ELSEVIER

Biochimica et Biophysica Acta 1565 (2002) 347–363

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Review

Structural aspects of oligomerization taking place between the transmembrane α-helices of bitopic membrane proteins

Isaiah T. Arkin*

Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Givat-Ram, Jerusalem, 91904, Israel

Received 12 December 2001; received in revised form 7 May 2002; accepted 7 May 2002

Abstract

Recent advances in biophysical methods have been able to shed more light on the structures of helical bundles formed by the transmembrane segments of bitopic membrane proteins. In this manuscript, I attempt to review the biological importance and diversity of these interactions, the energetics of bundle formation, motifs capable of inducing oligomerization and methods capable of detecting, solving and predicting the structures of these oligomeric bundles. Finally, the structures of the best characterized instances of transmembrane α-helical bundles formed by bitopic membrane proteins are described in detail.

Keywords: Oligomerization; α-Helix; Bitopic membrane protein

1. Introduction

A domain is normally defined as a protein segment capable of folding independently, that is, an independent folding unit. As such, a transmembrane α-helix, comprised of ca. 20 amino acids [1], is not only one of the smallest of all protein domains, but also the simplest, in which secondary and tertiary structures are one. Any protein that possesses a *single* transmembrane α-helix is designated as a bitopic membrane protein, whereas proteins with more than one transmembrane α-helix are termed polytopic. Genomic analyses employing various techniques (e.g. hydropathy algorithms) predict that 10–20% of all open reading frames in nearly all of the genomes studies so far, contain a single putative transmembrane α-helix [2–5]. However, it is important to note that such analyses do not detect a common feature of many single transmembrane α-helices: oligomerization. The process of oligomerization may transform a simple membrane anchor into a biologically *active* complex. This review will focus on the structural aspects of oligomerization taking place between the transmembrane α-helices of bitopic membrane proteins.

The driving force behind the formation of quaternary structure (i.e. oligomerization) can be of two, non-exclusive types:

- Formation of covalent bonds between the protomers, such as disulfide bonds.
- Non-covalent, specific interactions that take place between the protomers.

The oligomerization of a bitopic membrane protein that takes place due to interactions between its transmembrane α-helix is most likely non-covalent. The reason being, is that a prerequisite for disulfide bond formation, is the deprotonation of the thiol group. This charge separation is highly unlikely in the low dielectric environment of the lipid bilayer. Thus, it should not be surprising to realize that to the knowledge of the author, no evidence of native disulfide bonds has ever been recorded taking place in the membrane milieu.

In this review, we will only concern ourselves with non-covalent oligomerization that takes place between bitopic membrane proteins, through their transmembrane domains. Thus, for the sake of brevity, in this review, the term oligomerization will henceforth refer to the following:

Abbreviations: NMR, nuclear magnetic resonance; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; FTIR, Fourier transform infrared

* Tel./fax: +972-2-658-4329.
E-mail address: arkin@cc.huji.ac.il (I.T. Arkin).

0005-2736/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0005-2736(02)00580-1

"Non-covalent oligomerization taking place between bitopic membrane proteins, due to their transmembrane α-helices". It is important to note, however, that the oligomerization between polytopic membrane proteins is a very common phenomenon as well: nearly all helical membrane proteins whose structures have been solved are oligomeric.

I will begin by providing a brief overview of the biological importance and prevalence of bitopic transmembrane helix oligomerization, followed by a discussion of the energetic factors that may govern such interactions. Subsequently, methods used to detect non-covalent oligomerization events will be mentioned as they are key in identifying such phenomena before any structural characterization. Experimental methods capable of solving structures of transmembrane helical bundles will be discussed, alongside computational prediction approaches. The structure of the best characterized instance of an oligomeric helical bundle will be discussed in detail: the dimerizing human erythrocyte sialoglycoprotein, glycophorin A. Finally, two other extensively characterized helical bundles will be described: (i) the tetramerizing Influenza A M2 H^+ channel and (ii) the pentamerizing human cardiac sarcoplasmic reticulum protein, phospholamban.

1.1. Biological importance

While many examples of non-covalent oligomerization events taking place between bitopic membrane proteins, due to their transmembrane α-helices, are present in the literature, it is difficult to estimate how prevalent this phenomenon is, due to the experimental difficulty in detecting such events (see Section 1.4). Superficially, therefore, it is possible to distinguish between two kinds of oligomerization events: (i) those that we can experimentally detect and (ii) those that we know exist. Specifically, there are several examples in which the oligomerization between transmembrane helices can be observed experimentally [e.g. by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)]. Conversely, there are examples in which mutations in what would otherwise be considered a simple transmembrane anchor, lead to altered functionality of the protein. In such instances, one may surmise that the transmembrane α-helix is undergoing some sort of oligomerization event.

On a fundamental level, it is possible to classify all oligomerization events as belonging to one of the following two types: (i) homo-oligomers and (ii) hetero-oligomers. So far in the literature, more examples of homo-oligomers have been reported than that of hetero-oligomers. It is interesting to note that due to symmetry considerations (see below), homo-oligomers prove to be much easier subjects to study computationally, but are perhaps more difficult to study experimentally by techniques such as nuclear magnetic resonance (NMR) (see Section 2).

Below I briefly describe two representative examples of bitopic membrane protein families that oligomerize due to their transmembrane domains. The examples described do not imply any particular prevalence or prominent importance on the part of those families that were listed. I merely wish to provide key examples emphasizing the biological diversity and importance of these interactions.

1.1.1. Symmetry in homo-oligomers

Homo-oligomerization between transmembrane α-helices has been detected in many instances. Three prime examples are glycophorin A, phospholamban and Influenza A M2 H^+ channel, the structures of which will be described in detail in Section 2. One important consideration in analyzing the structure of such complexes is the assumption of symmetry. Symmetry results in obvious simplifications in computational analyses and as such, is a compelling restraint to employ. The general justification and validity of the symmetry assumption in homo-oligomeric bundles is based on two lines of evidence:

- Nearly all homo-oligomeric protein structures (membrane or water soluble) found in the protein data bank exhibit symmetry. Exceptions are rare and normally result from the binding of a ligand or substrate to only one of the monomers, thereby breaking the system symmetry (e.g. hexokinase [6]).
- Energetic considerations that govern the interactions between identical subunits favor a symmetric arrangement. The rationale being is that every non-symmetric arrangement can exist in more than one energetically equivalent configuration, leading to interconversion and system instability (see Fig. 1).

1.1.2. Receptor tyrosine kinases

Receptor tyrosine kinases (RTK) comprise an important class of cell surface receptors (see Refs. [7,8] for review). The archetypical RTK (e.g. epidermal growth factor receptor) is a bitopic membrane protein with an extracellular hormone binding domain, a single transmembrane α-helix and an intracellular region containing a tyrosine kinase domain. In general, activation of the receptor is thought to take place through ligand-induced dimerization of the receptor, leading to cross (auto) phosphorylation of the intracellular tyrosine kinase domains.

The role of the transmembrane α-helices in the dimerization process has so far been perceived as passive. Recently, however, several lines of evidence have provided data implying that the transmembrane domains may wield some of the dimerization *potential* for the interactions through the formation of ligand independent *pre-dimerization*. This tendency of the transmembrane domains of RTKs may explain several findings that were difficult to explain based on dimerization due solely to extracellular hormone binding:

- There is a growing body of evidence to support the notion that the transmembrane domains of several RTKs have the ability to dimerize (e.g. the erbB family of

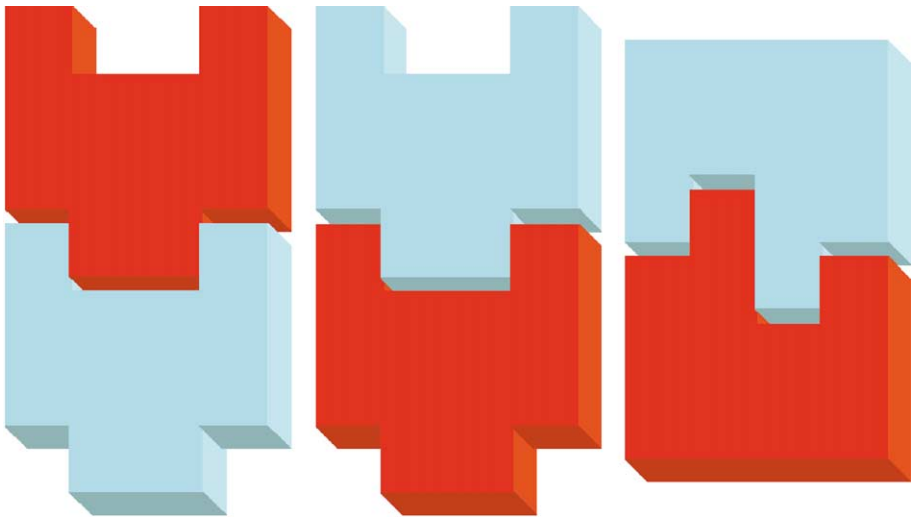

Fig. 1. Schematic representation of symmetric (right) and asymmetric (center and left) arrangements of a homo-oligomeric dimer. For clarity, each monomer is colored differently, although in reality, they are indistinguishable. Note that the two different kinds of asymmetric arrangements are energetically equivalent.

receptor [9–11]). Furthermore, the transmembrane domains of cytokine receptors (not true RTKs) such as the erythropoietin receptor have been shown to have similar tendencies [12,13]. Thus, ligand binding does not induce dimerization in these instances, but presumably results in changing the conformation of the pre-dimerized receptor structure. That ligand-induced conformational change (but not induced dimerization) is possible, and is known from several RTKs (e.g. insulin receptor [8]) that are known to be covalently bound dimers irrespective of hormone presence.

- An oncogenic mutation (Val⇒Glu) was identified in the transmembrane domain of the new (ErbB2) oncogene [14,15]. Furthermore, biophysical studies employing solid-state NMR spectroscopy were able to show that in a peptide encompassing the transmembrane domain of neu, hydrogen bonding was observed between the two glutamate protonated carboxylates [16].
- An oncogenic retrovirus contains a truncated human protein, ErbB, in which the entire extracellular domain is missing, yet is fully and constitutively active (see Refs. [9,10] for reviews). In this instance, dimerization of the receptor cannot be driven by the missing extracellular hormone binding domain.

1.1.3. Viral ion channels

As an initial step toward understanding the molecular biology that underlies the pathogenic activity of a virus, the entire viral genome is often sequenced. The research community will subsequently tend to focus on unique viral proteins, such as the spike proteins, nucleic acid polymerases and proteases. However, the genomes of many viruses may often contain in addition small hydrophobic (SH) proteins, including: 3A from Poliovirus [17], 6K from Semliki Forest virus [18], SH from Simian virus 5 [19], SH from Respiratory Syncytial Pneumovirus [20], M2 from Influenza A [21], NB from Influenza B [22], CM2 from Influenza C [23] and vpu from HIV [24].

