Chapter 10
Dynamics of Membrane Proteins

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Abstract Transmembrane proteins inhabit a highly asymmetric environment that is, to a first approximation, two-dimensional. Many of them serve to transmit information between aqueous compartments, while others serve as conduits for the transport of material between compartments. In serving these functions they have to adopt at least two stable structures and rapidly interconvert between them. The paucity of atomic resolution structures has been limiting in elucidating the mechanisms by which these proteins carry out their functions. However, this century has seen the determination of the three-dimensional structures of a number of membrane proteins, leading to the start of an understanding of the dynamics displayed by them within the bilayer. Without attempting to be exhaustive, we provide illustrative examples of dynamics in membrane proteins and review their underlying mechanisms as they insert, fold and function in biological membranes.

10.1 Introduction

Proteins can be broadly classified by the milieu they reside in. Those embedded in the non-polar environment of membranes and which have portions that traverse the membrane, are called integral membrane proteins. These proteins carry out a variety of functions ranging from providing a local structural framework at the membrane to the transmission of physiologically critical material and information across membranes. Inasmuch as they form the interface between the cell and its environment, plasma membrane resident proteins have been the subject of intense study. However, the detailed characterisation of these proteins and their mechanistic understanding has been impeded because of the challenge of obtaining purified protein in sufficient quantities. Nevertheless, there has been progress made into structure determination of membrane proteins over the past two decades. While only 1% or so of protein structures deposited in the Protein Data Bank (PDB) over this period are of membrane proteins, the number of membrane protein structures determined in the twenty-first century greatly exceed the total number determined

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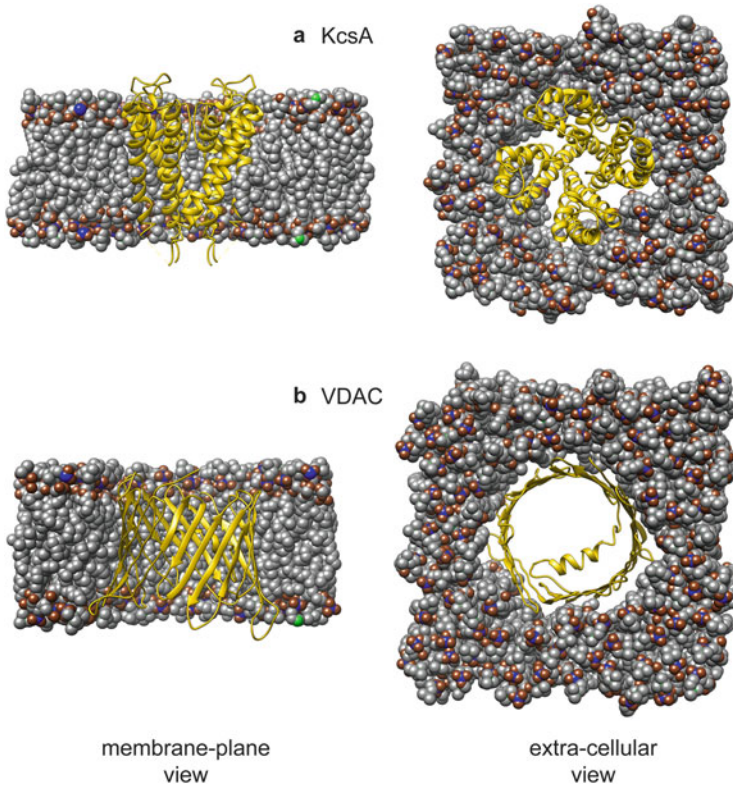e-mail: mathew@ncbs.res.in

Fig. 10.1 Two major categories of membrane proteins. Integral Membrane Proteins are either all-helical as in (**a**) or β-barrel as in (**b**). (**a**) KcsA—the first ion channel to be crystallised is an all helix protein; shown here embedded in the lipid bilayer. It is representative of the closed state of almost all potassium channels. The pore has a selectivity filter at the level of lipid head-groups in the upper leaflet visible in the cut-away model in the *left panel*. The C-terminus of the helices obstruct the channel. The *right panel* highlights the constricted channel aperture. On the other hand, there is Voltage Dependent Anion Channel (VDAC) (**b**). It is an interesting β-barrel protein found on the outer membrane of mitochondria. It posesses an atypical odd number of β-strands unlike other β-barrels which have an even number. The *right panel* shows the barrel from the top highlighting the lone helix that is hypothesised to alter the conductivity of the protein. Both (**a**) and (**b**) are drawn surrounded by a homogenous dipalmitoylphosphatidylcholine (DPPC) lipid bilayer modelled around the crystal structures available in PDB; [PDB id: 1BL8, 2JK4]

prior to this period. Armed with these static pictures, it is now becoming possible to understand the functional dynamics of membrane proteins.

The α-helix, first recognized by Linus Pauling 65 years ago [1] and the β-sheet, Pauling's "other" secondary structure [2], provided the only solutions to the problem of satisfying the hydrogen bonding requirements of the peptide bond within the bilayer (Fig. 10.1). The edges of a β-sheet would be unstable in a bilayer, hence it folds on itself to close and form a barrel in the membrane. β-barrels are seen primarily in proteins of the outer membranes of bacteria, mitochondria and plastids. While structures are available for a few β-barrel proteins (such as in Fig. 10.1),

considerably more structural and biochemical literature is available for proteins composed of transmembrane helices (TMHs). There is also a substantial bias towards information about plasma membrane proteins compared to those residing on endomembranes.

