Weakly Stable Regions and Protein-Protein Interactions in Beta-Barrel Membrane Proteins

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Running Title: WSRs and PPIs in Beta-Barrel MPs

Abstract

We briefly discuss recent progress in computational characterization of the sequence and structural properties of β-barrel membrane properties. We discuss the emerging concept of weakly stable regions in β-barrel membrane proteins, computational methods to identify these regions and mechanisms adopted by β-barrel membrane proteins in nature to stabilize them. We further discuss computational methods to identify protein-protein interactions in β-barrel membrane proteins and recent experimental studies that aim at altering the biophysical properties including oligomerization state and stability of β-barrel membrane proteins based on the emerging organization principles of these proteins from recent computational studies.

keywords: β-barrel membrane proteins — protein-protein interaction — weakly stable regions— VDAC — Tom40— OmpF

1 Introduction

Approximately 30% of all encoded proteins are membrane proteins [1, 2]. They are involved in a number of essential biological processes, including signaling of regulatory networks, transport of nutrients and metabolites, membrane anchoring, pore formation, and enzyme activity [3–6]. Due to the difficulty in experimental determination of their three-dimensional structures, the organization principles of membrane proteins remain relatively poorly understood compared to soluble proteins. β-barrel membrane proteins, one of the two major classes of membrane proteins, reside in the outer membrane of gram negative bacteria, chloroplast, and mitochondria. In addition to the diverse biological functions, they are also responsible for bacterial pathogenesis [7]. β-barrel membrane proteins have also been implicated in mitochondrial mediated apoptosis in recent studies [8–10]. In this review, we discuss recent progresses in computational studies on the sequence and structural properties of β-barrel membrane proteins. We then discuss computational and experimental studies that characterize the protein-protein interactions (PPI) of these proteins in the transmembrane (TM) domains, as well as recent efforts to engineer β-barrel membrane proteins with enhanced biophysical properties.

2 Sequence and Structural Properties of β-barrel Membrane Proteins

Identifying β-barrel membrane proteins from genomic sequences is a challenging task. Long stretches of hydrophobic residues, which are a hallmark of α-helical membrane proteins, are absent in β-barrel membrane proteins, due to the alternating hydrophobic (facing the lipids) and polar residues (facing the interior of the barrel), as a consequence of the β-sheet structure [11]. Nevertheless, a number of computational methods based on machine learning and empirical energy scoring functions can now identify and predict the topology of β-barrel membrane proteins accurately [12–17]. Computational studies have also identified a number of sequence and spatial motifs that have been implicated in structural stabilization and function of these proteins [18–20]. Further analysis revealed that selection pressure on proteins during the course of evolution gives rise to these sequence and structural motifs, reflecting structural or functional constraints [19–21]. A

recent review on the sequence and structural properties of membrane proteins can be found in reference [22].

Recent experimental measurement of insertion free energy of OmpLA, a β-barrel membrane protein, by substituting Ala with each of the other 19 amino acids [23], has renewed interest in the calculation of insertion free energies for membrane proteins and indirectly calculation of a universal hydrophobicity scale. A number of studies have been carried out to compute insertion free energy using molecular dynamics simulations on either small transmembrane peptides or whole proteins with some successes [24–26]. Hsieh *et al.* have developed an empirical knowledge based potential by analysing structures of known β-barrel membrane proteins. It captures the depth-dependent nature of the energetics of inserting amino acids in the bilayer, and is in good corelation with the experimental observations [27].

Identifying homologous sequences by sequence alignment is one of the most important tools for transferring functional and structural information from a known protein to an unknown protein. Specialized scoring matrices for β-barrel membrane proteins have been developed for such purposes, as standard scoring matrices are derived from soluble proteins and hence inadequate for β-barrel membrane protein studies [21, 28]. It was also found that other scoring matrices derived from α-helical membrane proteins were also inadequate for β-barrel membrane protein studies [21]. It is expected that using these customized scoring matrices, structures of about 5–10 times more β-barrel membrane proteins that are homologues of proteins with known structures can be modeled [21].