M2, the archetypical small hydrophobic viral protein (see Section 2.2), is by far the best characterized member of the family and exhibits properties that are thought to be representative:

- A small (100>) bitopic membrane protein.
- Non-covalent homo-oligomerization driven by the transmembrane α-helical domain. In some instances, the oligomer may be additionally stabilized by disulfide bonds between residues in the extramembranous regions of the protein.
- Ion channel activity that is entirely due to the transmembrane α-helical bundle.

Both the existence of anti-Influenza agents targeting and blocking the M2 channel, and the fact that classical ion channels have long been used as highly successful targets for point intervention by pharmaceutical agents, suggest that the SH viral protein family may represent a new and important target for viral therapy that has so far received little attention.

1.2. Energetic considerations

It is instructive to compare the features of water-soluble helical bundles with those of membrane helical bundles in terms of their stabilization energies. Both membrane and water-soluble helical bundles are proteins of similar fold, yet they exist in dramatically different environments. Comparative analysis of the packing interactions of water-soluble and membrane helical bundles by Eilers et al. [25] indicated that while the overall topology of both families of protein is similar, the packing density of membrane helical bundles is

significantly higher. This results from the large number of small residues (e.g. Gly) in the protein–protein interface, enabling close apposition of the helices [26].

Interestingly, recent studies by Frank et al. [27] and later on by Li et al. [28] have shown that a transmembrane helical bundle (phospholamban, see Section 2.3) can be converted into a water-soluble bundle by substituting apolar residues in the exterior of the protein to polar residues. Furthermore, Frank et al. were able to show that the resulting water-soluble bundle retained the same contact-specific interface [27]. Thus, the forces that govern the interactions inside the membrane may be of similar nature to those of water-soluble proteins.

Finally, one other component to take into consideration is the contribution of the lipids to the stabilization of the helical bundle. The reason being is that any oligomerization event results in a reduced number of helix–lipid interactions and increases the number of lipid–lipid interactions. There are several examples in the literature in which specific lipids are needed to maintain the protein structure and function (see Ref. [29] for review). However, more often than not, membrane proteins retain both their function and structure when moved from one lipid system to the other, or even into detergent micelles (albeit with a possible reduction in stability [30]). When the above is true, it is difficult to assume that lipids play a substantial role in the *specific* interactions taking place between transmembrane α-helices.

1.2.1. Water-soluble helical bundles

The energetic basis for the oligomerization of water-soluble α-helices (e.g. leucine zippers) has been characterized extensively [31]. In brief, the driving force for oligomerization is thought to derive mainly from the sequestration of hydrophobic residues from the aqueous environment. Additional contributions to the interaction specificity (at the possible expense of stability) are thought to arise from polar interactions such as H bonding [32].

1.2.2. Transmembrane helical bundles

Our understanding of the energetic basis of transmembrane α-helical bundle formation lags considerably behind that of water-soluble bundles, mostly due to experimental difficulties. The most fundamental measurement that is needed when studying oligomerization is missing: an oligomerization detection assay that takes place in the natural environment of the protein: the lipid bilayer (see Section 1.4). It is for this reason that theoretical considerations have in many cases advanced more rapidly than the gathering of basic thermodynamic data to substantiate such theories. Nonetheless, several theoretical considerations have proven very useful.

The two-stage model [33,34] for membrane protein folding and oligomerization has been particularly useful in forming a conceptual framework in which to analyze membrane protein oligomerization. The model states that membrane proteins oligomerize (or in the case of polytopic membrane proteins, fold) in two stages:

1. Formation of independently stable transmembrane α-helices.
2. Association of the independently stable transmembrane helices to form an α-helical bundle.

In conclusion, the two-stage model maintains that before oligomerization, the α-helices are independently stable. This fundamental assumption precludes any bitopic membrane protein from having a transmembrane α-helix that is substantially amphiphatic. This is indeed consistent with sequence analysis that identified a larger proportion of polar residues in polytopic putative membrane proteins versus bitopic proteins [35]. Based on the above considerations, it is unlikely that the driving forces behind the oligomerization of transmembrane α-helices is simply the reverse of that found in water-soluble helices: the sequestration of polar residues from the apolar environment [36]. Therefore, to understand the forces that bring together mostly apolar helices in a membrane environment, one should look at other interactions in addition to the formation of salt-bridges and inter-helix hydrogen bonding. Below I describe the most rigorous analysis of the energetics of transmembrane α-helix association, undertaken for human glycophorin A.

1.2.3. The energetics of glycophorin A dimerization

Glycophorin A was the first membrane protein whose sequence was determined, identifying in the process a long stretch of hydrophobic amino acids [37]. Furthermore, glycophorin A provided the first clear example of a bitopic membrane protein oligomerizing (more specifically, dimerizing) non-covalently due to specific interactions of its transmembrane α-helices (see Ref. [38] for review). As an example, the transmembrane domain of glycophorin A could be fused to a monomeric, water-soluble protein, resulting in the dimerization of the resulting chimera [39].

The dimerizing transmembrane α-helices of human erythrocyte sialoglycoprotein, glycophorin A, were extensively analyzed by saturation mutagenesis [40] and Ala insertions [41,42], assaying their ability to dimerize in SDS micelles by electrophoresis (i.e. non-equilibrium conditions). The sensitivity of some of the residues toward disruption was remarkably exquisite, whereby in some instances the addition or removal of a single methyl group was sufficient to prevent dimerization [40]. It was later on demonstrated [43] that the residues found to be sensitive toward substitution constituted the first known dimerization motif for transmembrane α-helices, as expanded in Section 1.3.

MacKenzie and Engelman [44] reported an insightful analysis relating the effects of each of the mutations to the nature of the substituted amino acid. Five empirical parameters were attributed to each substitution: (i) side-chain rotamer entropy change, (ii) increase of favorable van der

Waals contacts, (iii) introduction of steric clashes, (iv) hydrophobicity using the GES scale [45] and (v) side-chain volume. In their multivariant regression analysis of apolar substitutions, MacKenzie and Engelman [44] were able to show that the contributions toward dimerization were as follows:

- *Side-chain rotamer entropy*: The energetic contribution to dimer formation due to the change of side-chain entropy was found to be significant.
- *van der Waals contacts*: The formation of favorable van der Waals contacts was found to be essential to dimerization, as expected.
- *Steric clashes*: The introduction of steric clashes was drastically detrimental to dimer formation. Its magnitude indicated that its value superseded any other contribution.
- *Hydrophobicity and volume*: The hydrophobicity or volume of the particular side chain was found to be a very poor predictor of dimer stability, amongst the subset of apolar amino acids used in the study. The effect of strongly polar substitutions is thought to occur in stage I of the folding of the protein, through the decrease of the stability of the helix protomer (see Section 1.2.2 and Ref. [39]).

1.2.4. Contribution of polar interactions to oligomerization energetics

Recently, Fang-Xiao et al. [47] and Choma et al. [48] were able to demonstrate the possible importance of polar interactions toward the stability of an oligomeric complex. Both groups converted the water-soluble Leucine zipper into a hydrophobic transmembrane α-helix. Only upon the introduction of the polar amide group of Asn did the helices oligomerize, non-specifically. The authors thus indicated that groups capable of H bonding in the bilayer may provide opportunities for oligomerization stability at the expense of specificity [47,48].

In a more general study, Gratkowski et al. [49] and later on Fang-Xiao et al. [50] have investigated the effects of inserting a variety of polar residues in a background of a polyleucine transmembrane α-helix. The authors found that incorporation of a single Asn, Asp, Gln, Glu or His was capable of inducing homo- or hetero-oligomerization. On the other hand, the incorporation of Ser, Thr or Tyr was not able to induce oligomerization. The authors' rationale of why the first group was capable of inducing oligomerization while the latter was not, was that residues in the first group are capable of being simultaneous hydrogen-bond donors and acceptors. The latter group of residues is not able to.

Senes et al. [51] have more recently shown that the weak Cα−H···O hydrogen bond may contribute to the stability of transmembrane oligomers as well. In this instance, the close proximity between the juxtaposed helices needed for such a bond to take place maintains that close packing interactions precede such bonds forming.

The question then remains as to why strong interactions, such as (i) disulfide bonds, (ii) intermolecular hydrogen bonding and (iii) intermolecular salt bridges, are not commonly found [52,29]. Both Gratkowski et al. [49] and Fang-Xiao et al. [50] speculate that such interactions, while strong, may prove to be promiscuous in the membrane environment, resulting in non-specific aggregation. Furthermore, such strong interactions may negate any possibility for regulation of the oligomerization process that might be essential for function.

1.3. Oligomerization motifs

As stated in Section 1.2.3, it was shown that the residues found to be sensitive toward substitution in glycophorin A [40] constituted the first known dimerization motif for transmembrane α-helices: LIxxGVxxGVxxT [43] (L75-T87, see red and purple residues in the middle panel of Fig. 7). Langosch et al. [53] later on showed that when assayed for dimerization in a lipid bilayer, the motif could be further minimized to GxxxG (G79–G83, see red residues in the middle panel of Fig. 7). This finding is consistent with the fact that the potentially denaturing detergent micelle environment places more stringent restrictions on the dimerization process [30]. Thus, many interactions that are not observed in SDS-PAGE could still be taking place in a lipid bilayer and have so far escaped our attention.

Bioinformatics analysis by Arkin and Brunger [1] found the GxxxG motif to be common in putative transmembrane α-helices, pointing possibly to the prevalence of this sort of dimerization. Furthermore, the GxxxG motif was later on identified by Russ and Engelman [54] when screening random transmembrane α-helices on the basis of their ability to oligomerize. Another genetic screen aimed at identifying oligomerizing transmembrane α-helices was undertaken by Leeds et al. [55]. In this study, a library of protein fragments was tested, identifying several successful candidates. One such candidate was transmembrane helix 6 from the YjiO gene that contained a GxxxA motif [55] (similar to the GxxxG motif previously identified [43]). However, in this instance, Ala scanning mutagenesis studies pointed to the intervening residues as being more sensitive to substitution.

More recently, Kleiger et al. [56] have identified in the crystal structure of a water-soluble protein, the E1β subunit of *Pyrobaculum aerophilum* pyruvate dehydrogenase, a GxxxG motif that enables the protein to oligomerize. Thus, the GxxxG motif may be a general dimerization motif for transmembrane, as well as water-soluble helices.

Finally, the GxxxG motif is by no means the only oligomerization motif preset for transmembrane α-helices, since it is absent in many known cases of helices that are known to oligomerize. As an example, Laage et al. [57] were able to identify entirely different oligomerization motifs for transmembrane α-helices based on a heptad of leucine residues, similar to that found in water-soluble leucine zippers [31].

1.4. Oligomerization detection

Detecting the oligomerization of transmembrane α-helices in a lipid bilayer is by no means routine. Furthermore, even upon detection, accurate assessment of the oligomeric size (a difficult task on it sown) is needed to interpret the thermodynamic data. It is for the above reasons that oligomerization assays have been developed in membrane mimetic conditions.