Proteins that mediate transduction of information and exchange matter across the membrane have to adopt at least two forms: one that facilitates transmission and another that does not. The interconversion between these forms is controlled by an external signal such as a ligand, voltage, pressure, temperature, etc. However, the proteins first have to be made on ribosomes as linear polypeptides, folded into their final three-dimensional (3D) structure and then be transported to their location of action where they function. Hence, the dynamics of such membrane proteins can be studied broadly at three stages: (i) insertion into the membrane and folding therein, (ii) transport to the appropriate organelle and (iii) interconversion between a transmission competent state and the ground state.

10.2   Membrane Protein Translocation/Insertional Dynamics

Protein dynamics initiate right when a polypeptide starts emerging from the ribosomal exit tunnel. The highly hydrophobic nascent polypeptides have to be folded and transported to their site of function. While there has been considerable progress studying the folding of soluble proteins, membrane proteins pose major difficulties. Unfolding membrane proteins to structure-less entities generally results in irreversible denaturation, while refolding from partially folded states fails to capture the entire process (reviewed by Stanley and Fleming [3]). A further complication occurs due to the fact that the membrane ambient into which the protein has to fold is not homogenous, being polar at the head-groups and very non-polar at the mid-plane [4].

Khorana's group showed that individual helices of bacteriorhodopsin (bR) can insert and fold independently, then assemble into a functional entity capable of binding retinal and pumping protons on absorbing light [5]. This led to the postulation of a two-stage folding process with independent insertion of individual helices, followed by their assembly into a higher order structure within the membrane [6]. Kaback's group expressed lactose permease as contiguous polypeptides and if the breaks between fragments are introduced in loop regions, the peptides could associate to mediate active lactose transport [7]. Thus, the case for two-stage folding is strong, but the data is limited to very few membrane proteins.

Recent experiments have used cell free translation systems to extend this knowledge to insertion of freshly synthesised bacteriorhodopsin into nanodiscs. These data indicate that, in this system, retinal is required for formation of the fully folded and functional form of the protein [8]. However, for most membrane proteins, we are limited to the knowledge of the machinery that promotes insertion into the membrane.

10.2.1   Prokaryotic Translocation Machinery

10.2.1.1   α-Helical Proteins

SecYEG Translocase

α-helical proteins are predominantly found in the inner membrane of Gram nega-
tive bacteria. The growing hydrophobic chain emerging from the ribosome exit
tunnel is identified by a Signal Recognition Particle (SRP) [9]. A groove within the
SRP recognises the non-polar signal peptide that might be the first TM segment or a
region distal to it [10, 11]. The signal peptide loaded SRP is then transferred to the
SecYEG translocon by the SRP-FtsY composite. FtsY is a receptor for SRP and
utilises energy from GTP hydrolysis to load the nascent polypeptide on the SecYEG
complex. SecY is a 10 transmembrane helix protein that appears to have two exit
points [12]. One is a channel with a 3–5 Å hydrophobic constriction and the other
permits lateral entry to the membrane [13, 14].

An α-helix blocks the channel in SecY and the lateral entry port between
TM2-TM7 is also gated. The former is opened by signal peptide binding and the
latter by protein translocation through the channel [14]. This process is initiated
when the nascent polypeptide inserts into the channel as a loop. Signal peptide gets
intercalated into the walls of SecY while the distal region gets close to the pore
[15]. This binding moves the pore blocking helix by about 20 Å [16]. Subsequently,
the lateral entry gate opens into the membrane on protein translocation into the
channel, enabling insertion into the membrane. The signal sequence remains bound
till the entire protein has passed through [17].

Peptide propulsion through SecY requires the force generated either by the
ribosome itself [18] or by an ATPase-SecA [19] and a proton motif force
[20]. SecA has a two helix finger domain which is found near the entrance of the
SecYEG channel [21]. It is believed that this two helix finger pushes substrates in to
the channel [22]. Moreover, binding of SecA to SecY creates a 5 Å gap in the lateral
gate manned by TM2 and TM7 [21]. Hence, SecY in conjunction with SecA or the
ribosome can mediate co- or post-translational insertion, respectively, in the inner
membrane of Gram negative bacteria.

YidC Insertase

The YidC Insertase enables integration of membrane proteins in a SecYEG-
independent manner [23]. In contrast to SecYEG translocon that can mediate
both co- and post-translational translocation, YidC facilitates only post-
translational translocation [24]. How a substrate protein chooses between alternate
pathways to integrate into the membrane is not currently known. But, it is worth
noting that YidC can associate with SecYEG to form a supercomplex [25].

The YidC structure reveals a novel fold. It has a cytoplasmic helical hairpin and
a central membrane embedded groove that opens towards the cytoplasm and the

membrane. It is proposed that the cytoplasmic hairpin facilitates the entry of the substrate protein into the groove. The groove being blocked on the extracellular side, guides the processing TMH to slide into the bilayer core [26].

10.2.1.2 β-Barrel Proteins

β-barrel proteins are found in the outer membrane of Gram negative bacteria. The insertion of these outer-membrane proteins (OMPs) is mediated by a complex machinery composed of a Bam supercomplex and a few chaperones [27]. Upon synthesis in the cytoplasm, the polypeptide is carried across the inner membrane by the Sec translocon (Sect. 10.2.1.1). Once in the periplasmic space, it is bound by chaperones SurA and Skp which protect and escort the nascent OMP to the outer membrane [27]. A number of weak contacts mediate the transfer of the protein to the Bam complex where it is folded and passed into the membrane.

