STRUCTURAL SYMMETRY IN MEMBRANE PROTEINS

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Abstract

Symmetry is a common feature among natural systems, including protein structures. A strong propensity towards symmetric architectures has long been recognized for water-soluble proteins, and rationalized from an evolutionary standpoint. Proteins residing in cellular membranes, however, have traditionally been less amenable to structural studies, and thus the prevalence and significance of symmetry in this important class of molecules is not as well understood. In the past two decades great strides have been made in this area, providing exciting insights into the range of architectures adopted by membrane proteins. These structural studies have revealed a similarly strong bias toward symmetric arrangements, often unexpected, despite the restrictions imposed by the membrane environment on the possible symmetry groups. Moreover, membrane proteins disproportionately contain internal structural repeats resulting from duplication and fusion of smaller segments. Here, the types and origins of symmetry in membrane proteins are discussed, along with the implications for their function.

Keywords

oligomer; internal repeats; inverted-topology repeats; asymmetry; alternating access

REFERENCE ANNOTATIONS

1. Vinothkumar et al: A systematic and thorough review of structures and functions of membrane proteins up to 2009.

2. Myers-Turnbull et al: Recent assessment of internal pseudo-symmetry indicating that membrane proteins are enriched in symmetric structural repeats.

3. Goodsell & Olson: An excellent and comprehensive discussion of symmetry in all classes of proteins.

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6. Dutzler et al 2002: The first structure of a membrane protein containing inverted-topology repeats.

7. Baradaran et al 2013: The first structure of a face-to-back inverted-topology repeat.

8. Bowie, 2013: Insightful overview of membrane protein insertion and folding, particularly important for dual-topology proteins.

1 INTRODUCTION

Symmetry, defined as the property of having the same appearance from two or more vantage points, is an aesthetically appealing and common feature of natural systems. In the structure of macromolecules, and in particular, of proteins, a multitude of symmetries and pseudo-symmetries have been identified, and these appear to have a range of functional advantages (37). As we reach the milestone of 500 unique structures of membrane proteins (38, 109), it seems timely to review the prevalence and mechanistic significance of symmetry in this special class of proteins.

After briefly introducing the major functional classes of integral membrane proteins, I discuss the emergence of symmetry in their structures as a result of gene duplication or oligomerization. I then describe the specific types of symmetry observed thus far, and their mechanistic implications. This discussion is focused on membrane proteins whose chains span the entire lipid bilayer one or more times (i.e. not monotopic, membrane-associated proteins (11)). I conclude with open questions and exciting future directions for the field.

2 FUNCTIONS OF MEMBRANE PROTEINS

Around 25–35% of the genes in a genome encode for integral membrane proteins (55, 82). These proteins serve a wide variety of functions, and can be grouped into four types: receptors, channels and transporters, enzymes, and co-factor scaffolds.

2.1 Receptors

Lipid bilayers serve as hydrophobic barriers that protect the interior of cells and organelles, but also impede numerous essential processes. So-called receptor proteins facilitate the transmission of information across membranes. In response to light or to chemical signals from the cell exterior, these proteins adopt a different state or conformation and thereby modulate their ability to interact with other proteins in the interior. The family of seven-transmembrane (TM) helix G-protein coupled receptors (GPCRs) is the most prominent example, and constitutes the largest functional class in eukarya (2). Receptor tyrosine kinases (RTK) comprise another large and important family of membrane proteins in this class (57).

2.2 Channels and transporters

The second most abundant membrane proteins, accounting for 2–15% of the genes in a genome (2, 4), are those that facilitate selective passage of chemicals across the lipid membrane.

In the simplest case, proteins called channels create pores through which ions and other molecules diffuse passively, i.e., along their concentration gradients. To regulate this process, many channels incorporate 'gating' mechanisms that respond to environmental stimuli, such as voltage.

Cells also need to expel toxic compounds, and to take up rare nutrients, which typically requires movement against a concentration gradient. So-called primary transporters derive

the energy for such processes from ATP hydrolysis or light conversion. ATP hydrolysis is catalyzed by domains residing outside the membrane that are tightly coupled to the membrane-spanning domain through which the substrate passes.

Many primary transporters also serve as ion pumps, i.e. they accumulate e.g. H^+ or Na^+ ions on one side of the membrane and thereby generate an electrochemical gradient. Such concentration gradients are used as an energy source by membrane proteins known as secondary active transporters. Specifically, these proteins power the movement of one substrate against its gradient by harnessing the energy released from the dissipation of a gradient of a different substrate. The transport process may involve the substrates moving either in the same (symport) or opposite (antiport) directions. In all cases, these transporters function according to the so-called alternating-access mechanism (45), whereby the binding sites for the substrates are alternately exposed to one or other side of the membrane, but not both at the same time (reviewed in, e.g. (30)).

2.3 Membrane enzymes

A number of enzymatic reactions carried out by water-soluble proteins are also conducted by enzymes integrally embedded in the membrane. The membrane setting facilitates access to hydrophobic substrates, such as TM helices destined for proteolysis, but must also allow access to reactive water molecules. Indeed, recent structural studies have shown that membrane enzymes achieve this feat by creating an aqueous micro-environment within the membrane (24, 108).

2.4 Co-factor scaffolding proteins

The orientational confinement imposed by the lipid bilayer can also hold a functional advantage. During photosynthesis, for example, light is absorbed by co-factors in so-called light-harvesting complexes (LHCs) and, by way of resonance energy transfer, activates neighboring photosynthetic reaction centers (PRCs). By fixing the relative positions of their co-factors, LHCs and PRCs create optimal conditions for light absorption and transfer. Similarly, electron transfer reactions in the membranes of mitochondria and respiratory bacteria are facilitated by a series of scaffolding proteins. Ultimately, both photosynthesis and respiration result in a H^+ electrochemical gradient across the membrane, which is harnessed by the ATP synthase to energize the production of ATP.