Predicting three-dimensional structures from sequences is one of the most difficult problems in computational biology. Although the method transFold was able to predict the super-secondary features such as inter-strand contacts with considerable success [29], TMBpro was the only tool available until recently for predicting three dimensiaonl structure of β-barrel membrane proteins [14]. TMBpro is a template based method that requires the identification of a homologous protein whose structure has been resolved. The average all atom RMSD between the predicted and the native structures was about 7 Å for the transmembrane domains of the β-barrel membrane proteins. However, it is unable to model structures when no homologous structural template is known or when a protein adopts a novel topology, such as VDAC (19 transmembrane β-strands) [30] and PapC (24 transmembrane β-strands) [31] proteins. A recent study described a method than can model the transmembrane domains with an average RMSD of 4 Å for main

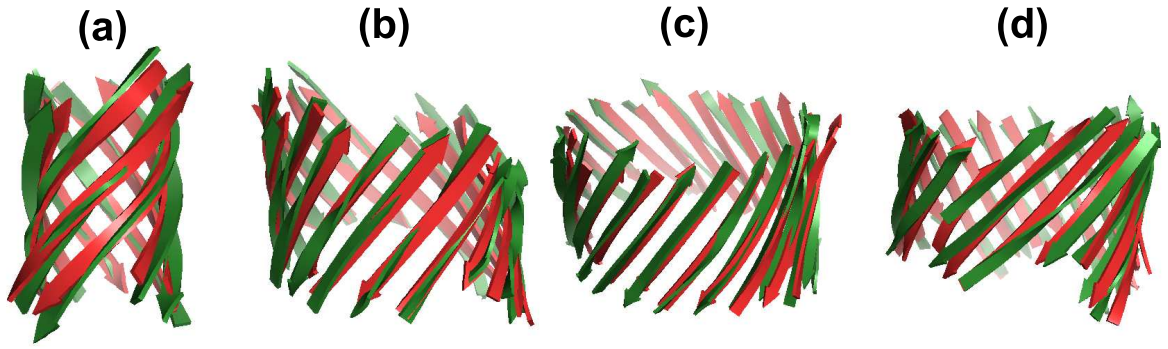

Figure 1: Predicted structures of the transmembrane domains (in green) superimposed on experimentally determined structures (in red): (a) OmpF (pdb id:2omf), (b) OmpA (pdb id:1bxw), (c) BtuB (pdb id:1nqe), and (d) VDAC1 (pdb id:3emn). The average RMSD between the modeled and experimental transmembrane domains of β-barrel membrane proteins is approximately 4 Å for main chain atoms. A template structure is not required to build the models.

chain atoms (5.6 Å for all atoms). This method does not require any template structure for constructing the three-dimensional model (Figure 1) [32]. It is based on a physical interaction model, a simplified state space for efficient enumeration of conformations, and an empirical potential function derived from detailed combinatorial analysis of available structures. It models the structures of β-barrel membrane proteins in two stages. In the first stage, it predicts the correct hydrogen bonding pattern between each pair of β-strands. In the second stage, it constructs the three-dimensional coordinates of the transmembrane segments of β-barrel membrane proteins using constraints from hydrogen bond geometry and an intertwined coil approximation of the β-barrel [32]. The accuracy of the method without the help of a template structure suggests that it captures the important elements of the organization principles of β-barrel protein assembly. The predicted structures can reveal important insight about the location of protein-protein interaction sites and weakly stable regions in these proteins. They can also be useful for re-engineering or designing β-barrel membrane proteins with enhanced stability, pore size, and geometry, which is of high importance in nanotechnology [33] and structural biology [34, 35]. Some of these aspects will be discussed below.

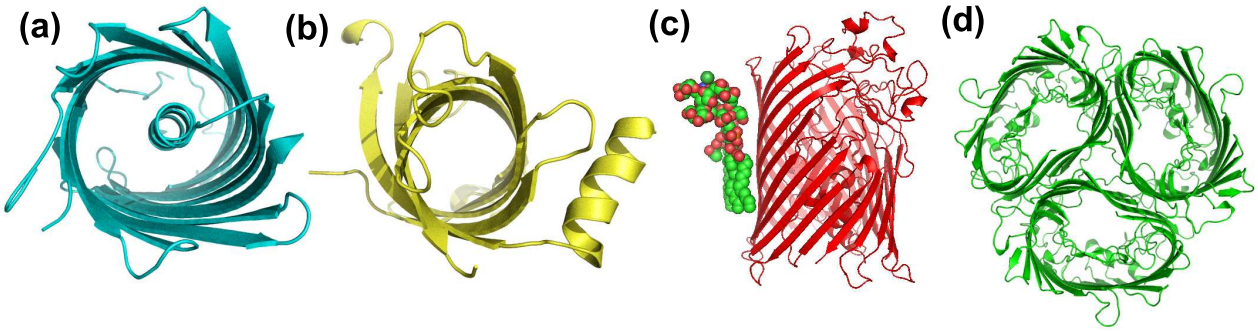

Figure 2: β-barrel membrane proteins employ four general mechanisms to stabilize weakly stable regions: (a) small helices and strands, called in-plugs, are packed inside the β-barrel to provide stabilizing interactions; (b) helices packed against the lipid accessible transmembrane β-strands provide stabilizing interactions; (c) specific lipid-protein stabilizing interactions, such as lipopolysaccharide binding in the FhuA β-barrel membrane protein; and (d) multiple weakly stable regions on separate proteins may interact through PPIs, resulting in stabilization of each of these regions.