Multiple crystal structures are available for the 5 Bam(A–E) individual proteins that make up the supercomplex (reviewed by O'Neil et al. [28]). Moreover earlier this year, multiple groups solved the structure of the complete complex [29–31]. This has brought substantial advance into understanding the molecular architecture that inserts and aids the formation of tertiary structure of an OMP.

10.2.2 *Eukaryotic Translocation Machinery*

10.2.2.1 α-Helical Proteins

Despite the large number of membrane bound organelles, eukaryotes have only three sites of TMH insertion. Co-translational insertion occurs at the endoplasmic reticulum (ER), inner mitochondrial and the thylakoid membrane. Sec61 which is present on the ER membrane is a homologue of SecY [32]. Though absent in the ER, Oxa1 in the inner mitochondrial membrane and Alb3/Alb4 in chloroplasts appear to be YidC homologues in eukaryotes [24]. Post-translational translocation has also been observed at the ER mediated by Sec62 and Sec63. There are other proteins in the ER that aid the translocation in specific ways (reviewed in Denks et al. [33]) and include TRAM, TRAP, oligosaccharyl transferase, BiP, etc. The process of targeting proteins to different membranes is beyond the scope of this article but the reader is referred to an excellent review [34].

10.2.2.2 β-Barrel Proteins

In Eukaryotes, β-barrel proteins are found predominantly on the outer membrane of mitochondria and plastids. Homologues of the Prokaryotic OMP insertion and translocation machinery are found in the Eukaryotic organelles [35]. In the mitochondrion, polypeptides are imported from the cytoplasm through the TOM

(Translocase of Outer Membrane) complex where TOM40, itself a β-barrel, forms the protein conduction channel. The imported protein is stabilised by small TIM (Translocase of Inner Membrane) chaperones—Tim9 & Tim10 and MIA (Mitochondrial Inner membrane Assembly) chaperones. The SAM (Sorting and Assembly Machinery) complex finishes the insertion of β-barrel precursors into the outer membrane. In the SAM complex, SAM50 is thought to perform the same role as the Prokaryotic protein—BamA [36]. The chloroplast membrane protein insertion pathway remains relatively obscure with only a few implicated proteins (Tic-Toc complex) that have been characterised [37].

10.3 Energetics Underlying Protein Dynamics in Lipid Bilayers

Integral membrane proteins, especially TM helices, upon reaching their destination are met with similar and dissimilar hydrophobic molecules. In other words, they reside in a complex milieu where lateral interactions between adjacent helices occur in competition with interactions with surrounding lipids and also with the encapsulating water. Furthermore, there are global bilayer effects which arise due to the electric field across the membrane and phase separation amongst lipids. Several studies have been undertaken to elucidate the forces stabilising the final 3D structures adopted by TMHs. Some understanding of the energetics of transitions between stable states has also been obtained in a few cases.

10.3.1 Van der Waal's Interactions

Van der Waals (VdW) packing interactions contribute significantly to the integrity of the core of membrane proteins. This is in contrast to soluble proteins where the core is stabilised by hydrophobic interactions [38]. However, membrane proteins cannot utilise the entropic gain of partitioning away from water if their interactions occur in the interior of the bilayer. The first example that emphasized the importance of VdW interactions in membrane protein dynamics came from studying the transmembrane helix (TMH) of Glycophorin A (GpA) [39]. Interhelical packing in GpATM was more stabilising compared to helix-lipid interactions, thereby promoting its homo-dimerization. The dimer interface in GpA is formed by a GxxxG motif where the Glycines occur on the same side of the lone TMH of GpA [40]. GxxxG has become probably the most studied dimerization motif in membranes and we take it up in more detail in Sect. 10.4.1. In addition, the VdW packing in membrane proteins is optimized for function. For instance, bR has only a fourth of void space in the interior of the protein when compared to mechanosensitive channels like MscL (Mechanosensitive Channel of Large conductance) which are

loosely packed [41]. The empty pockets in receptors and channels allows for conformational flexibility required to undergo gating transitions [41].

10.3.2 Hydrogen Bonding

Hydrogen bonding is widespread in membrane proteins. Apart from participating in secondary structure formation, hydrogen bonding is also critical for tertiary structure formation. However, experimental determinations of the contribution of hydrogen bonds to stability fall short by 3.5–4.5 kcal mol^{-1} of the expected value in several cases [42]. Nevertheless, a single polar residue has been shown sufficient to drive homo-dimerization of an otherwise hydrophobic model TMH [43, 44]. The contribution of Glycines is also particularly well studied in the context of hydrogen bonding in membranes. Especially, the GAS$_{Right}$/GxxxG/GG4 motif has been shown to be partly stabilised by hydrogen-bonding [45].

10.3.3 Salt Bridges

Salt bridge interactions are thought to contribute over long range [46]. Debye lengths in the interior of membranes can be very long compared to those in the aqueous phase. Further, the energy of all electrostatic interactions is enhanced in the low dielectric of the bilayer. Making a salt-bridge in a hydrophobic membrane is also energetically favoured when the contributing charged residues are in close proximity as seen in bacteriorhodopsin [47, 48]. Since burying a charged residue in the bilayer core is energetically costly, salt-bridges often contribute to function. There is experimental evidence supporting the role of specific salt bridges in altering the functional state of α-helical membrane proteins such as CFTR (Cystic Fibrosis Transmembrane conductance Regulator), Kcv (Potassium channel chlorella virus) [49], rhodopsin and TCR (T-cell receptor). In CFTR, a change from such coulombic interactions between R347-D924-D993 to R352-D993 can change its conductance [50]. Notably, rhodopsin becomes constitutively active upon removal of one salt-bridge [51] and salt bridge formation can promote homo/hetero-dimerization of TMH in TCR α, β and ζζ [52]. Interestingly, the dimerization in TCR occur through Aspartate and Threonine residues in preference to Glycines of GxxxG which are also present in the latter protein.