1.4.1. Oligomerization detection in membranes

As stated above, detecting oligomerization events in lipid bilayers is difficult. However, several approaches have been developed aside from the obvious in situ cross-linking followed by SDS-PAGE. Most notably is the ingenuous system developed by Langosch et al. [53] based on the *Vibrio cholerae* ToxR protein. In this system, transcriptional activation by ToxR is dependent upon membrane-induced dimerization [53]. When ToxR is fused to a transmembrane α-helix and a leader signal, the level of transcriptional activation will be proportional to oligomerization. Russ and Engelman [58] have recently utilized the system invented by Langosch et al. [53] to drive transcription of an antibiotic resistance gene, enabling genetic selection of the oligomerization process (this system is called TOXCAT).

Leeds et al. [55] have designed an alternative approach for genetic screening of transmembrane oligomerization based on the membrane-driven dimerization of the phage λ cI repressor. In this system, dimerization results in maintaining the phage's lysogeny.

Additionally, several biophysical methods have been developed, including fluorescence resonance energy transfer between fluorescently labeled peptides [59] (see below).

1.4.2. Oligomerization detection in detergents

There are multiple ways in which oligomerization can be analyzed in detergents:

- PAGE is a particularly simple, powerful, albeit a non-equilibrium method that can be used whenever the protein remains oligomeric in the SDS micelle (e.g. glycophorin A [40] or phospholamban [60]).
- Analytical ultracentrifugation is a very powerful method that can be used to gather exact thermodynamic data on transmembrane helix oligomerization [61].
- Fluorescence resonance energy transfer has been used to measure helix–helix interaction in detergents [30] as well as lipid bilayers [59]. While being a very sensitive method in terms of detection, it suffers greatly from the following two factors: (i) The need to chemically add two bulky and potentially artifact causing fluorescent groups to the peptides. (ii) The estimation of the oligomeric size is not very accurate leading to difficulties in the thermodynamic analysis.
- X-ray scattering has recently been shown to be capable of detecting oligomerization events [62]. However, due to the complicated nature of the measurements, it remains to be seen how popular the method will become.

1.5. Structural methods

Structural analysis of membrane proteins has lagged considerably behind that of water-soluble proteins. Conventional methods aimed at determining protein structure, such as X-ray crystallography and solution NMR spectroscopy encounter severe difficulties when analyzing membrane proteins:

- Crystallization of membrane proteins is notoriously difficult and as of yet, no X-ray structures of bitopic helical bundles have been solved.
- High-resolution solution NMR studies are hampered by the large size of the protein micelle complex. However, the monumental success of solving the structure of the glycophorin A transmembrane domain dimer by solution NMR in detergent micelles [63] (see Section 2) has indicated that such studies are feasible.

Finally, even upon success, both methods study membrane proteins not in their native environment, a lipid bilayer, but rather in detergent micelles.

Two alternative approaches exist that are capable of studying the structures of isotopically labeled peptides in lipid bilayers. Both methods derive a list of restraints that can be used as energy refinement factors in prediction algorithms (see Section 1.6). While the peptides are studied in lipid bilayers, the methods are currently limited in terms of possible candidates to study due to the necessity of specific isotope incorporation.

- Solid-state NMR methods are capable of yielding both spatial and distance restraints for membrane peptides (see Ref. [64] for review). The insensitivity of NMR and relatively small sample volumes result in peptides examined at relatively high protein-to-lipid ratios. Furthermore, distance measurements are normally obtained by magic-angle spinning techniques at low temperatures (ca. −10 °C).
  However, solid-state NMR has so far been able to solve structures of small integral membrane peptides that were inaccessible by any of the conventional methods (e.g. gramicidin [65,66] and M2 [67], see Section 2.2)
- Site-specific Fourier transform infrared (FTIR) spectroscopy is a very recent method that is capable of yielding high-resolution spatial restraints for transmembrane α-helices [68,69]. The sensitivity of FTIR and large sample volumes do not impose any limitations on the peptide-to-lipid ratios used. Furthermore, the experiments can be undertaken at any temperature desired, and the rapid nature of the measurements means that multiple con-

ditions (e.g. lipid nature or the presence of ligand) can readily be tested. One current limitation of the method is the need to avoid bulk water over the sample, since water infrared signals overlap those of the protein. Alternative vibrational spectroscopy approaches may be able to alleviate this limitation.

1.6. Prediction methods

Transmembrane helical bundles are relatively simple structures. Assuming the helices are canonical, a bundle of n helices can be described by a small set of parameters ($3n$), as shown in Fig. 2 [70]. Employing the assumption of symmetry on homo-oligomeric helical bundles (Section 1.1.1) results in only two global parameters that are needed to outline the structure. The relative helix tilt β, with respect to the bundle axis (related to the crossing angle) and the rotational angle about the helix director ϕ, which defines which side of the helix is facing toward the bundle core.

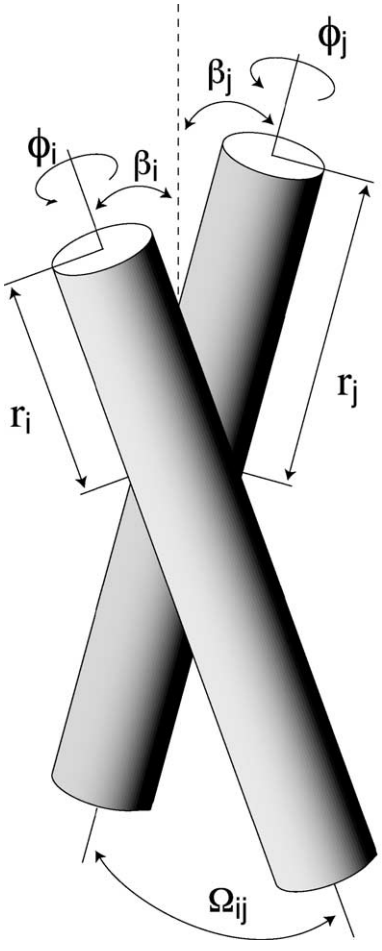

Fig. 2. In a bundle that contains n helices, $3n$ parameters are needed to describe the overall structure, assuming rigid helices: (i) the tilt angle with respect to the bundle axis, β_i, related to the commonly used crossing angle Ω [71], (ii) the rotational angle about the helix director, ϕ_i, which defines which side of helix i is facing toward the bundle core and (iii) the helix register, r_i, which defines the relative vertical position of the helix.

Based on this principle, Treutlein et al. [72] and Adams et al. [73,74] have developed a systematic algorithm termed global searching molecular dynamics, capable of searching this limited configuration space and retrieving possible solutions. Symmetric bundles are generated by replicating the helix and rotating it by $360°/n$ (where n is the oligomer size). An initial crossing angle of $25°$ for left-handed and $-25°$ for right-handed structures is introduced by rotating the helix with respect to the bundle symmetry axis. A symmetric search is carried out by applying a rotation to all helices simultaneously between $\phi = 0°$ and $\phi = 360°$ in given increments (e.g. $10°$).

Each of the above starting structures is subjected to several (e.g. 4) short molecular dynamics runs (each with a different initial random velocity) aimed at determining the stability of the starting position. Thus, at the end of the procedure, the resulting $288 = 36 \times 2 \times 4$ structures are compared, revealing local energy minima to which structures have clustered to (see Fig. 3). These clusters are then taken as candidate models, representative of the configuration space that was sampled.

As an example, Fig. 3 depicts the outcome of global searching molecular dynamics when implemented on glycophorin A, in which more than one cluster is present. How does one identify the correct structure? Two general approaches have been used:

- Evaluate each model's ability to *explain* external data, such as mutagenesis studies or evolutionary conservation. In other words, assume that (i) residues that were found important by mutagenesis or (ii) those that have been conserved throughout evolution lie in the protein–protein interface. This approach has been tried on glycophorin A with considerable success [40]. When applied to the pentamerizing phospholamban [60] (see Section 2.3), it has failed to produce the correct structure [75].
- A more recent approach utilizes evolutionary conservation and/or mutagenesis data as well, but in an entirely different way [76]. Rather than concentrating on conserved residues (either from mutagenesis or evolution) and make assumptions regarding their function, make use of *silent changes* (residues that when changed have no effect on the structure and function of the protein). The procedure then calls for applying global searching molecular dynamics simulations on multiple sequences, each containing different silent changes. When the resulting clusters from each of the different sequences are compared to one another, it is evident that only one cluster is common throughout all of the different homologs: the native structure. The rationale is that only the correct structure must *absorb* all of the silent changes. In other words, the silent mutations do not affect the native structure (by definition) but may not be compatible with non-native structures. This approach