3 Mechanisms of β-barrel Stabilization

The stability of β-barrel membrane proteins stems from the extensive intrastrand hydrogen bond network. As a result, their melting temperatures are typically around $80°C$ [36, 37]. Despite this extensive hydrogen bond network, recent computational studies on the transmembrane domains of β-barrel membrane proteins have found that weakly stable regions are present in these proteins [38, 39]. The stability of individual TM β-strands is calculated using an empirical potential function, TmSIP, which is derived from a combinatorial analysis of β-barrel membrane protein structures [18]. The energy for each residue consists of two terms. First, each residue is assigned an energy value of burying this residue type at a particular depth in the lipid bilayer and with the specific orientation of its side chain. Second, each residue interacts with two residues on separate neighboring strands through strong backbone H-bond interaction, side-chain interactions, and weak H-bond interactions. The overall strand energy is the summation of the above mentioned energy terms over all residues in the strand. Strands with energy higher than the mean energy of all the strands are regarded as "weakly stable regions". It was further discovered that β-barrel membrane proteins employ four general mechanisms to stabilize these regions (Figure 2): (1) small helices and strands, called in-plugs, are packed inside the β-barrel to provide stabilizing interactions [40]; (2) helices are packed against the lipid accessible transmembrane β-strands to provide stabilizing interactions [41]; (3) lipids bind specifically to these

weakly stable regions to provide stabilizing interactions, such as lipopolysaccharide binding in the FhuA β-barrel membrane protein [39, 42]; and (4) multiple weakly stable regions on separate proteins may interact through PPIs, resulting in stabilization of each of these regions [40]. Moreover, these structurally stabilizing mechanisms often have important functional roles, such as voltage sensing, flux control of metabolites, and ion sensing [40, 43].

4 Computational Methods to Detect Protein-Protein Interactions in Outer Membrane

It is important to identify weakly stable regions, which may indicate the presence of protein-protein interaction interfaces and provide an indication of the oligomerization state of the protein. Their identification can also provide rich information about the function of the proteins. Furthermore, such information will lead to greatly increased protein engineering efforts that construct mutants with desired protein-protein interaction interfaces and oligomerization states. A genomic scale survey of the combinations of different domains in α-helical membrane proteins suggested that membrane proteins in general exist as single domains, and that oligomerization might be the mechanism by which they gain new biological functions [44, 45]. Due to the sparsity of the structural data for β-barrel membrane proteins as well as the complexity in developing tools that are based on physical principles from complex structural data, computational studies have lagged behind in this area.

A recent study used an empirical energy function derived from detailed combinatorial analysis and a minimalistic structural model of β-barrel membrane proteins for predicting the oligomerization state and the protein-protein interaction interface of β-barrel membrane proteins with an accuracy of 100% and 82% respectively, from sequence information alone [38]. The study was also able to corelate the existence of weakly stable regions with the oligomerization state of the protein and the location of the protein-protein interaction interface [38]. This computational study was the precursor of a number of experimental studies summarized in the next section.

Another computational study used a random forest classifier to predict the protein-protein interaction

interface of membrane proteins [46]. This method is trained on residue type distributions and evolutionary conservation for individual surface residues. The prediction accuracy for membrane proteins is reported to be comparable to that of non-membrane proteins [46]. This study also shows that a predictor trained on non-membrane proteins produces poor results for membrane proteins, demonstrating that the driving forces behind the protein-protein interactions in membrane and non-membrane proteins are fundamentally different [46]. A drawback of this study is that the data set used contains only 37% β-barrel membrane proteins, the rest being α-helical membrane proteins and the results are not reported separately for each class. Therefore, it is difficult to draw specific conclusions about protein-protein interaction site predictions for β-barrel membrane proteins. Another study by Hsieh *et al.* for predicting the protein-protein interaction site in β-barrel membrane proteins is based on a statistical potential. Using the sum of radial moments from the barrel axis to the lipid facing amino acids, the approximate location of the protein-protein interaction interface in oligomeric β-barrel membrane proteins was predicted with reasonable success [27].