10.3.4 Aromatic–Aromatic Interactions

The WxxW, WxxY, YxxY motifs have also been shown to drive dimerization of TMHs through aromatic-aromatic interactions between Tryptophan and Tyrosine

residues [53]. These are long range (approx. 7 Å) interactions, usually considered in the context of strengthening the tertiary folded structure of integral membrane proteins [54], e.g. there are seven aromatic pairs that form in KcsA. Also, a ZAX motif, where Z = Alanine, Tryptophan, Phenylalanine, or Tyrosine and X = Alanine, Histidine, Lysine, Arginine show very high dimerization affinity in TMHs. This motif is stabilised by cation-π interactions [55]. It is the same cation-π interactions between protein and lipids that preferentially stabilise Tryptophan and Tyrosine residues at the membrane-water interface [56] and sometimes promote tilts in TM helices [57].

It should be noted that lipids in the bilayer have also been implicated in enabling interaction of TMHs and affecting function (reviewed in [58]). These interactions may be specific or non-specific and manifest themselves as change in helical tilts, perpendicular shifts of helices with respect to the membrane normal (reviewed by Lee [59]) or even inversion of helical topology post-insertion [60]. Lipids can also modulate the strength of existing dimers, shifting their dynamic equilibrium, like it is shown for the Glycophorin A TM dimer [61].

10.4 Dynamics of Helix Dimerization

The binding of ligands to signal transducing receptors on one side of the membrane, results in conformational rearrangements leading to changes in accessibility of critical residues on the other side. This alters protein-protein interactions with downstream binding partners that translates to a signal being transduced in the far compartment. Transmission of binding information across the membrane may be expected to implicate conformational rearrangements of transmembrane helices. The simplest case would involve proteins with a single transmembrane helix. We review instances of protein-protein interactions mediated by a single transmembrane helix (TMH) in different contexts focussing on well-characterised instances of bitopic proteins.

10.4.1 GxxxG Motif

The GxxxG/GG4 or the GAS$_{Right}$ motif is a *bona-fide* motif that induces dimerization of α-helices both in bilayers and micelles. This motif was first observed in Glycophorin A (GpA) [39, 62] which itself was one of the first membrane proteins to be sequenced [63]. Shortly after the protein was sequenced, it was observed that the TM region of GpA (GpATM) is responsible for dimerization of the protein [64]. This was inferred from Dodecyl Polyacrylamide gels (SDS-PAGE) of erythrocyte ghosts and it wasn't until a decade after, that dimerization in gels could be reproduced synthetically in liposomes [65]. On mapping the dimerization interface of GpATM, it was found that a minimal sequence of GxxxG, where the 'x'
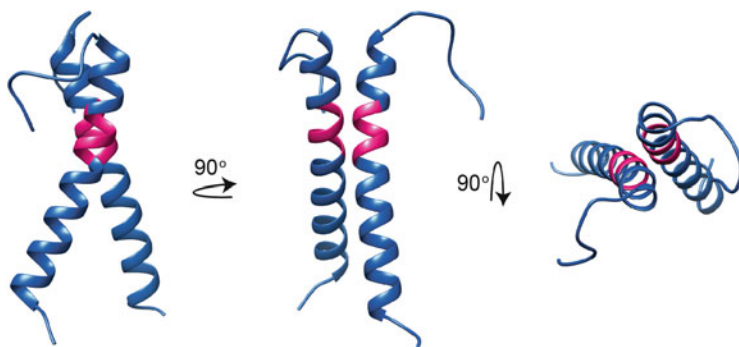
Fig. 10.2 Dimerization of Glycophorin A TM (GpATM) helix. GpATM is one of the most studied examples in the context of helical dimerization in membranes (see Sect. 10.4.1 in the main text for more details). The GxxxG motif (shown in *pink*) utilises hydrogen bonding and Van der Waals interactions to stabilise an SDS resistant helical dimer

represent dimerization insensitive amino-acids, could cause dimerization of unrelated transmembrane helices [66]. This discovery marked GxxxG as a common dimerization motif. Subsequent work which produced a NMR (Nuclear Magnetic Resonance) structure of the GpATM validated the biochemically identified interface (Fig. 10.2) [40]. Also, a search through the sequences of transmembrane helices available at the time revealed an enrichment of the said motif pointing to its physiological importance [67].

Proteins apart from GpA, like EGFR [68], ErbB4 [69], BNIP3 [70], etc. carrying a GxxxG motif form similar right handed helical TM dimers with a crossing angle of –40°. Of all the 3D structures of TM helices—bitopic and polytopic—in membrane proteins, when clustered pairwise, 12.8% had similar parallel, right-handed geometry as dimeric GpATM [71]. Proteins like RTKs (Receptor Tyrosine Kinases—ErbBs), neuropilins [72], immunologically important receptors like TLRs (Toll-like Receptors) [73], MHC (Major Histocompatibility Complex) [74] and Integrins [75, 76] have all been shown to use the GxxxG motif to dimerise. This geometry is stabilised by Van der Waals packing and hydrogen bonding (Sect. 10.3) [45].