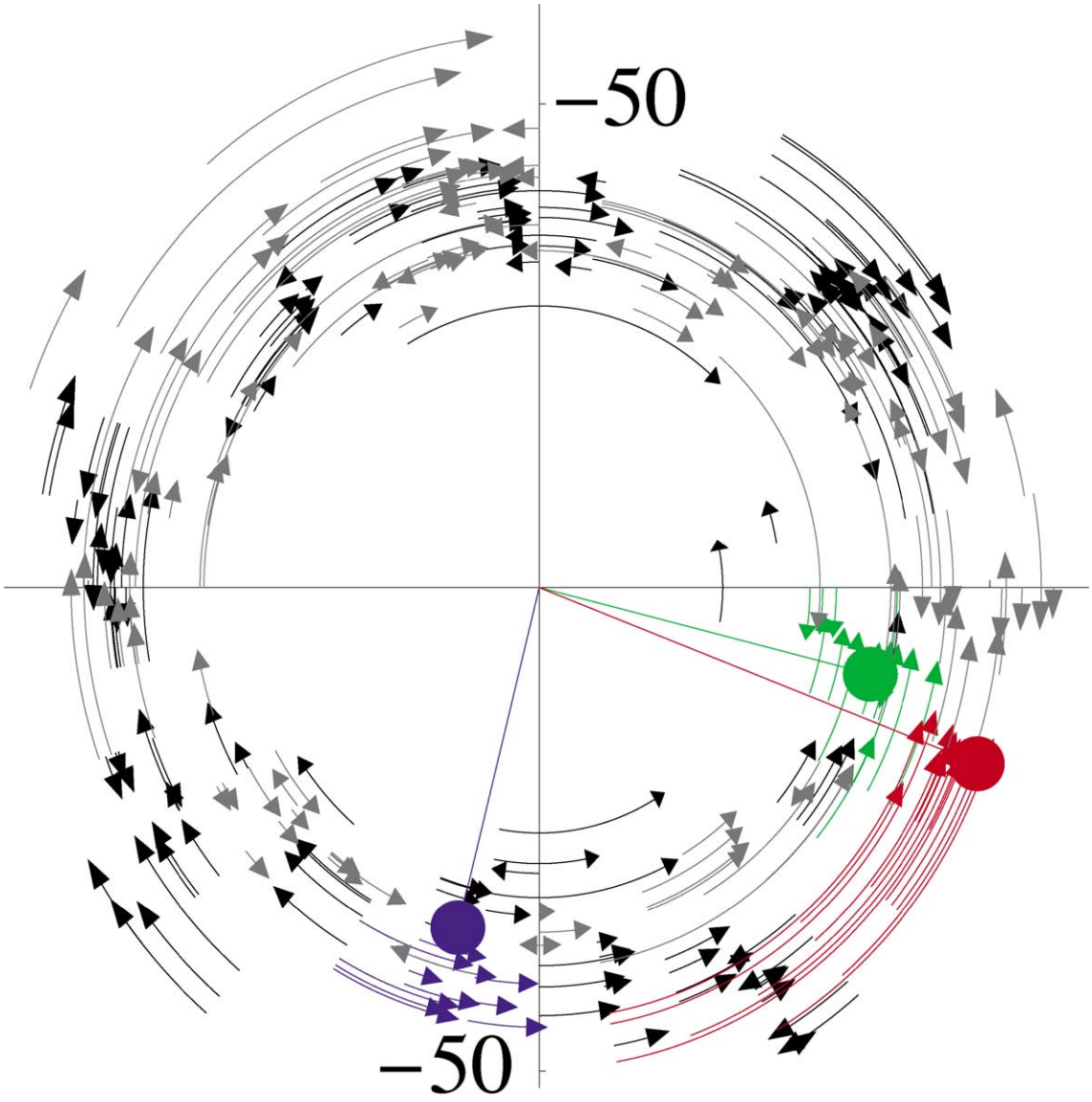

Fig. 3. Energy plots (in polar format) of all the structures obtained from the global search molecular dynamics protocol applied to the glycophorin A homodimer as a function of the helix rotation angle ϕ. The arcs represent each of the individual 288 starting structures in which the arrows designate the direction in which the starting structure moved during the simulation. The energy of each structure is measured as the distance from the origin. For clarity, the energy of the starting structure is not illustrated, rather the "energy" of the entire arc is equal to that of the final structure. Each of the structures is colored according to its cluster affiliation. Left-handed unclustered structures are depicted in black and right-handed unclustered structures in grey. The cluster averages are represented as azimuthal lines ending with a circle at the energy level of the average.

was capable of predicting the structure of glycophorin A [63] and more recently that of the oligomeric helical bundle formed by the transmembrane domain of TCR CD3ζ [77,78].

It is also possible to rigorously map the energy surface of transmembrane helix–helix interactions [70]. In this procedure, the energy of a helical bundle is calculated for every possible combination of tilt and rotational pitch angles at small intervals (ca. 1°). Fig. 4 depicts the result of such an analysis for the dimerization glycophorin A, in which a large energy minima is located at a tilt angle of −23° and a rotational angle of 260° [70]. Remarkably, these values are virtually identical to those obtained experimentally by solution NMR [63] (see Section 2). Note that in the case of a dimer, the inter-helix crossing angle can be derived directly from the tilt angle of the helices ($\Omega = 2\beta$).

2. Structural examples

2.1. Glycophorin A

Glycophorin A is undoubtedly the best characterized example of a bitopic membrane protein that oligomerizes

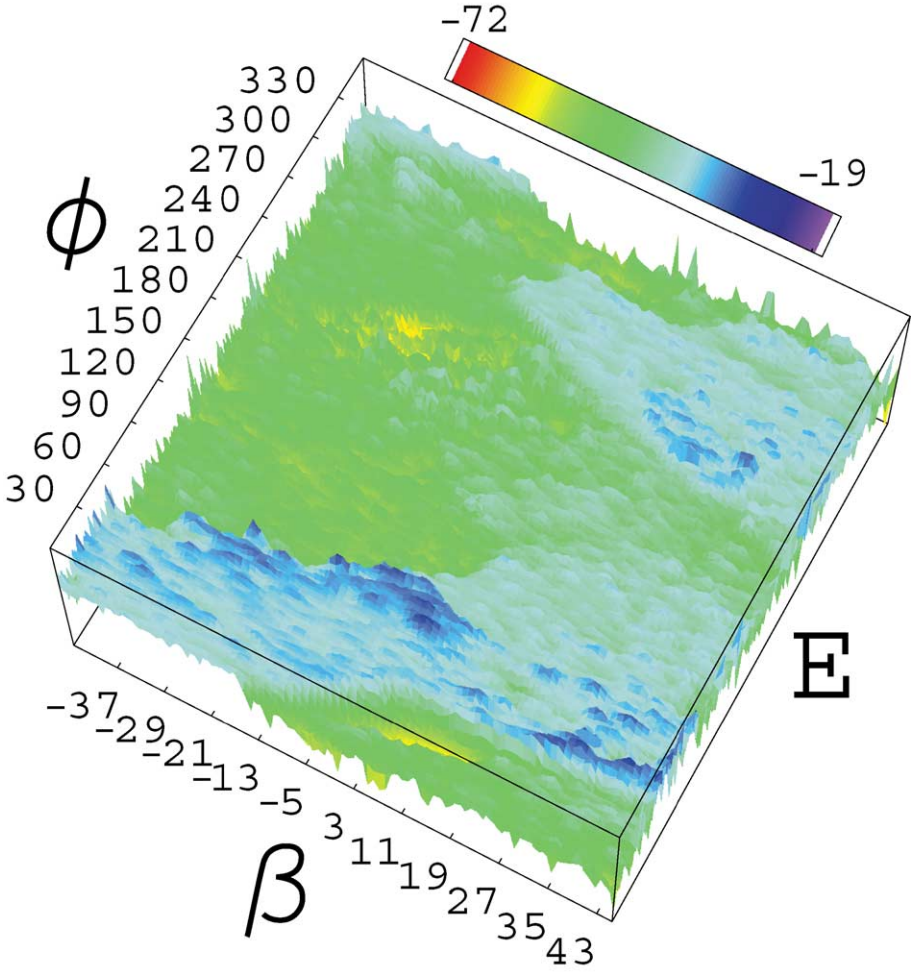

Fig. 4. Energy surface diagram of transmembrane helix–helix interactions for the dimeric human glycophorin A transmembrane domain as a function of the helix tilt, β and the rotational pitch angle ϕ. The color index corresponds to energy in units of kilocalories per mole.

through interactions of its transmembrane α-helix. Amazingly though, despite all of the scientific interest that it has sparked as a model system, glycophorin A still lacks an identifiable function. Below I describe the structure of the dimeric complex of the transmembrane domain of glycophorin A.

In a landmark study, MacKenzie et al. [63] employed solution NMR to study the structure of a peptide corresponding to residues 62–101 of glycophorin A, which encompasses the transmembrane domain, in dodecylphosphocholine micelles at 40 °C. The structure was solved using side-chain dihedral angle restraints from quantitative J couplings and NOE-based distance restraints. Since glycophorin A is a homo-dimer, difficulty arises when trying to differentiate between inter- and intra-helical distance restraints. Only six distance restraints could not have been accounted for by constructing a monomeric structure, and were therefore treated as exclusively intermolecular distance restraints (see Table 1). For all other NOEs, the authors have ingeniously "allowed" every distance restraint to be either intermolecular or intramolecular due to the ambiguity of the data. No explicit symmetry constraints were employed during the solution phase. Symmetry, however, was inherent in the system since each monomer contributed exactly the same signals and resulting constraints. On average, the NMR studies yielded eight experimental restraints per residue in the transmembrane region.

Using a novel method based on site-specific FTIR dichroism, Arkin et al. [68] analyzed the structure of isotopically labeled glycophorin A transmembrane peptide

Table 1
Unambiguous intermolecular NOE distance restraints derived from solution NMR 3D NOESY-HSQC spectra of the dimerizing glycophorin A transmembrane domains in dodecylphosphocholine micelles [63]

Helix 1	Helix 2	Distance (Å)
V80 Hγ2	G79 HN	2.0–3.5
L75 Hδ1	I76 Hβ	2.6–3.8
V84 Hγ2	T87 Hγ1	1.9–2.8
V84 Hγ2	G83 Hα	2.1–3.0
T87 Hγ2	I88 Hγ1	2.1–3.0
V84 Hα	T87 Hγ1	2.1–3.0

dimers in lipid (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) bilayers at room temperature. The structural parameters obtained from the aforementioned method include the helix tilt, β and the rotational position of the labeled residue about the helix axis (see Fig. 2). The results obtained are indistinguishable from those obtained from the NMR analysis in dodecylphosphocholine micelles.

The structure obtained for glycophorin A by solution NMR is that of a right-handed helical bundle with an inter-helix crossing angle of $\Omega = -40°$, as shown in Fig. 5. Contrary to the coiled coils that one observes in left-handed helical bundles, a right-handed bundle can form from straight helices, in which the contacts between the helices occur every 3.9 residues. This contact periodicity, and the subsequent assumption that glycophorin A forms right-handed dimers, was identified early on in mutagenesis studies of glycophorin A [40], whereby the residues that were found sensitive toward substitution exhibited the aforementioned periodicity.

The formation of a bundle from straight helices in which the crossing angle is $\Omega = -40°$ results in the fact that the contact area between the two helices is relatively small, 400 Å^2. The structure entails seven residues that make favorable inter-helix van der Waals contacts. These are the exact same residues shown by saturating mutagenesis in SDS-PAGE [40] to be sensitive toward substitution when assaying for dimerization (see Figs. 6 and 7). The contact between the two helices is very tight and the inter-helical separation is 7.0 Å.

As pointed out by MacKenzie et al. [63], close inspection of the structure reveals several interesting features. The side-chain rotamer angles are practically ideal, as expected. Out of the seven residues that are responsible for the intermolecular contacts (see Fig. 6), two are glycines and four are β-branched (Val and Ile) and can have only one χ_1 rotamer in an α-helix [46]. Therefore, the contact surfaces of the glycophorin A helix is preformed and does not significantly change upon dimerization. In other words, there is little, if any loss of conformational entropy upon oligomerization.

Another interesting feature of the dimer structure is that the governing interaction stabilizing the structure is van der Walls contacts between apolar side chains. The only probable intermolecular polar interactions are those of Thr87 O—H· ·O [80] and the Cα—H· ·O hydrogen bonds [51] mentioned above. Higher resolution structures will aid in the confirmation of these interactions.