5 Experimental Studies Complementing Computational Predictions

The emerging understanding of the organizational principles of β-barrel membrane proteins through computational studies has facilitated a number of experimental studies exploring a variety of questions. Here, we summarize three such studies that aim at altering the biophysical properties and/or identifying as yet unknown protein-protein interaction sites. These studies were carried out in bacterial OmpF β-barrel membrane protein, mitochondrial rat VDAC, and human Tom40 β-barrel membrane proteins.

5.1 OmpF Protein

OmpF from *Escherichia coli* belongs to bacterial porins, a well characterized protein family. It has a homotrimeric quaternary structure [47]. Porins allow diffusion of small solutes across the bacterial outer membrane. Bacterial porins can either filter solutes based on their molecular weight, as in OmpF protein, or have specific binding sites for certain solutes, as in Sucrose-Specific Porin (ScrY). Some porins form obligatory

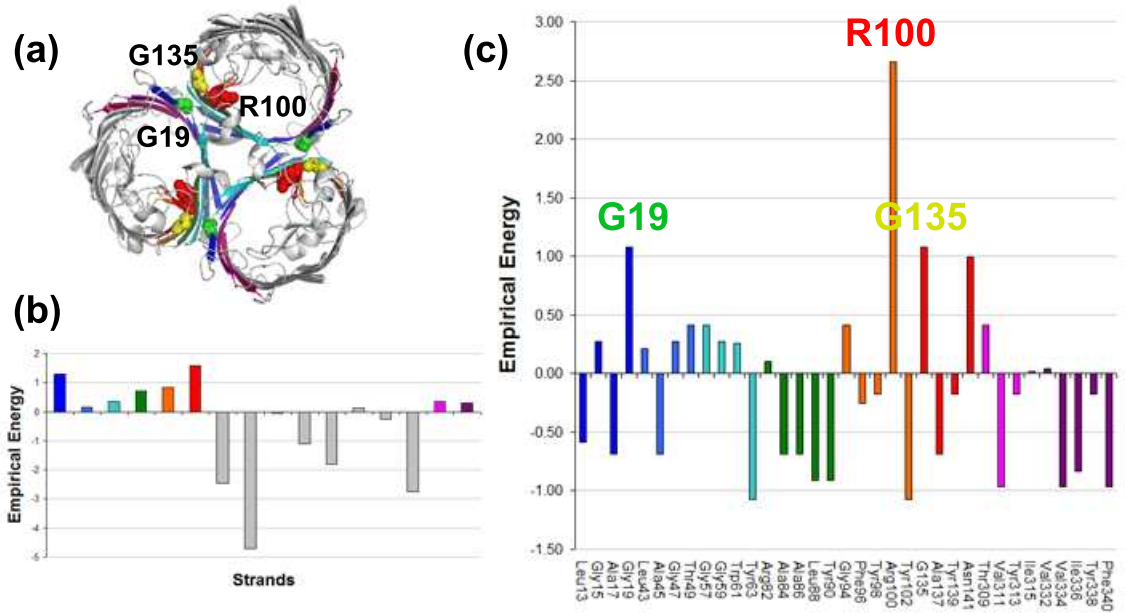

Figure 3: OmpF exists in trimeric form, with strands 1-5 and strand 16 forming the interaction surface in the crystal structure. The strands 1-6 and 15-16 also have the highest energy values according to the empirical energy function as shown in (b). These high energy strands largely coincide with the PPI interface and are termed weakly stable strands. The empirical energy profile of all residues facing the lipids in strands 1-6 and 15-16 are shown in (c). Residues G19, R100, and G135 have the highest empirical energy and are thus labeled weakly stable residues. The location of Residues G19, R100, and G135 in the PPI interface is shown in (a).

homotrimeric biological units, with significant in-plug domains in the interior of the barrel [47]. We have developed two computational approaches, namely, identification of weakly stable regions and identification of important residues through analysis of residue conservation in the extensive protein-protein interaction of OmpF protein. Using this design approach, we were able to engineer stable dimeric and monomeric mutants of OmpF protein that are not known to exist in nature [48]. These successes support the organizational principles identified in the earlier computational study [48]. Moreover, the study also demonstrates that oligomer disassociation in mutant OmpF protein can be separated from protein unfolding [48], in contrast to the long held belief that OmpF is an obligatory homo-trimer and that the processes of membrane insertion, folding, and oligomerization are coupled in OmpF protein [48, 49].

In bacterial porins, the exact protein-protein interaction site is known, but the purpose of oligomerization is not clear. For example, a functional monomeric form of PhoE porin, whose crystal structure is a trimer,

has been reported in *in vitro* and *in vivo* studies. [50, 51]. Stable dimeric OmpF has also been observed in both *in vitro* and *in vivo* experiments [49, 52–54]. Therefore, designing bacterial porins with different protein-protein interaction interfaces and oligomerization states can help in understanding why these proteins prefer a particular oligomeric state over the others.