10.4.1.1 GxxxG Is Not Sufficient for Dimerization

The experimental tools developed during discovery of GxxxG as a dimerization motif spawned a great deal of research on finding other such motifs. These validation tools utilise a similar concept of expressing TMH of interest fused with a protein that is active only as a dimer. First of these was ToxR, where the TMH dimerization induces ToxR dimerization leading to controlled expression of β-galactosidase enzyme which can be monitored [77]. Variations of this method include TOXCAT [78], POSSYCCAT [79], GALLEX [80], BACTH [81], AraTM [82] and MaMTH [83]. As a result, polar motifs (SxxxSSxxT and SxxxSSxxT) [84],

glycine zipper (GxxxGxxxG) and its variants with SAT substitutions [85], WxxW, WxxY and YxxY [53] have been shown to cause association of monomeric TMHs. Still, these dimer interfaces have limited associated literature which has been mentioned at the appropriate places.

These observations lead to the idea that any small-xxx-small motif could cause dimerization. However, this assumption was proved incorrect and believably so, as more than 55% of all predicted TM helices have a small-xxx-small motif [86]. On similar lines, it was also found that GxxxG in GpATM is also sensitive to the sequence context in which it occurs [87, 88]. Neither artificially enriching trans-membrane sequences from a randomised pool nor searching the entire sequence space of naturally occurring sequences carrying the dimerization motif, reveals any common contextual scaffolding pattern for GxxxG [86].

Studying GxxxG on more proteins revealed that the presence of GxxxG by itself does not cause dimerization. When recording dimerization status of peptides in denaturing polyacrylamide gels, unlike GpATM, a vast majority do not show association [89]. Even a high sequence conservation does not ensure that the GAS_{Right} motif will convey association of the TMHs [90, 91]. In the same vein, its corollary is also true; not every TM helical interaction relies on GxxxG or small-xxx-small motif. For instance, Dap12 which is an immunologically important, signalling competent receptor that associates with other proteins such as the T-cell receptor was shown to use a polar residue to dimerise in spite of the presence of a GxxxG motif [92] (See also Sect. 10.3.3). Another example illustrating the same is discussed in Sect. 10.4.2.1.

10.4.1.2   Ab Initio Prediction of Dimerization

Despite the advancement of technology, most simulations of transbilayer segments are computationally limiting. The application of force-field based modelling has remained the only option for membrane biologists trying to study helical interactions until recently. Nevertheless, simplified approaches are being developed to circumvent the issue. PREDDIMER is one such algorithm that is available through a web server [93]. Another method—CATM, is available as an open-source compilable C++ library on the internet [45]. CATM screens the helical interface with respective to a set of 463 geometries that any GxxxG motif can afford and then optimises the resulting structure using Monte Carlo simulations. While CATM presently only computes homo-dimeric interactions, PREDDIMER can be used for both homo- and hetero-dimeric structures. Both these solutions have stood validation against the experimentally solved available 3D structures for interacting TM helices.

In summary, we can explain some of the available structures containing the GAS_{Right} motif invoking hydrogen bonding and Van der Waals forces which has enabled respectable prediction of helical dimerization in membranes. However, more work is needed to have a unifying model to understand and predict the mechanism of GxxxG driven dynamics in natural membranes.

10.4.2   Beyond a Passive GxxxG Motif

A high resolution 3D structure alone isn't enough to furnish mechanistic information about a protein. Nevertheless, it allows for more guided experiments to be performed. More importantly, it provides researchers with a starting point for simulating the molecules. Once, arguably the largest barrier for computational biologists—*de novo* tertiary structure prediction is surpassed, molecular dynamics (MD) simulations can help in understanding their dynamics in nature [94]. This approach has helped gain deeper insights into GxxxG driven dimerization and glean more general principles which can be applied to complex polytopic transmembrane proteins such as ion channels (KcsA, MscL; Sect. 10.6.1) that have a glycine zipper motif.

10.4.2.1   GxxxG Motif as a TM Switch

The signalling proteins mentioned in Sect. 10.4.1 use as diverse a range of domains on extracellular and intracellular side of the plasma membrane as the number of signals themselves. Despite the multitude of folds and structures on either side of the membrane, all these proteins traverse the plasma membrane using a single TM helix. The GxxxG motif has been implicated in stabilising the dimerization of such TMHs. But there are instances, where using two GxxxG motifs, a TMH could go from one dimeric conformation to another. If two GxxxG motifs occur at the N- and C-termini of the same helix, then it becomes straightforward to imagine how two motifs can enable two structurally distinct forms to exist (Fig. 10.3). Also, one could hypothesize the introduction of an energy barrier between the two, such that interconversion between these two forms can be coupled to another process. Thus, hinting at the potential of GxxxG as a naturally occurring intramembranous molecular switch.