2.1.1. Solid-state NMR studies of glycophorin A in frozen membranes

Glycophorin A has also been a target of structural analysis by solid-state NMR undertaken by the Smith group [81,80]. Site-specific isotopically labeled peptides encompassing the transmembrane domain are reconstituted in lipid membranes, followed by freezing and magic-angle spinning solid-state NMR analysis. The distances between the ^{13}C isotopic labels can then be determined by several magnetization exchange techniques such as rotational resonance [64]. Once again, experimental complications arise due to the fact that glycophorin A is a homo-dimer. In this instance, the authors used two different kinds of peptides, each labeled in a different position, whereby the magnetization

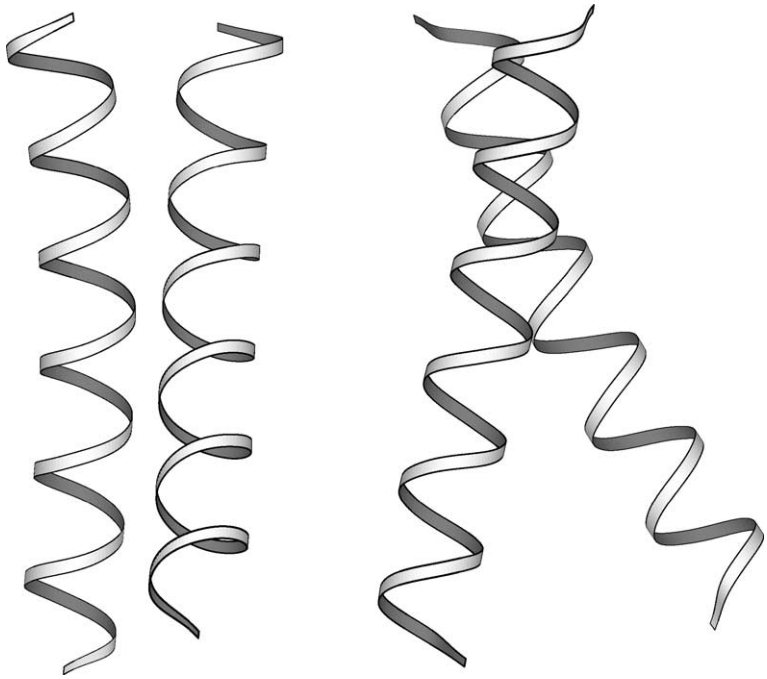

Fig. 5. Helical diagrams of the transmembrane dimer of glycophorin A in dodecylphosphocholine micelles [63] in orthogonal views. Note the formation of a right-handed contact between the virtually straight helices. Figure generated by molscript [79].

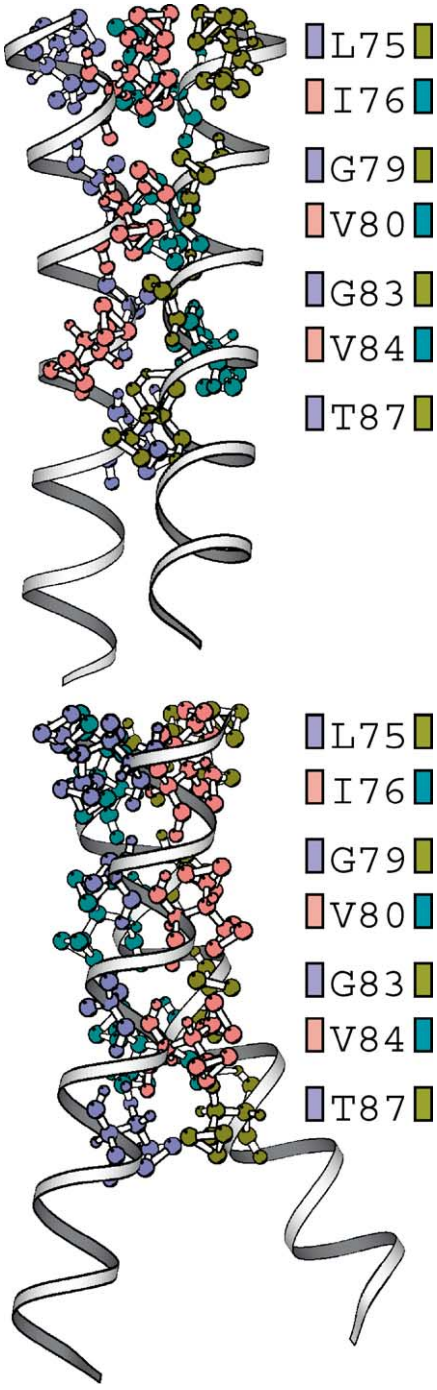

Fig. 6. Helical diagrams of the transmembrane dimer of glycophorin A in dodecylphosphocholine micelles [63], depicting the residues that make intermolecular contacts. The legend on the right identifies the vertical coloring of each residue. For clarity, residues of opposite molecules are colored differently. Figure generated by molscript [79].

is exchanged from one label to the other [64]. The peptides were then mixed in such ratios, such that all labels that ''donate'' magnetization are under populated and are assured to be near magnetization ''acceptors''.

The distance restrains are then employed as an energy refinement terms in a molecular dynamics protocol aimed at predicting the structure of the helical bundle, known as global searching molecular dynamics [73] (see Section 1.6). Thus, it is imperative to understand that any structural details on non-labeled groups is purely a result of the computational protocol used, due to chemical constraints and helical geometry. Table 2 lists the inter-helical distance restraints generated by the aforementioned method.

The structural model of Smith et al. [80] differs slightly from the structure determined by MacKenzie et al. [63] in solution, as shown in Fig. 7. The helices in the Smith model are rotated about their axes by $\sim 25^\circ$ relative to the NMR structure. Furthermore, the crossing angle between the helices is reduced relative to the solution NMR structure from 40° to 35°.

Smith et al. [80] point to several factors that might cause the differences between their structure to that derived from solution NMR. Namely, that the measurements are not undertaken under the same conditions, alluding to the fact that glycophorin A adopts a different structure in a lipid bilayer versus a detergent micelle. It is difficult, however, to reconcile these arguments with the site-specific FTIR dichroism data undertaken in a lipid bilayer [68] which yield exactly the same geometrical constants as the solution NMR data. However, it is possible to delineate between the solution NMR in detergents and the site-specific FTIR in membrane studies, versus the solid-state NMR in membrane study in that the first two measurements were undertaken at room temperature or above, while the solid-state NMR data are obtained at -10 °C.

The outcome of these differences is that in the Smith model, the Gly residues forming the GxxxG motif (Gly79 and Gly83) are in contact with one another (see Fig. 7). In other words, glycines 79 from each helix are in contact with each other, as are glycines 83. Additionally, the rotation enables Thr87 to hydrogen bond across the dimer interface.

2.2. Influenza A M2 H^+ channel

The M2 protein from Influenza A was the last step to be elucidated in the life cycle of the Influenza virus [82]. Viral attachment and entry is carried out through the activity of

Table 2
Intermolecular distance restraints derived from solid-state NMR magic angle spinning rotational resonance spectra of the dimerizing glycophorin A transmembrane domains in frozen lipid bilayers

Helix 1	Helix 2	Distance (Å)
G79 Cα	G79 C	3.8–4.4
I76 C	G79 Cα	4.5–5.1
G83 Cα	G83 C	4.0–4.6
G83 Cα	V80 C	3.9–4.5
G79 C	V80 Cγ	3.7–4.3
G83 C	V84 Cγ	3.7–4.3

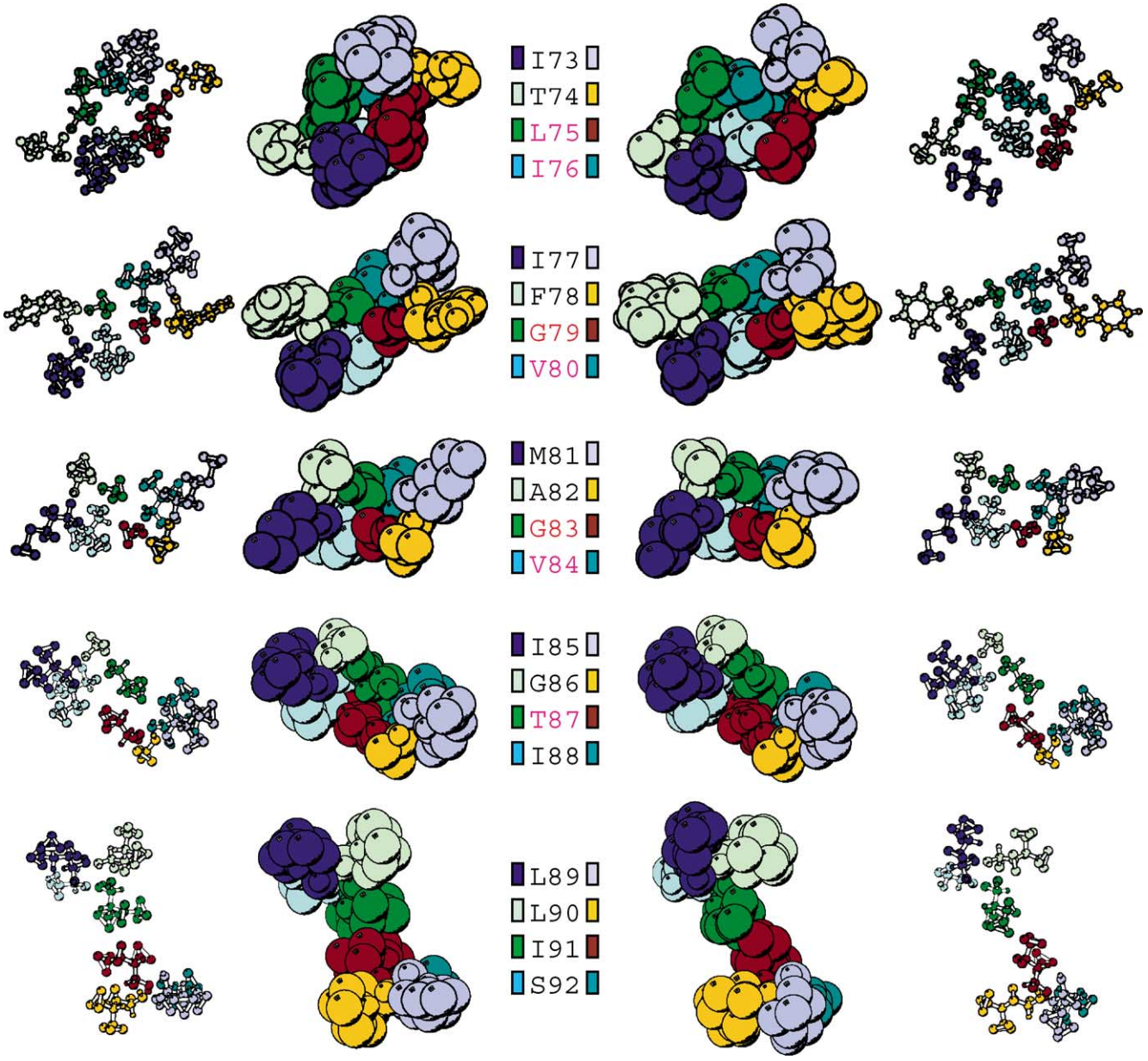

Fig. 7. Comparison between the solution NMR structure of glycophorin A in dodecylphosphocholine micelles [63] (right panel) and the solid-state NMR structure [80] (left panel). Five slices are presented each with four amino acids, whereby the different residues are color coded. For clarity, identical residues in different protomers are color coded differently according to the legend in the middle. Residues labeled in purple or red (e.g. L75 and G83) are those identified by mutagenesis to be sensitive to dimer disruption in SDS-PAGE [40]. Residues labeled in red (e.g. G79) are those identified by mutagenesis to be sensitive to dimer disruption in lipid bilayers [53]. Figure generated by molscript [79].

the major viral spike glycoprotein HA. Membrane fusion and viral genome release occurs after HA undergoes a pH-dependent irreversible conformational change in the acidic endosome, but it was not clear at first why HA did not change conformation in the Golgi secretory pathway where the pH is lower than that of the cytosol. The answer to this question came on identifying the pH-dependent ion channel activity of M2, which negates the activity of the Golgi H^+ ATPase [21].