3 FUNCTIONAL DIVERSITY, PROTEIN SIZE AND SYMMETRY

The assortment of functions described above appears to have required a very diverse array of protein architectures. A possible evolutionary strategy to achieve this diversity is to create larger proteins and complexes from smaller structural units (37, 59). This strategy appears to have a number of advantages: the creation of new protein surfaces capable of binding to other molecules; enhanced stability, e.g. shielding of hydrophobic surfaces too small to match the membrane width (8, 77); the conformational stability of supra-molecular complexes; and, the potential for cooperative or other regulatory mechanisms, e.g., by tethering distinct functions within hetero-oligomers (16). As explained below, larger proteins are created by the assembly of multiple subunits, or by gene fusion of duplicated or

dissimilar genes, and symmetry appears to be an intrinsic consequence of those processes (37, 59).

3.1 Oligomerization

The simplest mechanism by which larger proteins are formed is through assembly into homo- or hetero-oligomers. Oligomerization is remarkably common; Levy et al showed that between one-half and two-thirds of all proteins form obligate complexes (59). Unfortunately, the equivalent numbers for membrane protein structures have not been well documented, though a cursory analysis of the Protein Data Bank for TM proteins (PDB_TM (103), Figure 1) suggests that a similarly large fraction of membrane proteins (~65%) are obligate oligomers (59).

Symmetry is found in ~85% of protein complexes, and is therefore the norm (59). It has been proposed that symmetry arises naturally from the fact that symmetric protein-protein interfaces contain duplications of all pair-wise contacts. Thus, the most favorable interactions are also duplicated, leading to more stable interfaces than those achievable by non-symmetric complexes (3, 69). A simple survey of available structures (see below), suggests that membrane protein oligomers are also predominantly symmetric, though a statistical analysis would be desirable. It will also be important to assess whether the above arguments regarding interface energies also apply, and whether the reduced degrees of freedom in the membrane either enhance or diminish this inherent predisposition toward symmetry.

3.2 Gene fusion

A second solution to the need for larger and/or more complex proteins is to combine pre-existing domains by gene fusion (37). Indeed, the majority of all proteins (55–67%) contain multiple detectable subdomains (72). However, when considering only membrane proteins, the opposite trend is observed, as only about 30% contain multiple, independently-functioning domains (65). Notably, this lack of fusion between membrane protein domains is not due to an inherent inability of their genes to fuse: indeed, as many as 90% of membrane proteins contain water-soluble domains (65). Thus, it may be that the reduced dimensionality of the membrane enhances the stability of protein-protein interactions, reducing the need for fusion (65).

If fusion of membrane-spanning segments is indeed less common than fusion of water-soluble domains, then one must assume that more complicated membrane protein functions, such as cooperativity, occur preferentially via oligomerization. However, the fraction of membrane protein oligomers in PDB_TM (Figure 1) is similar to that found for all proteins (59). A more systematic analysis of available membrane protein structures may help clarify this apparent discrepancy.

3.3 Internal repeats

The above discussion pertains to the fusion of domains with independent functionality. However, larger proteins can also be constructed by fusion of genes encoding small protein segments, for example, after duplication of secondary structure elements (70), which results

in internal structural repeats. Of the proteins containing detectable internal repeats, about half are symmetric, suggesting that they originate from concurrent duplication and fusion of genes that encoded for homo-oligomeric complexes (1). In other cases, duplication and subsequent fusion of segments that did not previously form oligomers may have resulted in non-symmetric internal repeats (1). Either way, after fusion, internal repeat sequences are independently exposed to selective point mutations. Without a specific functional reason to maintain perfect internal symmetry, therefore, the primary sequences of the repeats are very likely to diverge, resulting in structures that are internally pseudo-symmetric, rather than symmetric.

The first studies of internal structural pseudo-symmetry in membrane proteins suggested that the proportion is as high as one-half (16, 40), while a recent study using more conservative repeat-detection strategy identified pseudo-symmetry in ~24% of membrane protein structures (80). This discrepancy suggests that a quarter of membrane proteins may contain harder-to-detect, highly divergent, internal repeats, although it is also possible that recently reported structures are less pseudo-symmetric. Either way, given that ~18% of all folds were found to be internally pseudo-symmetric, it is clear that available membrane protein structures are enriched in internal pseudo-symmetry relative to water-soluble proteins (80).

For a few membrane protein families, hints of these internal structural duplications were identified based on sequence analyses, long before structures were available, e.g. (84, 94). However, many other duplications are too distantly related (<10% identity) to be detected by sequence-based methods (16, 40, 51, 80).

4 SYMMETRY IN MEMBRANE PROTEIN STRUCTURES

Lipid bilayers themselves contain a planar symmetry that divides the hydrophobic core in half and reflects the two lipid head-group regions. One might therefore expect that some membrane proteins contain a similar structural symmetry, i.e., with an axis running along the mid-plane of the membrane. Nevertheless, the chemical environments at either side of the membrane are typically not equivalent.

4.1 Non-symmetric membrane proteins

By definition, the one-third of soluble and membrane proteins that are monomeric (37, 59) (Figure 1) cannot adopt oligomeric symmetry. Moreover, between one-half and four-fifths of individual domains also contain no internal repeats (16, 80) and therefore also lack any detectable symmetry. Such non-symmetric proteins ought to be well-suited to detecting differences between the environments at either side of the membrane (37). Indeed, none of the three classes of receptor with known structures, namely GPCRs, ligand-gated ion channels, and enzyme-linked receptors such as RTKs, exhibit any symmetry with respect to the membrane plane. Nevertheless, they all feature structural symmetry around an axis perpendicular to the membrane (see below).

Enzymatic reactions in the membrane appear to be accomplished readily by non-symmetric architectures. These include oligosaccharide transferase, OST (66), aspartate proteases (61), site-2-proteases (26), and rhomboid proteases (107, 110) (Table 1, Figure 2).

Non-symmetric folds are also found in the protein translocation systems YidC (56) and TatC (93), as well as in a cellulose synthesis and translocation system, the BcsA/BcsB complex (78). In the latter case, although the eight TM helices of BcsA are organized into pairs, they are not related by symmetry.

Finally, the P-type ATPases, one of the largest families of primary transporters (101) are clearly non-symmetric (Table 1), which is unusual among transport proteins (see below).