   It was in 2002 that the role of GxxxG as a switch was first formally hypothesised [95]. However, it was not until 11 years later that experimental evidence supporting the hypothesis was obtained in a plasma membrane bitopic receptor protein—EGFR (Epidermal Growth Factor Receptor) [68, 94]. EGFR or ErbB1 is one of the four Erb receptors of the Receptor Tyrosine Kinase family and being the cell surface receptor of the EGF peptide ligand, it has a crucial signalling role in cellular proliferation, migration and differentiation. But, its ligand-mediated activation was not clear until the TMH was studied. EGFR/ErbB1 was found to go from an active to inactive state utilising the two small-xxx-small motifs in its TMH (Fig. 10.3). Performing MD simulations on the structures of transmembrane and juxtamembrane segment obtained by NMR spectroscopy, it was demonstrated that the TMH of ErbB1/EGFR can indeed stabilise in either of the N- or C-terminal dimers. This change in the mode of dimerization controlled by EGF binding/unbinding modulates the activity of cytosolic kinase domains of EGFR [68] that has been implicated in certain cancers for over 30 years.
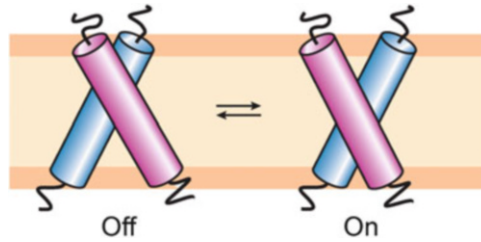
Fig. 10.3 Small-xxx-small motif in intra-membrane dynamics. A cartoon depiction of EGFR TM helices. The *helices* are proposed to switch from one conformation to another to shuttle between a signalling "On" state and an "Off" state. With both these interfaces stabilised by the small-xxx-small motif, the helices can lock-in either of them, affecting the interaction of the juxtamembrane region. This transduces the activation caused by extracellular ligand binding to kinase activity in the cytoplasm

Another notable example that demands a mention here is that of ErbB2 or HER2 (Human EGF Receptor 2). ErbB2, like EGFR, has two small-xxx-small motifs in its TMH [96]. It was shown that mutating the C-terminal GxxxG motif did not affect the dimerization potential to any significant degree [96]. Moreover, from the NMR structure it was found that ErbB2 used the N-terminal motif for homo-dimerization [97] This observation would appear confounding to the argument to have two dimerization motifs on a transmembrane helix for switching between active and inactive states. However, earlier this year another NMR structure of ErbB2 was solved [98]. Surprisingly, it was found that the TM helix of ErbB2 can indeed form a C-terminal right-handed dimer but without using the GxxxG motif. Instead, it uses an unusual (Sect. 10.4.1.1), highly hydrophobic stretch of residues IxxxVxxLLxxVLxxVFxxL. Thus, even ErbB2 can form both N- and C-terminal dimers with their potential use as a switch. Also, the C-terminal GxxxG motif of ErbB2 is suggested to be involved in hetero-dimerization [99].

10.5 TM Helices in Endomembranes

There is some evidence that STIM1 (Stromal Interaction Molecule 1) on the metazoan endoplasmic reticulum (ER) membranes can also form dimers with the small-xxx-small motif in its single transmembrane helix [100]. This dimerization has implications for the conversion of STIM1 from inactive to active state. The inactive to active state conversion is initiated by depletion of Ca^{2+} in the lumen of the ER. This signal is translocated across the ER membrane to activate Orai1, a highly selective, STIM1 gated Ca^{2+} channel. STIM together with Orai orchestrates the process of Store Operated Calcium Entry (SOCE) which is crucial for immunological signalling and cellular Ca^{2+} homeostasis; reviewed by Prakriya and Lewis [101].

ADCK3 (AarF Domain Containing Kinase 3) is a mitochondrial inner membrane protein which has been categorically studied in the context of helical dimerization. It also utilises a Glycine zipper motif (Sect. 10.4.1.1) to form homodimers [102]. ADCK3 protein has a role in Coenzyme Q (Ubiquinone) biosynthesis and a naturally occurring mutation in ADCK3 has been correlated to respiratory phenotypes and cerebellar ataxia [103]. However, the physiological relevance of homodimerization of ADCK3 TMH remains a speculation. Thus, despite the unarguable biological importance of integral proteins on endomembrane organelles, the transmembrane region of these protein remain understudied. This is exemplified by almost no literature highlighting more than a passive role for the TM region of such proteins.

10.6   Dynamics of Ion Channels

The extension of dimerization studies to multi-pass membrane proteins has been limited, due in part to the relatively small number of well characterized instances where a reversible equilibrium association can be observed and manipulated in native membranes. One system that has been studied, is the homo-dimeric ClC-ec1 Cl^-/H^+ antiporter of *Escherichia coli*. This 50-kDa membrane protein dimerizes via a membrane embedded, non-polar interface lined mainly by Isoleucines and Leucines. Chadda et al. [104] have diluted this protein into liposomes to the point where monomers and dimers can both be observed by single molecule fluorescence. Such an approach has allowed the extraction of thermodynamic parameters, including the free energy of dimerization, but fails to provide atomic level information of the dimerization process [104].

There are relatively few multi-pass membrane proteins for which a mechanism of transition between stable states has been worked out at atomic resolution. Of these, ion transporters are probably the best understood and have been extensively studied for four decades. The first structure of a membrane protein [105] was of bacteriorhodopsin (bR)—a light-driven H^+ pump—which is also the first membrane protein to have essentially all its characterised intermediates crystallised. These structures and a vast body of biophysical and mutagenesis studies have revealed individual steps of proton translocation through the protein coupled to movements of transmembrane helices. These include long range proton migration using a hydrogen-bonded chain, proton transfer between protonable groups within the membrane and also proton transfer utilising water molecules present between protein residues [106]. Consequently, it is the best understood membrane protein today. After bR, the protein that attracted concerted attention was the voltage gated potassium channel that is involved in the generation of action potentials in excitable tissues. Ion channels constitute a class of transporters that stabilize an aqueous pore across the membrane through which ions can diffuse down their electro-chemical gradient. These pores are responsible for electrical signalling in the nervous system, volume regulation and other critical physiological processes. The appearance of a

series of high resolution structures of ion channels makes it possible to infer the stimulus-driven conformational changes underlying the transition from conducting to non-conducting states and *vice-versa*.

