a structure of oligomeric α-toxin (PDB: 7ahl), to highlight the organization of β strands in the final pore [67]. All structural images were prepared using PyMol (http://www.pymol.org).

and its relatives generally have a helical hydrophobic C-terminal extension, which is missing in colicins, that performs membrane binding and is different from the use of lipid-binding loops by β-sandwich proteins. In addition, some members of the Bcl family show even greater differences from pore-forming colicins (Figure 4). Recently, a viral protein, which inhibits the protective response of apoptosis during cell infection, was shown to have a Bcl-2-like fold with no sequence homology [47]. Although membrane binding is one aspect of the activity of this family, they also bind a variety of ligands, so the retention of the fold might have been ensured by these functions. Bax (Bcl2-associated X protein), a proapoptotic family member, can oligomerize in the membrane and form large pores [48–50], which is a feature that is not observed in colicins (all measurements of colicin activity indicate a single protein per pore). The small pores that are formed by colicins might consist of three or four transmembrane helices, whereas oligomeric Bax might use just one helix from the hydrophobic hairpin pair to form the pore [51,52]. Bid (BH3-interacting domain death agonist) and Bad (Bcl2-antagonist of cell death) lack the colicin fold motif entirely and contain only a single Bax homology domain, BH3 [53] (Figure 4). Both pore-forming and Bcl families dimerize, although the functional formation of homo- and heterodimers is only implicated in Bcl-family activity [54,55]. The extended group of colicin-like helical