Given that a third of membrane proteins are monomeric (Figure 1), one might expect this list of non-symmetric proteins (Table 1) to be longer. Could it be that most monomeric proteins contain internal symmetry? It has been estimated that as many as ~25–50% of domains contain no internal pseudo-symmetric (16, 80). However, it is possible that such automated approaches underestimate the occurrence of internal repeats, because their divergence makes them difficult to detect (1, 80). As a telling example, most GPCRs have been classified as non-symmetric (80), even though rhodopsin (also a GPCR) contains a clear structural duplication (16). Thus, it seems that the repeats in GPCRs have diverged significantly. An effective strategy might therefore be to assign pseudo-symmetry to a given structural class based on analysis of all known structures in that structural class, rather than using representative folds.

4.2 Cyclic Symmetry

When surveying the most common symmetry groups in oligomeric structures, Levy et al found that 80% contained dihedral symmetry, while only 20% were cyclic (59). In contrast, internally-duplicated segments are >90% rotationally symmetric (80). A survey of available symmetries in membrane proteins (both internal and oligomeric; Tables 1–3, Figure 2) corroborates previous observations that membrane proteins differ from water-soluble proteins in that the vast majority of membrane protein symmetries are cyclic (16, 80), even for oligomers. Indeed, only two cases with dihedral symmetry were found (see section 5.3). In contrast, almost all imaginable cyclic symmetry groups are found in the available membrane protein structures (Figure 2).

4.2.1 Symmetry with the axis perpendicular to the membrane plane—The majority of symmetric membrane proteins contain a rotational symmetry whose axis runs perpendicular to the membrane plane (Figure 2). This type of symmetry axis implies that the N-terminal and C-terminal ends of all involved chains are located on the same side of the membrane, which presumably simplifies the insertion process.

4.2.1.1 Take your partner by the hand: cyclic two-fold (C_2) symmetry and pseudo-symmetry with a perpendicular axis: The simplest and among the most common symmetric arrangement in membrane proteins involves a 180° rotation around an axis perpendicular to the membrane (C_2, Table 1). As described below, perfect C_2 symmetry is found in homo-oligomeric complexes while C_2 pseudo-symmetry is observed both in hetero-oligomeric complexes and in internal repeats (Figure 2). In cases where this association is required for function, the symmetric elements almost always create a binding site or pathway at their interface (Table 1).

Signaling receptors such as RTKs, for example, create a ligand binding site at the dimer interface (57, 60). Each protomer contains extracellular and intracellular domains, connected by a single TM helix. Binding of the ligand to the extracellular domains causes dimerization, or triggers a conformational change within a pre-existing dimer (57). Recent structures obtained by nuclear magnetic resonance (NMR) of isolated TM helix homo-dimers for, e.g., ErbB2 (9), and heterodimers of, e.g., ErbB1/ErbB2 (75), are consistent with C_2 symmetry or pseudo-symmetry extending into the membrane.

Primary transporters of the ATP-binding cassette (ABC) transporter family assemble using C_2 perpendicular symmetry, creating a substrate pathway as well as ATP binding sites at the dimer interface (90). All ABC transporters contain two TM domains (TMDs) and two nucleotide-binding domains (NBDs, or ABCs), assembled from either separate chains or fused domains. Notably, the NBDs create two, off-axis binding sites for ATP using a head-to-tail arrangement, whereas the substrate pathway follows the symmetry axis.

Four different classes of ABC transporter have been identified (67), namely ABC exporters, and three types of ABC importer called type I, type II, and energy coupling factor (ECF) importers. Simple homo-dimers are found in the first three classes, e.g., forming local C_2 symmetry in the TM domains of the type I importer $ModB_2$ ((43), Figure 2), and of the type II importer $BtuC_2$ (68). Hetero-dimeric ABC transporters, in contrast, come together in pseudo-C_2-symmetric complexes, as exemplified by MalF and MalG, which form the TM segments of the ABC importer MalFGK ((83), Figure 2). In some cases, one NBD has lost the ability to hydrolyze ATP. A structure of an ABC exporter with one of these so-called "degenerate" NBDs contains nucleotide bound only to one site, creating an asymmetry in these soluble domains (42), with intriguing functional implications (34).

Perpendicular pseudo-C_2-symmetry is also seen in some secondary transporters (Table 1, Figure 3), notably in the largest class, the major facilitator superfamily (MFS, (85)). The MFS fold was confirmed by structural studies (41) to contain two lobes of six-TM helices each lining a central pathway. Interestingly, the multidrug and toxin extrusion (MATE) transporters, such as NorM (39), and the resistance-nodule-division (RND) transporters exemplified by AcrB (23), also contain two domains of six TM-helices lining a central pathway. Nevertheless, the topological arrangement of the helices differs between the three folds.

Beyond the aforementioned functional roles, dimerization of membrane proteins also appears to be a common strategy for regulation (Table 1), e.g. by enhancing stability or introducing allostery. Dimerization of class C GPRCs allows them a unique mode of activation (53). In addition, the occurrence and functional relevance of homo- and hetero-oligomerization by class A GPCRs is the focus of intense study (27).

4.2.1.2 Three-fold symmetry is often found in regulatory roles: As discussed below, homo-trimeric assemblies are sometimes found to create channels. These include the P2X ATP-gated ion channels (48) and the outer membrane protein component of the RND efflux systems (20). In addition, membrane proteins with diverse functions are assembled from triplicated internal repeats with pseudo-C_3 symmetry (Table 2). These include ion-pumping

pyrophosphatases (Figure 2) (49, 63), and mitochondrial carrier proteins (MCP, Figure 3) (86).

Strikingly, no perfectly three-fold-symmetric structures have been reported for transporters, at least not for their core transport units (Table 2). However, homo-trimeric assemblies of secondary transporters and channels are common, apparently for regulatory reasons. For example, trimerization of the Na^+-coupled aspartate transporter, a homolog of the excitatory amino acid transporters (EAAT), may help stabilize the protein during its large elevator-like conformational change (17, 92).

Interestingly, asymmetry between protomers is also seen in trimeric transporter assemblies, such as AcrB (Figure 3). In each protomer, two repeats of five TM-helices cycle through three distinct conformational states, resulting in H^+ uptake (23). These changes in the membrane domains are mechanically transduced to a periplasmic domain, which also cycles through three distinct states resulting in drug extrusion. The asymmetry in the trimer results from coupling of the transport cycles of the three protomers, owing to an extensive interface between the periplasmic domains. This asymmetric coupling mechanism may minimize the extent of drug backflow (23).