Ion channels stabilise columns of water that traverse the membrane and hence the functional channel has multiple pore-lining helices. For a given stoichiometry of ideal helices lining the conducting pore, the enclosed pore radius depends on the tilt of the helices with respect to the membrane normal. Conversely, for a given helical tilt, the pore radius increases with the number of constituent helices. Thus, gating transitions from conducting to non-conducting states can be achieved by altering helical tilt or by changing the number of constituent helices. The disposition of side chains that project into the pore lumen is another critical feature determining effective pore radius.

10.6.1 Voltage Gated K^+ Channels

Voltage gated ion channels are tetrameric proteins contributing one helix per subunit to the aqueous pore. These channels are strongly ion-selective. Selectivity is conferred by a selectivity filter which is non-helical and tightly packed against the pore helix. This ensures that interatomic distances within the filter remain constant so as to achieve selectivity between ions as similar as Na^+ and K^+ (Fig. 10.4). Much of our knowledge of ion channel structure starts with a series of crystal structures of prokaryotic channels solved by Rod MacKinnon. The first such structure was that of the KcsA, a homo-tetrameric K^+ channel that crystallised in the Closed State [107] (Figs. 10.1 and 10.4). The protein has two transmembrane helices, one of which contributes to the lining of the aqueous pore. The re-entrant loop connecting these helices contains the selectivity filter. The pore-lining helices are at an angle to the membrane normal, and the point of closest approach or the "bundle crossing" point between them leaves insufficient room for a hydrated K^+ ion to pass. The MacKinnon group subsequently solved the structure of the MthK channel, which is also K^+ conducting, but crystallised in the Open State [108]. A comparison of these structures revealed that channel opening and closing probably proceeds through an iris-type opening, involving bending of the four pore-lining helices so that the diameter at the narrowest point is about 12 Å (Fig. 10.4).

Voltage gated ion channels undergo a transition between the Open (or conducting) and Closed (or non-conducting) States in response to changes in transmembrane electric field. These two stable states differ by about 10 Kcal mol^{-1} in energy at resting membrane potentials. For simplicity, we will not consider other states such as an activated state or any of the inactivated states, all of which are non-conducting. Each subunit of voltage gated K^+ channels (K_v channels) has six transmembrane helices with a re-entrant loop, containing the selectivity filter, connecting the last two helices. These last two helices and included loop are analogous to the KcsA and MthK channels. The last helix, S6, lines the pore while the positively charged fourth helix, S4, constitutes the voltage sensor. This
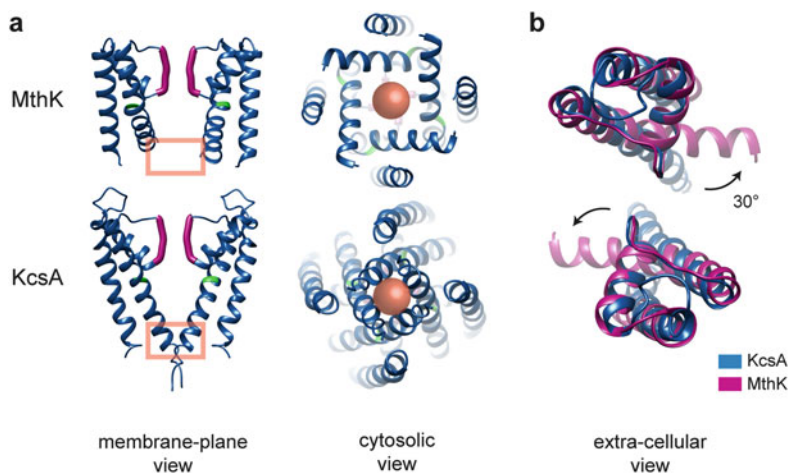
Fig. 10.4 The Open ↔ Closed transition in ion channels. The bacterial potassium channels KcsA and MthK crystallised in the Closed and Open States respectively. (**a**) Views of the two tetrameric structures without lipid and with two chains omitted for clarity. *Top*: MthK which crystallised in the Open State. Highlighted in *green* is the conserved Glycine residue that allows a kink in the pore lining helix. *Bottom*: KcsA which crystallised in the Closed State. Note the bundle crossing of the helices highlighted by a rectangle in the view at *left*. The non-helical, tightly-packed selectivity filter is shown in *magenta*. The *orange spheres* represent the space between the helix backbones towards the cytosolic end of the pore as viewed from a cytosolic vantage point. The bend in the helix in MthK that occurs around the highlighted Glycine is responsible for opening of the pore by about 12 Å to allow for a hydrated K^+ to pass. The pore lining helices splay about 30° to open the channel as represented in (**b**); [PDB id: 1BL8, 3LDC]

basic architecture of the channel is shared by all voltage gated channels whose structure has been solved to date.

The determination of crystal structures of rat $K_v1.2$ in the Open State [109] provided a starting point to understand the dynamics of the voltage driven Open to Closed transition of the channel. Deducing a model for the Closed State starting from an open structure requires integrating the vast body of biochemical and electrophysiological work, coupled with data on the mutagenesis of a significant fraction of the residues to elucidate conformational switching in these proteins. This challenge has been approached by several groups [110–112]. Unfortunately, in the absence of an experimentally determined structure for the Closed State, there is no final answer at this time. Upadhyay et al. [111] started with the crystal structure of $K_v1.2$, which has a bent S6 as in MthK (Fig. 10.4) and modelled the Closed State of the pore based on the structure of KcsA [107]. The pore lining S6 helix had to be remodelled by the action of the electric field so as to achieve the narrow "bundle crossing" seen in the KcsA structure. This involves straightening of the S6 helix, resulting in closure of the channel inasmuch as the C-γ of V408 in diagonally opposite subunits are positioned around 5.3 Å apart. This spacing generates a constriction too small to be negotiated by a hydrated K^+ ion (which is over 8 Å in diameter).