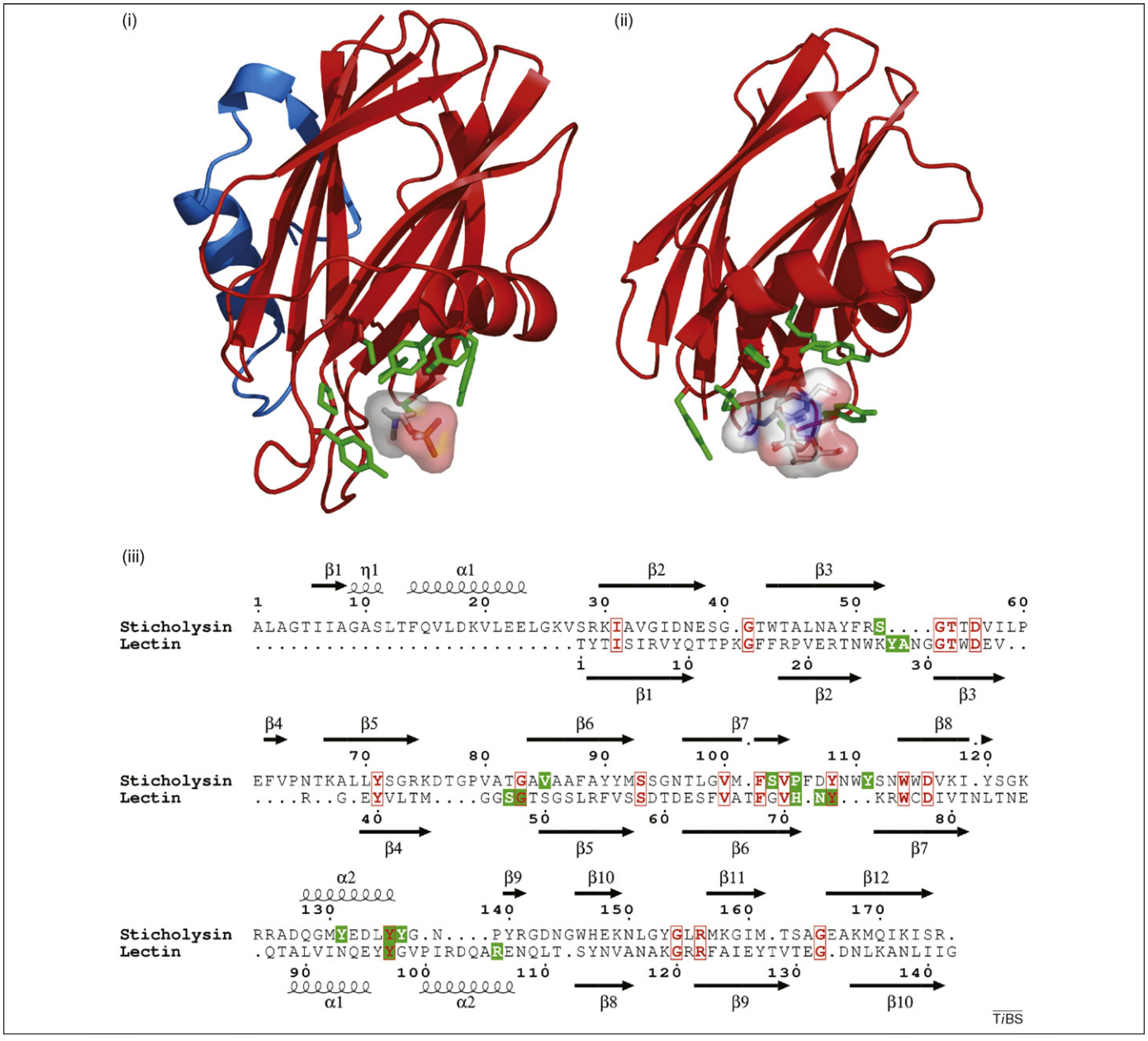

Figure 3. The β-sandwich fold directs the binding of diverse molecules. A structural comparison between actinoporin sticholysin II (PDB: 1o72) (i) and a fungal lectin from *Agaricus bisporus* (PDB:1y2v) (ii) is shown. The β-sandwich is shown in red, the amino acids that participate in binding (actinoporins bind phosphocholine and the fungal lectin binds the disaccharide Galβ1–3GalNAc) are shown as sticks in green. Both ligands are shown with sticks and surface representation. The N-terminal region that participates in the formation of the actinoporin transmembrane pore is shown in blue [25,35]. An alignment based on the structural elements is also shown (iii) to highlight that amino acids (shown in green) on the equivalent sites enable ligand binding. Amino acids that are shared between both proteins are red and boxed. The secondary structures of sticholysin II and lectin are shown above and below the alignment, respectively. α-helices are shown as coils, and β-strands are shown as arrows. The alignment was prepared using ESPript 2.2 [68].

membrane-binding proteins also includes the uteroglobins, which are homodimeric four-helix proteins with similarities to colicins. Their functions are complex and include lipid binding and phospholipase A_2 (PLA_2) inhibition [56]. The eukaryotic pore-forming proteins ameobapore, granulysin and NK-lysin families have a helical bundle fold that is similar to the pore-forming colicins, but lacks the buried helix. As members of a larger family of helical-bundle lipid-binding proteins that use their hydrophobic cavities in a wide variety of cellular functions [57], these proteins participate in host defence and pathogenesis, and possibly form hexameric pores.

Forming β-structures in membranes

β-PFT-mediated membrane penetration involves only a small proportion of the protein; the remainder of the membrane-binding fold, and any associated domains, remain outside of the lipid membrane hydrophobic core (Figure 2). The free energy of transfer for a non-hydrogen bonded peptide bond into this core is about the same as that for burying a charged side-chain [15], so the β-strands in the membrane must form continuously hydrogen-bonded structures. Thus, a single β-hairpin cannot be used, and it seems that six hairpins might be the minimum requirement (although in those proteins with defined