Interactions between the cytoplasmic tails of neighboring protomers (91) also create an asymmetry in the trimeric Na^+-coupled betaine transporter, BetP (102), which may contribute to increasing its transport rate in response to osmotic stress (95).

4.2.1.3 Higher cyclic symmetries in channels & other systems: The creation of a central pathway is a common feature of parallel oligomers of membrane proteins (Figure 2), resulting in hollow rings with between three and twelve-fold symmetry (see above; Tables 2–3). Tetramers include the H^+ channel M2 from influenza virus (44) and the ionotrophic glutamate receptors such as the Glu2A receptor (98). Many channels are pentameric, e.g., the anion channel TehA from the plant SLAC family (14), although hexamers, like synaptophysin, a MARVEL-domain channel (5), heptamers, e.g., the small mechanosensitive channel, MscS (7), and octamers (19) have been observed. The largest pore-forming oligomers are constructed from toxins such as the cytolysins (88) (Table 3). Indeed, a toxin called perfringolysin O may form a pore-forming complex with 40–50 protomers, which would undoubtedly represent the highest symmetry order in a membrane protein (88, 97).

Rings of membrane proteins perform roles other than pore formation (Table 3). During photosynthesis, for example, LHCs, form ovals or rings in order to transfer the energy harnessed to PRCs located in the center (Table 3)(74, 81). Another example is the rotor ring of the F-type ATP synthases. These rings are very likely plugged by lipids (87) and the ring facilitates H^+ or Na^+ permeation as it rotates against an adjacent, static subunit (46, 87). Because three ATP molecules are synthesized for every revolution of the ring, the number of subunits (which in most cases equals the number of transported ions) determines the thermodynamic capacity of the enzyme to synthesize ATP (73).

Pseudo-symmetry is less common in α-helical complexes with higher order number (Tables 2–3), although a number of channels and receptors are known to form heteromers (e.g. (47)), as do some of the membrane rings of rotary ATPases (73). The β–strands of outer-membrane β-barrel proteins (OMP) are typically related by an 8- to 24-fold rotational pseudo-symmetry (25) (Table 3).

4.2.2 The membrane-bisecting axis of rotation—A surprising discovery of recent years is of rotational symmetries whose axes run along a plane that bisects the membrane (Figure 2). This symmetry raises energetic conundrums because the protein must either insert non-helical segments deep into the bilayer and risk exposing polar groups to the hydrophobic core, or insert the entire protein with dual TM topologies, seemingly a challenge for the insertion machinery (10).

Most of these pseudo-C_2 symmetries relate internal repeats with an odd number of TM helices (Figure 3). As a result, the topologies of these repeats are inverted with respect to one another, placing their N-termini on opposite sides of the membrane (Figure 2, Table 1). The small multidrug resistance (SMR) transporters (Figure 2), and the FluC fluoride channel (99) represent the only well-characterized homo-or hetero-dimers with a symmetry axis oriented in this way.

When comparing inverted-topology folds, an underappreciated distinction emerges, namely the position of the symmetry axis relative to the repeats. The symmetry axis may lie in between the two repeats, in which case each repeat has an independent fold (see EmrE, Figure 2). Alternatively, the symmetry axis may pass through the center of both repeats, and then the helices of the two repeats must interdigitate (see NCX, Figure 2).

<u>**4.2.2.1 Adjacent inverted-topology repeats:**</u> Interestingly, inverted-repeat folds in which the repeats are adjacent to the axis are mostly found in channels (Table 1). Many of these channels are specific for small polar molecules such as water (Aqp1 (76)), urea (dvUT (58)), or ammonia (AmtB (50)). In addition, adjacent inverted-topology repeats are found in channels and transporters for small anions (e.g., CLCs (21)) and cations, as in the Bax inhibitor homolog, BsYetJ (Figure 2) (13). Unusually, the two three-TM-helix repeats in BsYetJ surround the seventh TM helix, through which the pseudo-symmetry axis also passes (Figure 2).

With the exception of BsYetJ, the interface between adjacent repeats defines two symmetry-equivalent pathways that lead from either side of the membrane (31). This seems an evolutionarily parsimonious strategy for channel formation because a single duplication leads to a narrow pathway that may be especially suitable for small molecule conductance (99).

<u>**4.2.2.2 Interdigitating inverted-topology repeats:**</u> Proteins containing interdigitating inverted-topology structural repeats have arguably the most complex of the known membrane protein folds (Figure 2), and are mainly involved in secondary transport (Table 1). However, interlocking repeat elements also contribute to subdomains in larger transporters, e.g., in type II ABC importers, four TM helices within each lobe contribute to a pseudo-

symmetric inverted repeat (16, 68). In addition, in the MFS fold, each two-fold symmetric lobe is itself composed of interdigitating three-TM-helix inverted-topology repeats (41) (Figure 3). As a result, the MFS fold can be considered to contain interlocking inverted-topology repeats of 6-TM helices (89). As described below, a functional advantage of such interlocking inverted-repeats may be an ability to adopt asymmetric states that fulfill the requirements for alternating-access.

Interdigitation leads to wide spacing between contiguous helices (in sequence), implying that the isolated repeats are unlikely to be well-folded. The resultant interlocked interfaces are indistinguishable from the interior of proteins (37), with minimal hydration and extensive hydrophobic cores, in stark contrast to interfaces of typical oligomers. The folding of these proteins must therefore be a complex process. It would be interesting to examine whether individual inverted-topology repeats can exist as independently-folded units.

The possible evolutionary origins of such a complex fold are also enigmatic. However, their interfaces are reminiscent of domain-swapping water-soluble proteins (64), which may provide a useful starting point for further inquiry.

4.2.2.3 Asymmetry in membrane proteins: Although symmetric and pseudo-symmetric arrangements predominate, a number of membrane proteins exhibit a structural asymmetry that is essential for function (blue in Table 1). Asymmetry in a homo-oligomer has the evolutionary handicap that the asymmetric interface must evolve to optimize interactions with two different environments simultaneously (37); this disadvantage explains their rarity, but appears in some cases to be compensated by the functional advantages.