Like the potassium channels (K_v), voltage gated Na^+ channel and Ca^{2+} channels, are Closed at the resting potentials of neuronal cells, around -60 mV. They open on depolarising the cells—i.e. going to less negative potentials. The early view of channel gating was that electric field actively forced the channels into opening from a "ground" Closed State. On the other hand, Upadhyay et al argued that since the crystals were formed in the absence of a bilayer, they are perforce zero-field structures. Which is to say that the Open State of the channel is the "ground state" of the channel. This is supported by the observation that all available structures of voltage gated channels are of the Open State. The application of an inside-negative electric field applies force on the voltage sensing helix, S4, moving it towards the cytoplasmic side of the plasma membrane. S4, in turn, tugs on the S4–S5 linker so that S5 prods S6, resulting in its "unkinking" and straightening, which constricts the spacing at the bundle crossing point. The Closed State is thus a "cocked gun" kept closed by action of the electric field. Once this is relieved by depolarising the membrane, S5 (by the action of S4) moves away from S6 allowing it to swing open rapidly resulting in channel opening. Since the electric field is used to constrain S6 into the Closed State, an S4 with significantly lower charge would require a greater electric field to apply the same pressure on S6 to keep it closed. The most dramatic instance of such a shift in the voltage of channel opening is probably that reported by Miller and Aldrich [113] for the double mutant R365N: R371I of the Shaker channel which has a mid-point of activation at -180 mV as compared to the wild type channel which opens at -20 mV.

10.6.2   Ligand Gated Ion Channels

Ligand gated channels can be broadly classified, for the purposes of this review, into two classes—those with four subunits and those with five. Glutamate receptor channels are the prototypic tetrameric neurotransmitter receptors while serotonin, GABA, glycine, and acetylcholine gate pentameric receptors. Though structures of ligand binding domains have been available for some time in both liganded and free states, an understanding of how ligand binding leads to channel opening requires the structure of the full length channel. These have been elucidated over the past few years allowing the examination of plausible pathways to ligand driven transitions in these proteins.

Glutamate receptors come in several flavours, named after ligands that can activate the channel (apart from glutamate, which opens all GluRs). Structures of full length AMPA channels have been solved with either an antagonist bound [114] or with an auxiliary subunit called TARP bound to it [115]. In both cases, the channel is in a closed state. Here the narrowest point of the channel is the bundle crossing point of the M2 helices from each of the subunits, which is structurally similar to the bundle crossing seen in the S6 helices of voltage gated K^+ channels. Thus, channel opening is presumed to follow an iris opening transition as inferred for the K^+ channels. The conformational changes in the ligand binding domain effected by binding of ligand

have been observed in crystal structures of the isolated domain and the manner in which this is likely to affect the transmembrane helices is apparent [116].

The pentameric ligand gated receptor family includes receptors for nicotinic acetylcholine (nAChRs), serotonin (5-HT3Rs), glycine (GlyRs) and GABA (GABAARs). X-ray structures of the ancestral prokaryotic receptor channels were solved almost a decade ago [117–119]. These channels are symmetric homo-pentamers, whereas eukaryotic channels are often hetero-multimeric. Nonetheless, the prokaryotic channels probably represent a common minimal core. Indeed, more recent structures of a mouse serotonin receptor [120], a human GABA receptor [121] and glycine receptors from humans [122] and zebrafish [123] confirm this conservation.

The transition between Open and Closed States can be clearly seen in comparing crystal structures of the channels in the respective states. Fortunately, there are structures of the Closed State of unliganded channels (GLIC at pH 7 and the Glutamate gated Chloride channel [117, 119, 124]) as well as the strychnine-bound state of the Gly receptors [123]. In all of these structures, side chains of long, hydrophobic amino acid residues point into the lumen of the pore restricting it to under 2 Å radius precluding the transport of hydrated ions like Na^+. The presence of the hydrophobic side chains prevents passage of desolvated ions, which would need compensatory ligands from the channel lining residues. Open State structures of GLIC at pH 4 [117, 119, 124], glutamate activated chloride channel in the presence of agonists [125, 126] and the glycine receptor bound to glycine [122] are also available. The pore-lining M2 helices move outwards towards the M3 helices of the same subunit and also undergo an anticlockwise rotation which move the constricting Isoleucine side chains away from the pore and towards the helical interface. This results in an opening of the pore to allow the passage of hydrated ions. Such a model of channel opening is consistent with a variety of mutagenesis studies which implicate an activation-gate hydrophobic block in the nAChR [127], as well as the finding that mutation of the critical hydrophobic resides to polar residues results in stabilisation of the Open State.

10.7   Concluding Remarks

There are several stages in the life of a membrane protein where dynamics play a critical role. We have very little information on the folding of membrane proteins, although the machinery involved has been identified. It may be, that the process will have to be studied with the full panoply of the cellular machinery intact. Trafficking of the membrane integrated protein to its target location is well studied. Operation of the protein in its native location requires the elucidation of structures of the protein in the various stable states that it adopts. The gradually increasing repository of atomic resolution structures provide a starting point for understanding how these remarkable proteins do what they do.

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