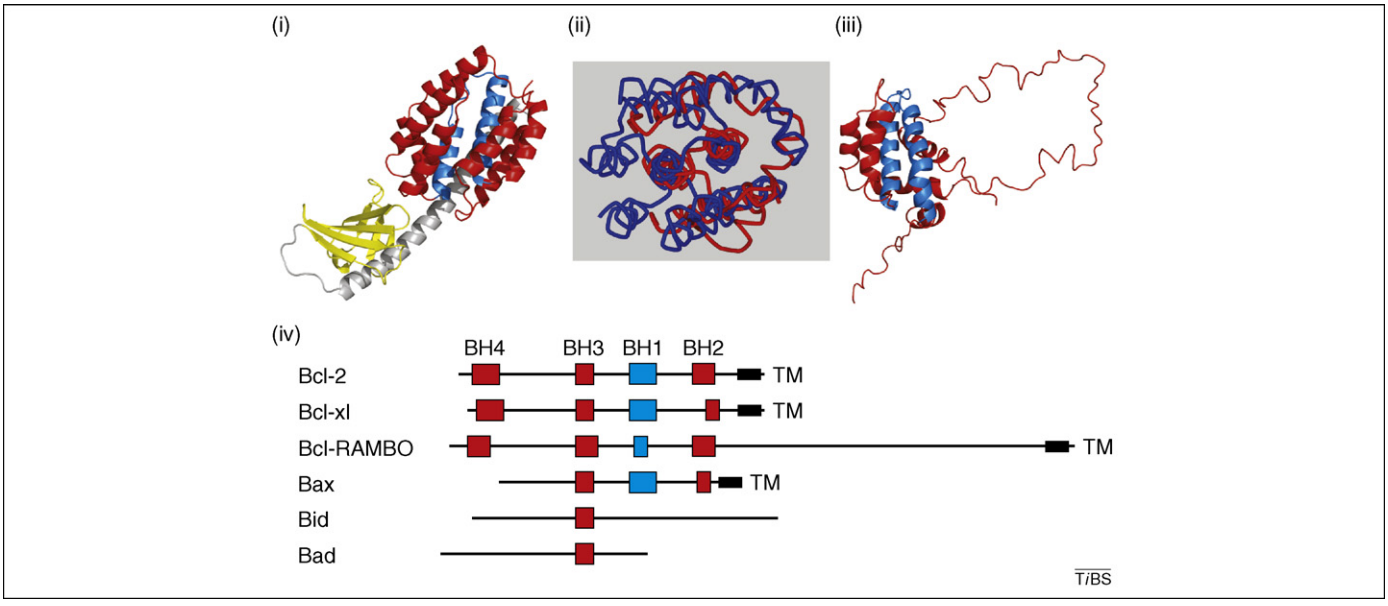

Figure 4. The varieties of colicin fold. Colicins usually contain three domains. On the left is shown the structure of colicin N (i) (PDB: 1a87) [69]: the helical hairpin is shown in blue, and the remainder of the pore-forming domain is shown in red. The yellow region is the receptor-binding domain, which interacts with the *E. coli* outer membrane protein F (OmpF). The third, N-terminal, domain is not visible in the X-ray structure because it is natively unfolded. Other colicins contain a variety of domains, but the toxic domain is always C-terminal (Box 2), and the N-terminal domain is usually unfolded [41]. The central image (ii) shows a superposition of the pore-forming domain of colicin A (blue; PDB: 1col) [19] and Bcl-xl (red; PDB: 1maz) [1]; the central hydrophobic helical hairpin is shown end-on. The second helix of the hairpin is more water-exposed in Bcl-xl than in colicin A, but the overall similarity is clear [40]. The solution structure of Bcl-xl (iii) (PDB: 1lxl) [1] reveals additions to the core fold, and a large flexible loop is visible within the structure. The Bcl family is defined by Bax homology (BH) regions; these are shown boxed in the sequence alignment schematic (iv). The blue box corresponds to the BH1 domain that corresponds to the first helix of the hydrophobic hairpin. To the left, the first red box is BH3, a surface helix, which is the heterodimerization site for BH3-only proteins (e.g. Bid). The C-terminal transmembrane anchor (TM) is also found in some BH3-only proteins, so, although they lack a colicin-like BH1 domain, they can still bind membranes. Thus, whereas colicins add N-terminal sections to a largely invariant pore domain, the Bcl family show greater diversity in the core structure. Indeed, Bcl-RAMBO contains a 250-residue insertion, the largest known [53].