M2 also participates in the virus uncoating process after viral uptake by endocytosis. The passage of H^+s from the acidic environment of the endosomal lumen into the virion lumen (through M2) weakens the interactions between the matrix protein (M1) and the ribonucleoprotein (RNP) core, enabling the release of the viral genome into the cytoplasm [83].

The ion channel activity of M2 has been investigated in some detail and has been shown to be blocked by amantadine and BL-1743 which are both potent anti-Influenza agents [84]. Amantadine-resistant strains of Influenza exhibit mutations in the M2 proteins that render them insensitive to amantadine ion channel blockage.

Furthermore, the channel is activated by low pH, and the His residues that reside in the transmembrane segment have been shown to be essential for this function [85,86].

Interestingly, a homolog of M2 is not found in Influenza B or Influenza C, both of which contain additional proteins (NB and CM2, respectively) with similar structural and functional characteristics, but no sequence similarity [87]. Much less is known about the CM2 protein of Influenza C virus [88] and NB from Influenza B [89,90].

M2 was shown to be a homo-tetrameric membrane protein, linked by disulfide bonds [84]. Mutation of the cysteine residues does not affect the channel activity of the protein and synthetic peptides corresponding to the transmembrane domain alone exhibit similar channel activity and

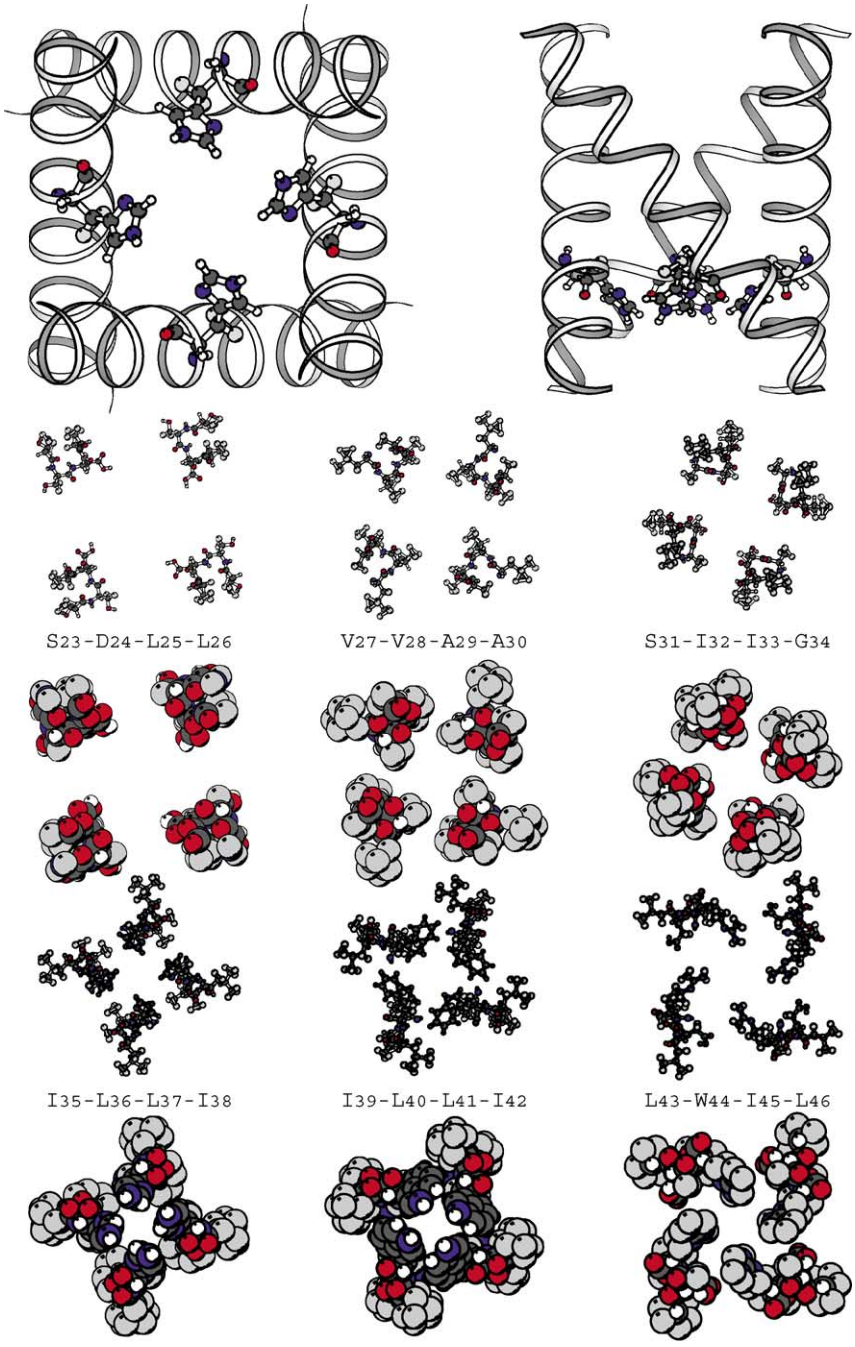

Fig. 8. Solid-state NMR structure of the transmembrane region of the Influenza A M2 H^+ channel [67]. The upper panel depicts two orthogonal views of the helical assembly including the His residue implicated in pH channel activation [85,86], whereas the bottom panel represents slices of space filling representations of the channel. Figure generated by molscript [79].

amantadine sensitivity [84]. Taken together, the data suggest that tetramerization is initiated by the transmembrane domain and subsequently stabilized by cytoplasmic disulfide bonds.

Both solid-state NMR [91,92] and site-specific FTIR [69] studies of a transmembrane domain of M2 were able to show that the transmembrane helices of M2 are tilted from the membrane normal by about 30°–40° and that the rotational pitch angle (see definition in Fig. 2) about the helix axis of A29 is −60°. Wang et al. [67] have recently reported a detailed solid-state NMR study of M2 in which spatial restraints were gathered for every amino acid in the transmembrane peptide. The spatial restraints resulted in a promoter structure of M2 in which the helices are nearly perfectly canonical and are tilted from the membrane normal by 38°. Constructions of a tetrameric assembly from the monomer structure by Wang et al. [67] resulted in the structure shown in Fig. 8. The structure places the His

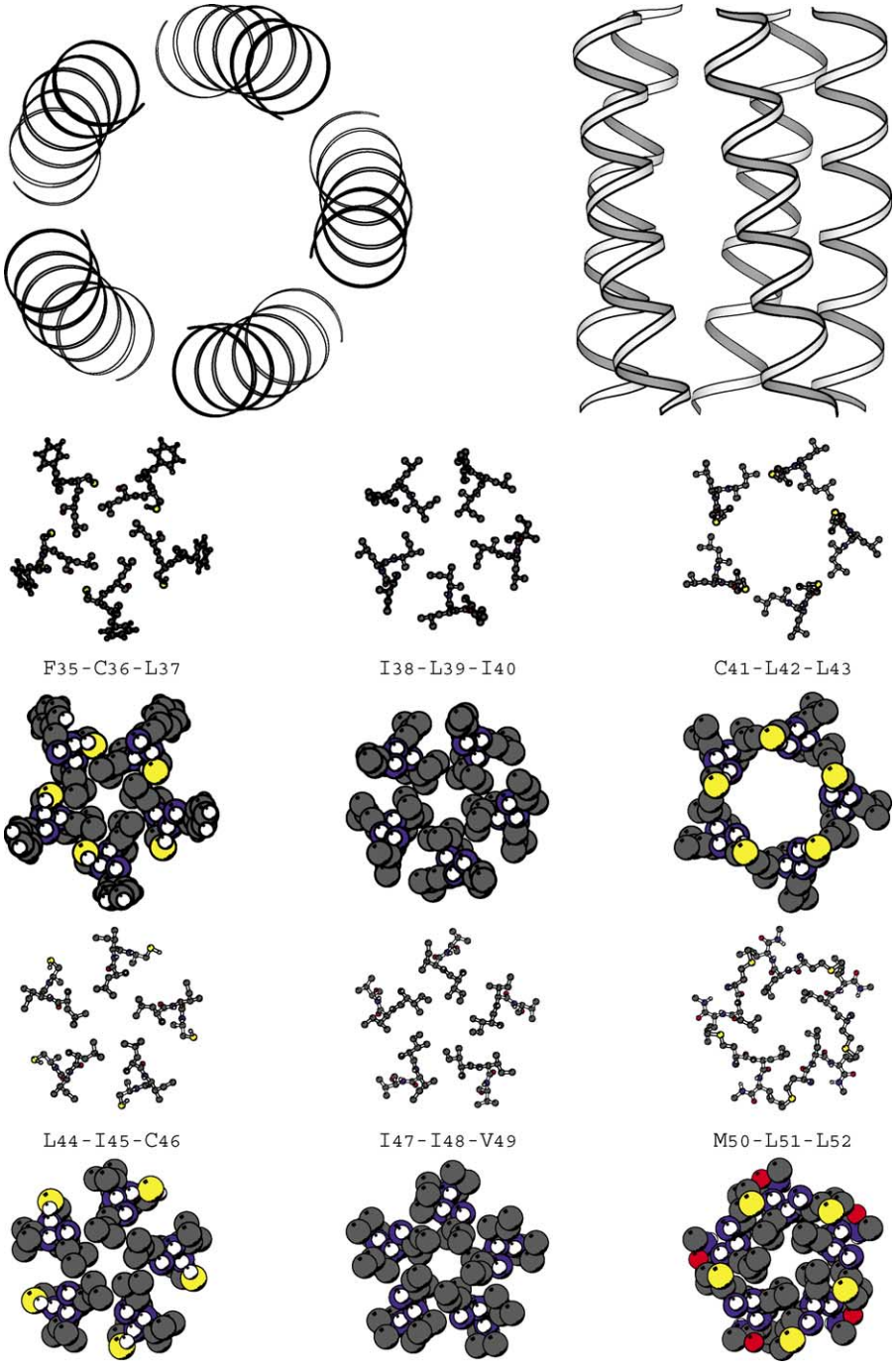

Fig. 9. Site-specific FTIR structure of the transmembrane region of phospholamban [75]. The upper panel depicts two orthogonal views of the helical assembly, whereas the bottom panel represents slices of space filling representations of the channel. Figure generated by molscript [79].

residues implicated in the pH activation of the channel [85] in the bundle core. Furthermore, residues implicated in binding the anti-Influenza A channel blocking drug amantadine are located in the channel lumen as well.

2.3. Phospholamban

Phospholamban is a 52-amino-acid protein resident in the cardiac sarcoplasmic reticulum (see Refs. [93–95] for reviews). Its role is the regulation of the Ca^{2+} ATPase by way of an inhibitory association. β-Adrenergic stimulated phosphorylation of phospholamban relieves this inhibition, enabling the Ca^{2+} pump to restore the Ca^{2+} gradient across the sarcoplasmic reticulum faster, leading to a more rapid heart rate.