5.2 VDAC Protein

The voltage dependent anion channel (VDAC) is the only β-barrel mitochondrial protein with a known structure [30]. It mediates the crosstalk between the cytosol and the mitochondria, and regulates the mitochondrial permeability to small molecules and ions [8, 9, 55]. Moreover, it is also implicated in Ca^{+2} homeostasis [8, 9] and apoptosis [8–10]. VDAC is known to form a number of oligomeric states (dimers, hexamers, higher order oligomers containing perhaps up to 20 molecules) [56–58]. In order to identify the protein-protein interaction sites of these oligomers, Guela *et al.* identified four weakly stable regions in the rat VDAC protein. Site-directed mutagenesis, combined with cysteine substitution and chemical cross-linking verified that the predicted weakly stable regions indeed form protein-protein interaction sites in VDAC oligomers under physiological and apoptopic conditions [59]. The study also suggest that the VDAC dimer undergoes conformational changes upon apoptosis induction to assemble into higher oligomeric states [59]. This investigation provides insights into the underlying mechanism of apoptosis and proposed a model describing the process of translocation of cyrochrome c from the inner membrane space of the mitochondria to the cytosol. This translocation of cytochrome c is thought to be responsible for the initiation of apoptosis [60, 61].

5.3 Tom40 Protein

Another mitochondrial β-barrel membrane protein, Tom40, is responsible for the translocation of many unfolded mitochondrial proteins from the cytosol into the mitochondria [62–64]. The structure of Tom40 is not known, but it is shown to be evolutionarily related to the VDAC family and is predicted to have a similar topology [65]. A number of other proteins interact with Tom40 and combine to form the TOM machinery [66]. Based on computational predictions, Gessmann *et al.* identified three unstable residues

in the weakly stable region of the human Tom40 protein. To test to what extent these unstable residues determine the conformational stability and oligomerization state of Tom40, these residues were substituted to leucines, which was predicted to be the most stabilizing amino acid when facing the lipid within the core region of a transmembrane β-strands. Site-directed mutagenesis with thermal and chemical denaturation experiments confirmed that the resistance of the mutant proteins to external perturbation was increased [67]. Specifically, the triple mutant with all three unstable residues substituted with leucine showed an increased resistance of $11°C$ to thermal denaturation, when compared to the wild type Tom40 ($84°C$ vs $73°C$) [67]. This stabilization was also accompanied by a change in oligomerization state, *i.e.* the triple mutant existed in primarily monomeric states compared to the wild type that could form dimers and higher order oligomers [67].

6 Outlook

The development of computational models and tools for the study of sequence and structural properties of β-barrel membrane proteins has led to significant insight into their organization principles. The discovery of the existence of weakly stable regions in bacterial OmpF, mitochondrial rat VDAC, and human Tom40 β-barrel membrane proteins seems to suggest that such regions are conserved across prokaryotes and eukaryotes. The functional significance of these regions appear to be different for homo-oligomers and hetro-oligomers. In the case of homo-oligomers, weakly stable regions arise as a result of random mutations, but in order to maintain biological fitness, these regions are stabilized by protein-protein interactions with other weakly stable regions. Oligomerization in this case may not directly confer new functionality, but instead provides structural stabilization. This is consistent with similar recent observation for soluble proteins [68]. In the case of hetro-oligomers, weakly stable regions provide an opportunity for the protein to interact with new partners and hence gain new functionality. This is consistent with the general hypothesis on the function of oligomerization in membrane proteins [44, 45].

A number of β-barrel membrane proteins are used as biological nanopores (e.g., porins and α-hemolysin) [69]. Due to the strong substrate specificity and an abundance of control points, biological nanopores have recently been adapted for novel applications, including reagentless DNA sequencing, monitoring of single-molecule chemical reactions, bioalarm systems, bio-inspired batteries, and nanotransistors [69–72]. However, their

limitations, such as lack of stability, non-flexible pore size, nonspecific binding, and undesirable oligomeric states, have hampered their applications in the uncontrolled environment of the real world compared to the controlled laboratory environment, where extreme temperature and denaturing conditions are often encountered. Recent studies have provided useful additions to the nanobiotechnician's toolbox by demonstrating how to alter the oligomeric state, stabilize the transmembrane domain, identify unknowm PPI interaction sites, and remove non-specific interactions of β-barrel membrane proteins [48, 59, 67]. These studies are likely to accelerate the efforts for designing novel biological nanopores.

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