The first example of functional asymmetry in membrane proteins was provided by the homo-dimeric SMR transporter EmrE (15, 28, 104). In structures of EmrE, the two identical protein chains adopt an antiparallel orientation with different conformations, and as a result create a pathway to one side of the membrane only (Figure 2). This fold is an example of classical asymmetry. Around the same time, the structure of an amino-acid transporter, LeuT in an outward-facing conformation, i.e., also containing one access pathway (111), was found to contain a distinctive asymmetry underlying the formation of the outward-facing state (31, 32). The asymmetry in LeuT was obscured by the pseudo-symmetry of the repeats (111) which contain <10% identical residues, and therefore also exhibit some level of inherent structural divergence. Once detected, however, the asymmetry could be taken advantage of, in a strategy to model an alternate state. Specifically, by threading the sequence of the first repeat onto the structure of the second repeat, and vice versa, the two halves of the protein 'swap' their conformations. For LeuT this resulted in a model of an inward-facing state (32), whose overall features are remarkably consistent with structures determined subsequently (54).

The implication of these findings for EmrE and LeuT is that these proteins can adopt two states, consistent with alternating-access, i.e., with pathways leading to one or other side of the membrane, by creating an asymmetry in the two repeats. During the cycle, therefore, the first repeat (or protomer) must adopt the conformation of the second repeat (or protomer), and vice versa (28, 32). Given that physiologically, substrate accumulation is driven only by

the balance of substrate concentrations and the associated membrane potentials, all conformations of the transporter need to be accessible from an energetic standpoint, without any additional input (i.e., in contrast to primary transporters). The degeneracy of structural states implied by the asymmetry-exchange mechanism is an elegant solution to this requirement.

The 'repeat-swapping' modeling strategy has since been applied to proteins with diverse structural folds (Figure 3) including Glt_{Ph} (17), NCX from the Ca^{2+}:cation exchanger family (CaCA (62)), lactose permease from the MFS (89), and NhaA from the Na^+-H^+ antiporter (NPA) family (96). Notably, all these folds comprise inverted-repeats with interdigitating helices (Table 1, Figure 2). In each case, a model of an alternate state was generated, and the predicted global conformational changes have since been validated by structures (29, 30, 92, 106) or other evidence (89, 96). Importantly, NMR spectroscopy data supports the proposal that antiparallel EmrE functions by exchanging between degenerate states (79). Thus, the asymmetry-exchange mechanism underlies alternating-access by both homo-dimers and pseudo-symmetric interdigitating inverted-repeats.

How do asymmetric transporters optimize their pathways to interact equally favorably with two different environments (37), i.e., to participate in protein-protein packing as well as interact with substrate and/or aqueous solution? In Glt_{Ph} these interfaces comprise smooth surfaces with both polar and hydrophobic character (92), thereby preventing the protein from becoming trapped in one state.

A notable conundrum posed by the asymmetry-exchange mechanism is the role of symmetric states. Let us consider two subunits A and B that can both adopt two conformations, i and j. The asymmetric states of the transporter can thus be defined as A_iB_j or A_jB_i. What then prevents the formation of A_iB_i or A_jB_j? Might one of these arrangements correspond to an occluded state, or possibly, a leaky state (71)?

Intriguingly, asymmetry has recently been detected in the antiparallel homo-dimeric fluoride channel, FluC (C. Miller, personal communication), although the reason why asymmetry is required by a channel is unknown. Still, pure asymmetry as in EmrE and FluC is rare, and the inverted repeats of asymmetry-exchanging transporters are typically divergent in sequence. Pseudo-symmetry may therefore play an important role in adapting secondary transporters to diverse substrates and conditions. In some cases, the breakdown in symmetry may also reduce the free energy of one state over the other, just enough to provide preferential conformations of the transporter, e.g., while awaiting substrate binding. Analysis of possible common ancestors of asymmetry-exchanging transporters may therefore provide useful insights into the minimal requirements for secondary transport.

4.2.2.4 Face-to-back inverted-topology-repeats: Remarkably, a two-fold screw-axis pseudo-symmetry with a membrane-bisecting axis was recently identified within a membrane protein subunit related to Na^+-H^+ antiporters of the Mrp family (22)(Table 1). Specifically, the multimeric NADH:ubiquinone oxidoreductase, or complex I of the respiratory chain, was found to contain an five-TM helix internal repeat within each of three subunits (Nqo12, Nqo13 and Nqo14) responsible for proton pumping (Figure 4). However,

because they are related by a pseudo-twofold screw axis (2_2) rather than a rotational axis (C_2), the repeats are oriented face-to-back, rather than adjacent or interdigitating (22). Moreover, the three subunits are arranged linearly, and capped at one end by an additional domain (Nqo8), which also contains a repeat (6). It is therefore tempting to speculate that the screw-axis is well suited to creating a linear (in effect, helical) array of subunits with repeated interactions. That is, the interfaces between repeats in Nqo14 and Nqo13 are similar to those between repeats in each subunit (Figure 4). The mechanistic relevance of this face-to-back symmetry remains to be established.

4.3 Dihedral and plane symmetries

Dihedral and planar symmetries contain within them a two-fold symmetry axis, and are therefore potentially compatible with membranes. However, in dihedral symmetry, there must be rotational two-fold axes both perpendicular and parallel to the membrane (105). Therefore, dihedral symmetry within a protein spanning a single membrane potentially exposes polar groups to the hydrophobic membrane core. Consistent with this constraint, the only two known structures with dihedral symmetry are proteins that span two membranes. In gap junctions, two hemichannels, one per membrane, are stacked up to create a pore crossing the two membranes. Each hemichannel is formed by a hexamer of connexin proteins with C_6 symmetry, and the entire gap junction exhibits a dihedral (D_6) symmetry (Table 3, Figure 2). Aquaporin-0 (AQP-0) tetramers pack head-to-head into octamers that span the membranes of the eye lens fiber cells creating a dihedral symmetry ($_4$, Table 2) (36).

Plane symmetries, created by translations in two directions, although rare, are found in arrays of membrane proteins at specific cellular structures. These include ribbons of claudin proteins at epithelial tight junctions (33, 100), and arrays of AQP-0 octamers (12). Both specific and non-specific properties of the lipids may be critical for the formation of such arrays (18, 35).