Structurally, phospholamban is known to be a non-covalent, homo-pentameric protein in which the pentamerization is driven solely by the transmembrane domains. The pentamerization of phospholamban persists in SDS-PAGE enabling mutagenesis analysis to outline which residues are critical for pentamerization. Work by Arkin et al. [60] was able to identify the following key residues as being critical for pentamerization: LxxIxxxLxxI (Leu37–Ile47). Model building efforts by the Engelman and Brunger groups [60,73] using global searching molecular dynamics simulation predicted a structure for the protein that was consistent with the aforementioned mutagenesis data. However, work by Simmerman et al. [96] suggested an alternative structure of the pentameric complex that was consistent with the mutagenesis data as well. Finally, Torres et al. [75] employing site-specific FTIR data were able to obtain a structural model of the transmembrane domain of phospholamban that was consistent with the Simmerman model [96]. This model of phospholamban is depicted in Fig. 9.

One of the most interesting aspects of the model is that structure of phospholamban is similar to the pentamerization of the water-soluble COMP protein elucidated by Malashkevich et al. [97]. In fact, as stated in Section 1.2, Frank et al. [27] were able to covert the pentamerization sequence of phospholamban into a water-soluble protein similar to COMP.

Another intriguing feature of the phospholamban sequence are the three Cys residues interspersed amongst the Leu and Ile residues. Mutagenesis studies [98] were able to show that none of these Cys residues are essential for pentamerization, nor for the function of the protein. However, while replacement of any two cysteines did not result in pentamer disruption, replacement of all three residues did [98]. Their role in the structure is not clear although FTIR analysis [99] indicated that all three residues are hydrogen bonded to the carbonyl residues at position $i-4$ in the sequence.

Finally, it is noteworthy that phospholamban transmembrane domain sequence does not end with any polar residue aside from the terminal carboxyl. It is not clear if this has anything to do with the protein's function or rather with the incorporation of the protein in the lipid bilayer as phospholamban does not have a signal sequence.

Acknowledgements

Support is acknowledged from the Wellcome Trust (UK), the Biotechnology and Biological Sciences Research Council (UK) and The Israel Science Foundation (grant no. 784/01-16.1). The author is grateful to Professors Cross, Lemmon and MacKenzie for helpful discussion and communicating results before publication.

References

[1] I.T. Arkin, A.T. Brunger, Statistical analysis of predicted transmembrane α-Helices, Biochim. Biophys. Acta, Biomembr. 1429 (1998) 113–128.
[2] D.T. Jones, W.R. Taylor, J.M. Thornton, A model recognition approach to the prediction of all-helical membrane protein structure and topology, Biochemistry 33 (10) (1994) 3038–3049.
[3] I.T. Arkin, A.T. Brunger, D.M. Engelman, Are there dominant membrane protein families with a given number of helices? Proteins 28 (4) (1997) 465–466.
[4] E. Wallin, G. Von Heijne, Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms, Protein Sci. 7 (4) (1998) 1029–1038.
[5] T.J. Stevens, I.T. Arkin, Do more complex organisms have a greater proportion of membrane proteins? Proteins 39 (2000) 417–420.
[6] T.A. Steitz, C. Anderson, W. Bennett, R. McDonald, R. Stenkamp, Protomer structure of oligomeric enzymes: symmetry and allosteric interactions in yeast hexokinase, Biochem. Soc. Trans 5 (3) (1977) 620–623.
[7] S.R. Hubbard, J.H. Till, Protein tyrosine kinase structure and function, Annu. Rev. Biochem. 69 (2000) 373–398.
[8] J. Schlessinger, Cell signaling by receptor tyrosine kinases, Cell 103 (2) (2000) 211–225.
[9] M.A. Olayioye, R.M. Neve, H.A. Lane, N.E. Hynes, The ErbB signaling network: receptor heterodimerization in development and cancer, EMBO J. 19 (13) (2000) 3159–3167.
[10] N.E. Hynes, K. Horsch, M.A. Olayioye, A. Badache, The ErbB receptor tyrosine family as signal integrators, Endocrinol. Cancer 8 (3) (2001) 151–159.
[11] T. Moriki, H. Maruyama, I.N. Maruyama, Activation of preformed EGF receptor dimer by ligand-induced rotation of the transmembrane domain, J. Mol. Biol. 311 (5) (2001) 1011–1026.
[12] S.N. Constantinescu, X. Liu, W. Beyer, A. Fallon, S. Shekar, Y.I. Henis, S.O. Smith, H.F. Lodish, Activation of the erythropoietin receptor by the gp55-P viral envelope protein is determined by a single amino acid in its transmembrane domain, EMBO J. 18 (12) (1999) 3334–3347.
[13] S.N. Constantinescu, T. Keren, M. Socolovsky, H.S. Nam, Y.I. Henis, H.F. Lodish, Ligand-independent oligomerization of cell-surface erythropoietin receptor is mediated by the transmembrane domain, Proc. Natl. Acad. Sci. U. S. A. 98 (8) (2001) 4379–4384.
[14] C.I. Bargmann, M.C. Hung, R.A. Weinberg, Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185, Cell 45 (5) (1986) 649–657.
[15] D.B. Weiner, J. Liu, J.A. Cohen, W.V. Williams, M.I. Greene, A point mutation in the neu oncogene mimics ligand induction of receptor aggregation, Nature 339 (6221) (1989) 230–231.
[16] S.O. Smith, C.S. Smith, B.J. Bormann, Strong hydrogen bonding interactions involving a buried glutamic acid in the transmembrane

sequence of the neu/erbB-2 receptor, Nat. Struct. Biol. 3 (3) (1996) 252–258.
[17] J.R. Doedens, K. Kirkegaard, Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A, EMBO J. 14 (5) (1995) 894–907.
[18] M.A. Sanz, L. Perez, L. Carrasco, Semliki forest virus 6K protein modifies membrane permeability after inducible expression in *Escherichia coli* cells, J. Biol. Chem. 269 (16) (1994) 12106–12110.
[19] S.W. Hiebert, R.G. Paterson, R.A. Lamb, Identification and predicted sequence of a previously unrecognized small hydrophobic protein, SH, of the paramyxovirus Simian virus 5, J. Virol. 55 (3) (1985) 744–751.
[20] P.L. Collins, G. Mottet, Oligomerization and post-translational processing of glycoprotein G of human respiratory syncytial virus: altered O-glycosylation in the presence of brefeldin A, J. Gen. Virol. 73 (4) (1992) 849–863.
[21] L.H. Pinto, L.J. Holsinger, R.A. Lamb, Influenza virus M2 protein has ion channel activity, Cell 69 (3) (1992) 517–528.
[22] N.A. Sunstrom, L.S. Premkumar, A. Premkumar, G. Ewart, G.B. Cox, P.W. Gage, Ion channels formed by NB, an Influenza B virus protein, J. Membr. Biol. 150 (2) (1996) 127–132.
[23] S. Hongo, K. Sugawara, Y. Muraki, F. Kitame, K. Nakamura, Characterization of a second protein (CM2) encoded by RNA segment 6 of influenza C virus, J. Virol. 71 (4) (1997) 2786–2792.
[24] E.A. Cohen, E.F. Terwilliger, J.G. Sodroski, W.A. Haseltine, Identification of a protein encoded by the vpu gene of HIV-1, Nature 334 (6182) (1988) 532–534.
[25] M. Eilers, S.C. Shekar, T. Shieh, S.O. Smith, P.J. Fleming, Internal packing of helical membrane proteins, Proc. Natl. Acad. Sci. U. S. A. 97 (11) (2000) 5796–5801.
[26] M.M. Javadpour, M. Eilers, M. Groesbeek, S.O. Smith, Helix packing in polytopic membrane proteins: role of glycine in transmembrane helix association, Biophys. J. 77 (3) (1999) 1609–1618.
[27] S. Frank, R.A. Kammerer, S. Hellstern, S. Pegoraro, J. Stetefeld, A. Lustig, L. Moroder, J. Engel, Toward a high-resolution structure of phospholamban: design of soluble transmembrane domain mutants, Biochemistry-us 39 (23) (2000) 6825–6831.
[28] H. Li, M.J. Cocco, T.A. Steitz, D.M. Engelman, Conversion of phospholamban into a soluble pentameric helical bundle, Biochem. 40 (2001) 6636–6645.
[29] J.L. Popot, D.M. Engelman, Helical membrane protein folding, stability, and evolution, Annu. Rev. Biochem. 69 (2000) 881–922.
[30] L.E. Fisher, D.M. Engelman, J.N. Sturgis, Detergents modulate dimerization, but not helicity, of the glycophorin A transmembrane domain, J. Mol. Biol. 293 (3) (1999) 639–651.
[31] E.K. O'Shea, J.D. Klemm, P.S. Kim, T. Alber, X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil, Science 254 (5031) (1991) 539–544.
[32] P.B. Harbury, T. Zhang, P.S. Kim, T. Alber, A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants, Science 262 (5138) (1993) 1401–1406.
[33] J.L. Popot, S.E. Gerchman, D.M. Engelman, Refolding of bacteriorhodopsin in lipid bilayers. A thermodynamically controlled two-stage process, J. Mol. Biol. 198 (4) (1987) 655–676.
[34] J.L. Popot, D.M. Engelman, Membrane protein folding and oligomerization: the two-stage model, Biochemistry 29 (17) (1990) 4031–4037.
[35] I.T. Arkin, A.T. Brunger, Statistical analysis of predicted transmembrane α-helices, Biochem. Biophys. Acta 1429 (1998) 113–128.
[36] T.J. Stevens, I.T. Arkin, Are membrane proteins "inside-out" proteins? Proteins Struct. Func. Gen. 36 (1999) 135–143.
[37] M. Tomita, V.T. Marchesi, Amino-acid sequence and oligosaccharide attachment sites of human erythrocyte glycophorin, Proc. Natl. Acad. Sci. U. S. A. 72 (8) (1975) 2964–2968.
[38] B.J. Bormann, D.M. Engelman, Intramembrane helix–helix association in oligomerization and transmembrane signaling, Annu. Rev. Biophys. Biomol. Struct. 21 (1992) 223–242.
[39] M.A. Lemmon, J.M. Flanagan, J.F. Hunt, B.D. Adair, B.J. Bormann, C.E. Dempsey, D.M. Engelman, Glycophorin A dimerization is driven by specific interactions between transmembrane alpha-helices, J. Biol. Chem. 267 (11) (1992) 7683–7689.
[40] M.A. Lemmon, J.M. Flanagan, H.R. Treutlein, J. Zhang, D.M. Engelman, Sequence specificity in the dimerization of transmembrane alpha-helices, Biochemistry 31 (51) (1992) 12719–12725.
[41] I. Mingarro, P. Whitley, M.A. Lemmon, G. Von Heijne, Ala-insertion scanning mutagenesis of the glycophorin A transmembrane helix: a rapid way to map helix–helix interactions in integral membrane proteins, Protein Sci. 5 (7) (1996) 1339–1341.
[42] I. Mingarro, A. Elofsson, G. Von Heijne, Helix–helix packing in a membrane-like environment, J. Mol. Biol. 272 (4) (1997) 633–641.
[43] M.A. Lemmon, H.R. Treutlein, P.D. Adams, A.T. Brunger, D.M. Engelman, A dimerization motif for transmembrane alpha-helices, Nat. Struct. Biol. 1 (3) (1994) 157–163.
[44] K.R. MacKenzie, D.M. Engelman, Structure-based prediction of the stability of the transmembrane helix–helix interactions: the sequence dependence of glycophorin A dimerization, Proc. Natl. Acad. Sci. U. S. A. 95 (7) (1998) 3583–3590.
[45] D.M. Engelman, T.A. Steitz, A. Goldman, Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins, Annu. Rev. Biophys. Biophys. Chem. 15 (1986) 321–353.
[46] R.L. Dunbrack Jr., M. Karplus, Backbone-dependent rotamer library for proteins. Application to side-chain prediction, J. Mol. Biol. 230 (2) (1993) 543–574.
[47] Z. Fang-Xiao, M.J. Cocco, W.P. Russ, A.T. Brunger, D.M. Engelman, Interhelical hydrogen bonding drives strong interactions in membrane proteins, Nat. Struct. Biol. 7 (2) (2000) 154–160.
[48] C. Choma, H. Gratkowski, J.D. Lear, W.F. DeGrado, Asparagine-mediated self-association of a model transmembrane helix, Nat. Struct. Biol. 7 (2) (2000) 161–166.
[49] H. Gratkowski, J.D. Lear, W.F. DeGrado, Polar side chains drive the association of model transmembrane peptides, Proc. Natl. Acad. Sci. U. S. A. 98 (3) (2001) 880–885.
[50] Z. Fang-Xiao, H.J. Merianos, A.T. Brunger, D.M. Engelman, Polar residues drive association of polyleucine transmembrane helices, Proc. Natl. Acad. Sci. U. S. A. 98 (5) (2001) 2250–2255.
[51] A. Senes, I. Ubarretxena-Belandia, D.M. Engelman, The Cα—H· · ·O hydrogen bond: a determinant of stability and specificity in transmembrane helix interactions, Proc. Natl. Acad. Sci. U. S. A. 98 (16) (2001) 9056–9061.
[52] J.U. Bowie, Helix packing in membrane proteins, J. Mol. Biol. 272 (5) (1997) 780–789.
[53] D. Langosch, B. Brosig, H. Kolmar, H.J. Fritz, Dimerisation of the glycophorin A transmembrane segment in membranes probed with the ToxR transcription activator, J. Mol. Biol. 263 (4) (1996) 525–530.
[54] W.P. Russ, D.M. Engelman, The GxxxG motif: a framework for transmembrane helix–helix association, J. Mol. Biol. 296 (3) (2000) 911–919.
[55] J.A. Leeds, D. Boyd, D.R. Huber, G.K. Sonoda, H.T. Luu, D.M. Engelman, J. Beckwith, Genetic selection for and molecular dynamic modeling of a protein transmembrane domain multimerization motif from a random *Escherichia coli* genomic library, J. Mol. Biol. 313 (1) (2001) 181–195.
[56] G. Kleiger, J. Perry, D. Eisenberg, 3D structure and significance of the GΦXXG helix packing motif in tetramers of the E1β subunit of pyruvate dehydrogenase from the archeon *Pyrobaculum aerophilum*, Biochem. 40 (2001) 14484–14492.
[57] R. Laage, J. Rohde, B. Brosig, D. Langosch, A conserved membrane-spanning amino acid motif drives homomeric and supports heteromeric assembly of presynaptic SNARE proteins, J. Biol. Chem. 275 (23) (2000) 17481–17487.
[58] W.P. Russ, D.M. Engelman, TOXCAT: a measure of transmembrane helix association in a biological membrane, Proc. Natl. Acad. Sci. U. S. A. 96 (3) (1999) 863–868.
[59] B.D. Adair, D.M. Engelman, Glycophorin A helical transmembrane