stoichiometry, the overwhelming majority have seven hairpins from seven monomers and 14 β-strands) [32]. In *Staphylococcus aureus* α-toxin, binding by the rim domain is followed by strand formation from existing β-strands in the pre-pore structure [32] (Figure 2). The anthrax toxin protective antigen (PA83) contains a loop in domain 2 that refolds to form the β-strands [39], whereas in the aerolysin family, the transmembrane β-hairpin originates from a thirty-residue-long loop from domain 3 [58,59] (Figure 2). Here, it is noteworthy to mention that aerolysin domains 3 and 4 are apparent only in the 3D structure. The same β-strands criss-cross between the two domains so that, unlike the other examples, the domains cannot be delineated on the primary structure. This renders the classification of domain 4 difficult using CATH methods but additionally makes any homology to other families of β-PFT particularly unlikely. In fact, the similarity among aerolysin-like membrane-binding proteins was revealed only at the structural level [3,4,6]. Interestingly, the aerolysin-membrane interaction is mediated by domains 1 and 2, which bind a glycosylphosphatidylinositol (GPI)-anchored receptor protein [60]. The CDC transmembrane β-barrel consists of large numbers (>40) of monomers, each inserting a pair of β-hairpins [23,30]. With >160 strands, these are the largest transmembrane β-barrels known (Figure 1). Each strand is formed partly from existing strands and partly from short helices that connect them in domain 3. This process occurs during extensive conformational rearrangement of the aggregated membrane-bound monomer (Figure 2, encircled). Surprisingly, recently determined structures of MACPF domains from bacteria (*Photorhabdus luminescens*) and human complement components revealed a similar organization [61]. A clear similarity was observed between the folds of the MACPF domain and domain 3 of CDC [7–9] (Figure 2). MACPF domains were initially recognized in complement and perforin from the immune system but were later found in other proteins. At least one of the characterized MACPF proteins is haemolytic and is thought to form transmembrane pores [62]. For years, the understanding of membrane pore formation by the complement membrane-attack complex or perforin was hampered by the lack of structural data. Therefore, this unexpected finding implies that pore formation by MACPF proteins involves β-barrel formation by the same well-studied mechanism as in CDC.

The common use of a β-sandwich-binding domain followed by the insertion of newly folded β-strands could indicate that the small and large β-barrels share common origins. However, they form distinct groups and no intermediate forms have been identified at the structural level. The strands penetrate the membrane and have a short turn on the far side, which does not emerge from the surface and does not seem to have any function in the cytoplasm of the targeted cells. So far the only described function for the turn, as described for aerolysin, is the anchoring of the β-barrel in the membrane in a rivet-like fashion [59]. Thus, a structurally well-defined barrel, which obeys physico-chemical rules, is a common feature for all β-PFTs. It is important to note that although *de novo* β-hairpin formation during membrane insertion is ubiquitous, the process is highly variable between different families (Figure 2).

Concluding remarks

Despite the large number of folds that are known in protein structure, it is curious that many known membrane-binding domains across biology are either β-sandwiches or helical bundles. This finding indicates that these structures are consistently effective in membrane-binding, regardless of their cellular environment. The extensive data available from the PFT field can be used to direct research on newly discovered or less-well-known intracellular proteins. β-sandwiches provide a rigid scaffold that enables tryptophan-rich loops to insert and bind to membrane interfaces and can provide a well-defined lipid-binding site. As a result, the β sandwich is the only fold to show specific lipid binding as a precursor to pore formation. Pore formation occurs via β-strand insertion, except in the case of actinoporins, which insert an amphipathic helix. Because actinoporins are related to a lectin that binds glycosylated proteins or lipids, they might represent another origin for β-sandwiches in membrane attachment and, thus, have acquired a different form of pore.

The pore-forming colicins and Bcl proteins are the most complex members of a group that could have its origins in the simple pore-forming lytic peptides, which form pores by self-association in the membrane plane. The dynamic and flexible properties of helical proteins could indicate that the options for pore formation and membrane binding are also more flexible. Nevertheless, a defining step occurs when buried hydrophobic helices are added, thus providing a level of stability and membrane binding that is impossible with amphipathic sequences. With surprising similarities still being reported from new structures, it is clear that the trend of discovering shared structural designs in membrane binding is likely to continue. It is, therefore, important to place each new structure within the wider biological context because this can save experimental effort and indicate modes of action that are not apparent from the known literature for the protein in question.

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