4.4 High-order cubic and space-group symmetries

The planar nature of lipid membranes renders the three-dimensional arrangements with cubic or space group symmetries unavailable to membrane proteins. However, attachments to scaffolding or anchoring proteins may result in assemblies of membrane proteins forming spatially ordered complexes.

Acknowledgments

Acronym list

TM	transmembrane
PDB	protein data bank

GLOSSARY

Non-symmetric proteins

Those that show no apparent symmetry-related internal duplications, nor any symmetry relationship between chains in a multi-subunit complex.

Symmetric proteins

Those composed of identical sequences replicated around the symmetry axis, therefore invariably homo-oligomeric complexes.

Pseudo-symmetric proteins

Two or more protein segments whose sequences differ, but share similar topological arrangements (folds) of their backbones.

Inverted-topology repeats

Membrane protein segments inserted in opposite orientations, and related by a two-fold axis running parallel to the membrane.

Internal repeats

Duplications of a structural element within a single polypeptide chain.

Asymmetric proteins

Protein segments with similar folds adopting distinct conformations of their backbones within the context of that same fold.

Topology

The direction of threading of protein segments back and forth across the membrane.

Oligomers

Assemblies of two or more protein chains, with either the same (homo-oligomers) or different (hetero-oligomers) protein sequences.

Domain

A functional and structural unit of protein, typically between 100 and 250 amino-acids in length.

Domain swapping

The positional exchange of one or more secondary structure elements from neighboring protomers in an oligomeric complex.

Protomer

A single subunit of a homo-oligomeric subunit, as distinct from a monomer, which is a non-oligomeric entity.

Dihedral symmetry with order N

contains $2 \times N$ units related by N 2-fold rotational axes and one N-fold rotational axis.

Dual-topology

The ability for a protein to insert into the membrane in both orientations with equal probability. Also known as undecided.

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SUMMARY POINTS

1. Membrane protein structures to date exhibit a wide range of symmetries and pseudo-symmetries from two-fold to planar arrays, but the vast majority conforms to cyclic point group symmetry.

2. Available membrane protein structures apparently constitute a similar fraction of oligomers as water-soluble proteins, but contain a higher proportion of internal repeats. Overall, membrane proteins may exhibit symmetry more frequently than water-soluble proteins.

3. Inverted-topology (pseudo-)symmetries create channels and pathways through the membrane; the MFS family is a notable exception where 3-TM helix inverted-topology repeats create the two halves that themselves line the substrate pathway.

4. Asymmetry has been observed in secondary transporters, and in one channel, whose folds contain inverted topologies.

5. Both identical (homo-oligomeric) and divergent (pseudo-symmetric internal-repeat) protein sequences use asymmetry-exchange to create degenerate alternate states consistent with alternating-access transport mechanisms.

FUTURE ISSUES

1. Systematic analysis of the growing number of membrane protein structures will be needed to further classify symmetries, pseudo-symmetries, oligomerization, and internal-repeats in membrane proteins. Continued efforts in structural biology will be needed to fill in gaps in fold space.

2. Which factors govern asymmetry in secondary transporters, and how is the balance of asymmetric states affected by substrate binding? How do those factors change for different transporter architectures, and how are they affected by single point mutations?

3. Do symmetric (or pseudo-symmetric) states play a role in secondary transport by asymmetry-exchange mechanisms, e.g. as occluded/closed or leak states?

4. What factors define the boundary between channel and transporter functions (with symmetric and asymmetric functional states, respectively), especially in folds such as the CLCs?

5. What was the evolutionary pathway of inverted-topology interlocking repeats? Are the individual repeats stable as separate entities? Bioinformatic and folding studies of the simplest cases, e.g. NCX, may help identify contributions from, e.g., circular permutation or domain swapping.

6. Did all inverted-topology folds arise from divergent evolution of a single ancestor, or from convergent evolution from many dual topology proteins ?
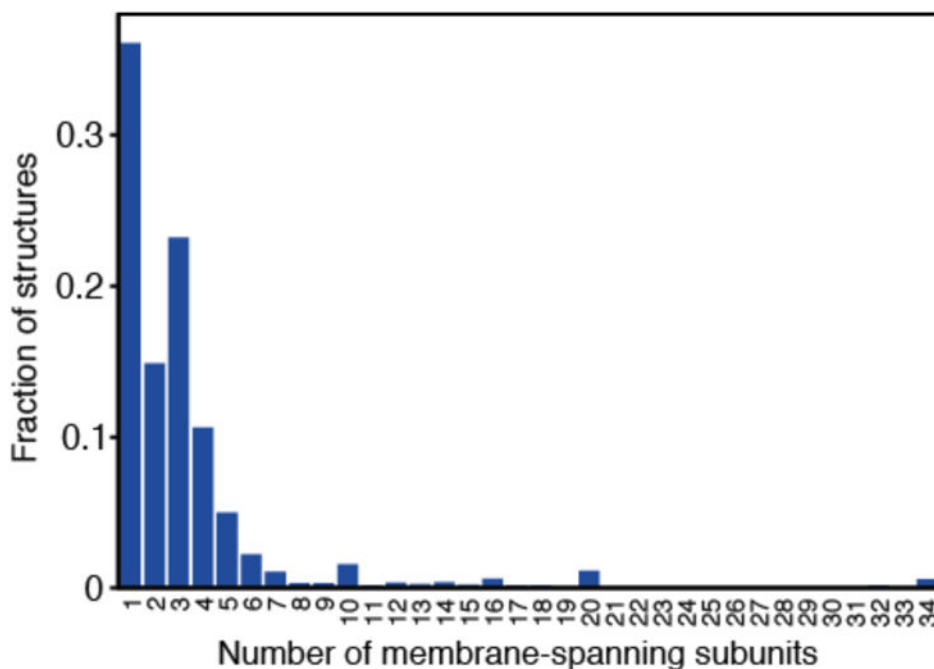
Figure 1.
Degree of oligomer formation in membrane protein structures. Data was taken from the PDB_TM database (103) dated 2014.08.01 containing 2241 proteins. No filtering of low-resolution models or redundancy was applied. Thus, each data-point may contain a number of representatives from the same structural family. This analysis is necessarily biased toward those proteins that crystallize and are well studied. Oligomers are formed via interactions between membrane domains as well as between fused water-soluble domains.
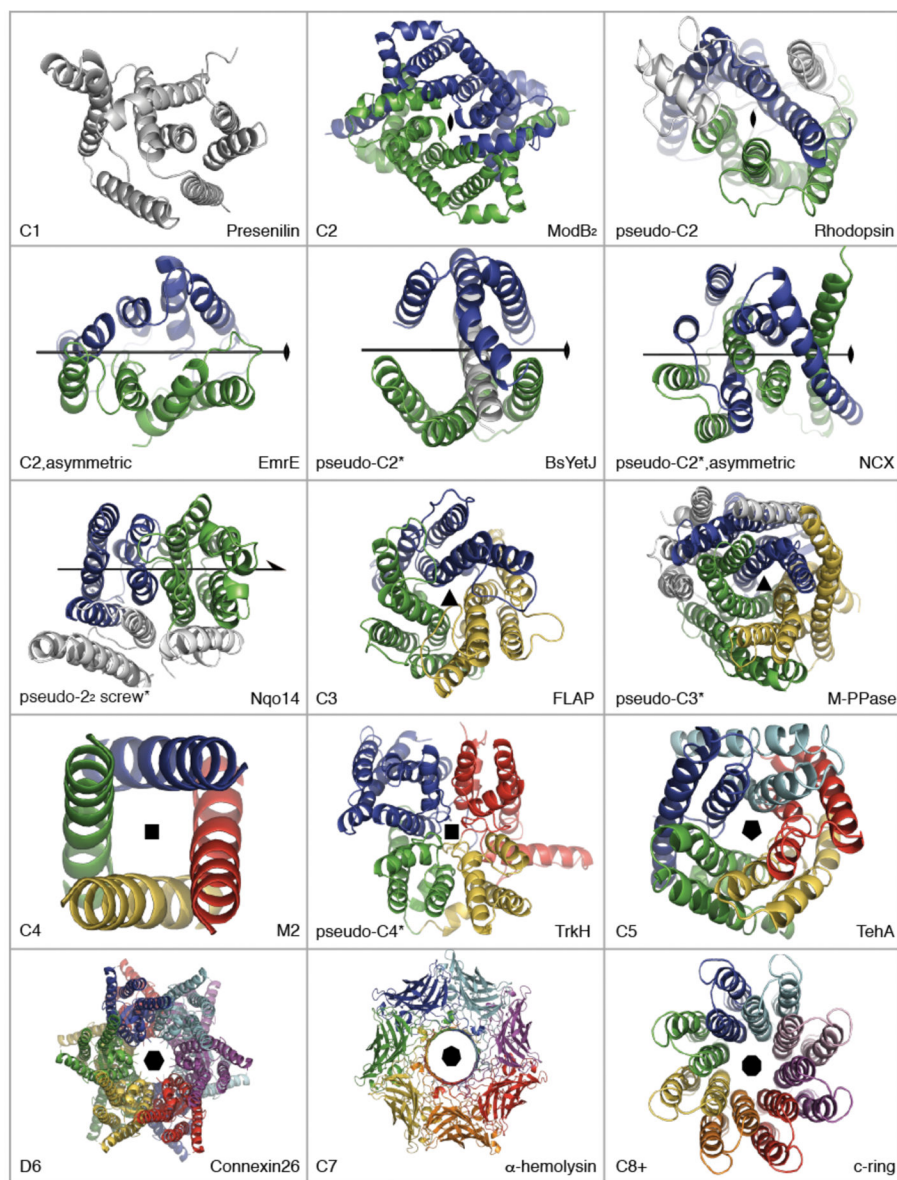
Figure 2.
Point symmetry types in membrane protein structures. Structures are shown as cartoon helices, viewed down onto the membrane. Different colors are used to indicate symmetric elements, i.e., independent chains or (*)internal repeats. Non-symmetric elements are in gray. EmrE is an asymmetric homo-dimer (PDB entry: 3B5D), while other transporters are asymmetric as well as pseudo-symmetric (e.g., NCX, 3V5U). Two-fold screw axis (2_2)-pseudo-symmetry is seen in the Mrp antiporter-like subunits of complex I (see Figure 4). Presenilin is an aspartate protease (4HYG). $ModB_2$ is from the homodimeric molybdate type I ABC importer (2ONK). MalFG is from the heterodimeric $MalFGK_2$ type I ABC importer (2R6G). BsYetJ is a pH-dependent Ca^{2+} channel from the TM Bax inhibitor motif (TMBIM) family (4PGW). FLAP, or five-lipooxygenase-activating protein, is a member of the family of membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG,

2Q7M). M-PPase is a membrane pyrophosphatase (4AV3). M2 is a H^+ channel from influenza A (3LBW, TM domains only). TrkH is a K^+ channel from the superfamily of K^+ transporters (SKT, 3PJZ). TehA is a SLAC anion channel homolog (3M73 chain A). Connexin-26 is a gap junction (2ZW3); each hemi-channel exhibits C_6 symmetry. α-hemolysin is a pore-forming toxin (7AHL). The c_8-ring is a F-type ATP synthase membrane rotor (2XND). Symmetry axes were defined using SymD v1.3 (52) and figures were made with Pymol v1.7 (Schrödinger Ltd).

Figure 3.
Transmembrane topologies of secondary transporters of known structure. The outside of the cell or organelle is oriented to the top. Protein name, family name, human solute carrier (SLC) nomenclature, and representative PDB identifiers (in parentheses) are given. Helices are represented as cylinders, and strands as arrows. Each inverted-topology repeat is highlighted using a triangle whose base is on the side of the N-terminus. Abbreviations: Vc, *Vibrio cholerae*; CNT, concentrative nucleoside transporter; APC, amino acid/polyamine/organocation superfamily; AAC, ADP-ATP carrier; PDs, periplasmic domains.
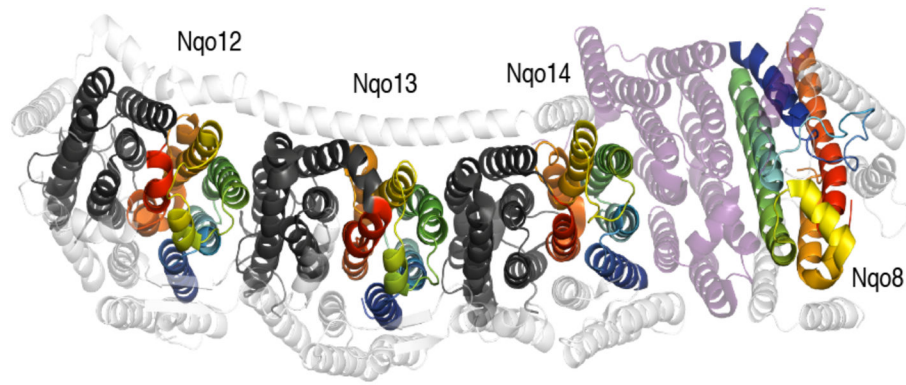
Figure 4.
The membrane domain of complex I from *T. thermophilus* (PDB entry 4HE8). Inverted-topology repeats related by a two-fold (2_2) screw-axis are colored blue to red for the first repeat or dark gray for the second repeat. The single 'repeat' in subunit Nqo8 has same TM topology as the three 'first repeats', but is rotated by 180° around an axis perpendicular to the membrane plane (cf. dark blue helices), and is more tilted. Nqo8 is separated from Nqo14 by a number of additional subunits (purple). Other non-symmetric segments and subunits are colored white.

Table 1

Membrane proteins of known structure with non-symmetrical or two-fold symmetrical folds

Order	None	2-fold				
Axis		**Perpendicular**		**Planar**		
Group		C_2	[c] pC_2	[d] aC_2	pC_2	2_2
Receptor, Enzyme	YidC, TatC, OST, proteases,	RTK, sulfur-transferase, **cyt c ox aa$_3$, cyt c$_1$, PS-II SR-II, class C GPCR, glycophorin A**	BR, PRC, complex I, thiol oxidase, rhodopsin, UbiA prenyl-transferase			
[a] Primary	P-type ATPase	ABC Export, ABC Import I, ABC Import II	ABC export, ABC Import I		*ABC Import II[l]*	
[b] Second-ary	CDF, **MFS, APC, EIIC, DASS, CLC**		MATE, MFS, RND[l]	SMR	CLC, *APC.NPA[l], CaCA.EAAT CNT.DASS, MFS.NCS2*	Mrp[l]
Channel	BcsA, PorA[l]	MgtE, **SKT**			MIP, Amt, SecY, FNT, UT, TMBIM	

Protein families (or one subunit of a larger complex) are organized by symmetry order, axis orientation (perpendicular or parallel to the membrane) and symmetry group. **Bold** indicates a regulatory role (e.g. stability, cooperativity).

[a] Primary transporters,

[b] secondary transporters.

[c] pC2 indicates pseudo-C2 axis,

[d] asymmetry. *Italics* indicates that the corresponding symmetry axis runs through the helices of the two repeats (interdigitating repeats). Blue text indicates asymmetry (synonymous with homo-oligomer, while pseudo-symmetry includes both hetero-oligomers or internal repeats).

[l] Indicates symmetry is within a protomer in an oligomeric complex.

Abbreviations not in main text: PS-II, photosynthetic reaction center II; CDC, cholesterol-dependent cytolysin; CNT, concentrative nucleoside transporter; DEG-eNaC, degenerin epithelial sodium channel family; BR, bacteriorhodopsin; SR-II, sensory rhodopsin II; DASS, divalent anion:Na$^+$ symporters.

Table 2

Membrane proteins of known structure with three- or four-fold symmetrical folds

Order	3-fold		4-fold		
Group	C_3	pC_3	C_4	pC_4	D_4
Receptor, Enzyme	DgkA, MAPEG, plant LHC-II, pMMO, **BR**	Nitric oxide reductase, cyt c oxidase, pMMO[1]			
Primary		M-PPase			
Secondary	**BCCT, RND, EAAT, MFS**	MCP			
Channel	P2X-R, DEG-eNaC, RND, dermicin, U**T, SLAC1, Amt, OMP**	UreI[1]	KcsA, NavAb, AMPA-R, M2, **MIP**	SKT, NMDA-R	AQP-0

See legend to Table 1 for details. Abbreviations not elsewhere in text: DgkA, diacylglycerol kinase A; MAPEG, membrane associated proteins in eicosanoid and glutathione mechanism; pMMO, particulate methane mono-oxygenase; P2X-R, P2X receptor; UT, urea transporter; OMP, outer membrane protein (β-barrel); cyt c oxidase, cytochrome c oxidase; cASIC, chicken acid sensing ion channel; AMPA-R, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; MIP, major intrinsic proteins; NMDA-R, N-methyl-D-aspartate receptor.

Table 3

Membrane proteins of known structure with five-fold or higher symmetrical folds

Order	5-fold		6-fold		7-fold	8+-fold
Group	C_5	pC_5	C_6	D_6	C_7	C_{8+} or pC_{8+}
Receptor, Enzyme						PmLH-II (C_8), RaLH-II (C_9), TtLH-I (C_{16})
Primary						F/V/A-ATPase c-rings (C_8, C_{10}–C_{15} & pC_{11})
Secondary						
Channel	cys-loop, CorA, SLAC, MscL, **FNT**	cys-loop	MARVEL, CRAC, **UreI**	Cx	MscS, CDC	PFT (C_{12}), OMA (C_8), OMP (pC_8–pC_{24})

See legend to Table 1 for details. Higher-order symmetry groups are given in parentheses. Abbreviations not used elsewhere in text: cys-loop, cys-loop receptor; CorA, Mg^{2+} channel family; SLAC, slow anion channel; FNT, formate/nitrite transporter; MARVEL, myelin and lymphocyte (MAL) and related proteins for vesicle trafficking and membrane link family; CRAC, Ca^{2+}-release activated Ca^{2+} channel; Cx, connexin; CDC, cholesterol-dependent cytolysin; PmLH2, *Phaeospirillum molischianum* light-harvesting complex (LHC)-II; RaLH2, *Rhodoblastus acidophilus* LHC-II; TtLH-I, *Thermochromatum tepidum* LH-I/PRC complex; OMA, outer membrane auxiliary protein; PFT, pore-forming toxin.