domains dimerize in phospholipid bilayers: a resonance energy transfer study, Biochemistry 33 (18) (1994) 5539–5544.
[60] I.T. Arkin, P.D. Adams, K.R. MacKenzie, M.A. Lemmon, A.T. Engelman, D.M. Engelman, Structural organization of the pentameric transmembrane alpha-helices of phospholamban, a cardiac ion channel, EMBO J. 13 (20) (1994) 4757–4764.
[61] K.G. Fleming, A.L. Ackerman, D.M. Engelman, The effect of point mutations on the free energy of transmembrane alpha-helix dimerization, J. Mol. Biol. 272 (2) (1997) 266–275.
[62] Z. Bu, D.M. Engelman, A method for determining transmembrane helix association and orientation in detergent micelles using small angle X-ray scattering, Biophys. J. 77 (2) (1999) 1064–1073.
[63] K.R. MacKenzie, J.H. Prestegard, D.M. Engelman, A transmembrane helix dimer: structure and implications, Science 276 (5309) (1997) 131–133.
[64] S.O. Smith, K. Aschheim, M. Groesbeek, Magic angle spinning NMR spectroscopy of membrane proteins, Q. Rev. Biophys. 29 (4) (1996) 395–449.
[65] R.E. Koeppe II, J.A. Killian, D.V. Greathouse, Orientations of the tryptophan 9 and 11 side chains of the gramicidin channel based on deuterium nuclear magnetic resonance spectroscopy, Biophys. J. 66 (1) (1994) 14–24.
[66] W. Hu, T.A. Cross, Tryptophan hydrogen bonding and electric dipole moments: functional roles in the gramicidin channel and implications for membrane proteins, Biochemistry 34 (43) (1995) 14147–14155.
[67] J. Wang, S. Kim, F. Kovacs, T.A. Cross, Structure of the transmembrane region of the M2 protein H^+ channel, Protein Sci. 10 (11) (2001) 2241–2250.
[68] I.T. Arkin, K.R. MacKenzie, A.T. Brunger, Site-directed dichroism as a method for obtaining rotational and orientational constraints for oriented polymers, J. Am. Chem. Soc. 119 (38) (1997) 8973–8980.
[69] A. Kukol, P.D. Adams, L.M. Rice, A.T. Brunger, I.T. Arkin, Experimentally based orientational refinement of membrane protein models: a structure for the Influenza A M2 H^+ channel, J. Mol. Biol. 286 (1999) 951–962.
[70] J. Torres, A. Kukol, I.T. Arkin, Mapping the energy surface of transmembrane helix–helix interactions, Biophys. J. 81 (2001) 2681–2692.
[71] C. Chothia, M. Levitt, D. Richardson, Helix to helix packing in proteins, J. Mol. Biol. 145 (1981) 215–250.
[72] H.R. Treutlein, M.A. Lemmon, D.M. Engelman, A.T. Brunger, The glycophorin A transmembrane domain dimer: sequence-specific propensity for a right-handed supercoil of helices, Biochemistry 31 (51) (1992) 12726–12732.
[73] P.D. Adams, I.T. Arkin, D.M. Engelman, A.T. Brunger, Computational searching and mutagenesis suggest a structure for the pentameric transmembrane domain of phospholamban, Nat. Struct. Biol. 2 (2) (1995) 154–162.
[74] P.D. Adams, D.M. Engelman, A.T. Brunger, Improved prediction for the structure of the dimeric transmembrane domain of glycophorin A obtained through global searching, Proteins 26 (3) (1996) 257–261.
[75] J. Torres, P.D. Adams, I.T. Arkin, Use of a new label, $^{13}C{=}^{18}O$, in the determination of a structural model of phospholamban in a lipid bilayer. Spatial restraints resolve the ambiguity arising from interpretations of mutagenesis data, J. Mol. Biol. 300 (2000) 677–685.
[76] J.A.G. Briggs, J. Torres, I.T. Arkin, A new method to model membrane protein structure based on silent amino-acid substitutions, Proteins 44 (3) (2001) 370–375.
[77] J. Torres, J.A.G. Briggs, I.T. Arkin, Multiple site-specific infrared dichroism of CD3-ζ, a transmembrane helix bundle, J. Mol. Biol. 316 (2002) 365–374.
[78] J. Torres, J.A.G. Briggs, I.T. Arkin, Convergence of experimental, computational and evolutionary approaches predicts the presence of a tetrameric form for CD3-ζ, J. Mol. Biol. 316 (2002) 375–384.
[79] P.J. Kraulis, Molscript: a program to produce both detailed and schematic plots of protein structures, J. Appl. Crystallogr. 24 (1991) 946–950.
[80] S.O. Smith, D. Song, S. Shekar, M. Groesbeek, M. Ziliox, S. Aimoto, Structure of the transmembrane dimer interface of glycophorin A in membrane bilayers, Biochemistry-us 40 (22) (2001) 6553–6558.
[81] S.O. Smith, B.J. Bormann, Determination of helix–helix interactions in membranes by rotational resonance NMR, Proc. Natl. Acad. Sci. U. S. A. 92 (2) (1995) 488–491.
[82] A. Helenius, Unpacking the incoming influenza virus, Cell 69 (4) (1992) 577–578.
[83] M. Bui, G. Whittaker, A. Helenius, Effect of m1 protein and low pH on nuclear transport of influenza virus ribonucleoproteins, J. Virol. 70 (1996) 8391–8401.
[84] Q. Tu, L.H. Pinto, G. Luo, M.A. Shaughnessy, D. Mullaney, S. Kurtz, M. Krystal, R.A. Lamb, Characterization of inhibition of M2 ion channel activity by BL-1743, an inhibitor of influenza A virus, J. Virol. 70 (7) (1996) 4246–4252.
[85] C. Wang, R.A. Lamb, L.H. Pinto, Activation of the M2 ion channel of influenza virus: a role for the transmembrane domain histidine residue, Biophys. J. 69 (4) (1995) 1363–1371.
[86] D. Salom, B.R. Hill, J.D. Lear, W.F. DeGrado, pH-dependent tetramerization and amantadine binding of the transmembrane helix of M2 from the influenza A virus, Biochemistry-us 39 (46) (2000) 14160–14170.
[87] A. Pekosz, R.A. Lamb, The CM2 protein of influenza C virus is an oligomeric integral membrane glycoprotein structurally analogous to influenza A virus M2 and influenza B virus NB proteins, Virology 237 (2) (1997) 439–451.
[88] S. Hongo, K. Sugawara, H. Nishimura, Y. Muraki, F. Kitame, K. Nakamura, Identification of a second protein encoded by influenza C virus RNA segment 6, J. Gen. Virol. 75 (12) (1994) 3503–3510.
[89] R.A. Lamb, L.H. Pinto, Do Vpu and Vpr of human immunodeficiency virus type 1 and NB of influenza B virus have ion channel activities in the viral life cycles? Virology 229 (1) (1997) 1–11.
[90] T. Betakova, M.V. Nermut, A.J. Hay, The NB protein is an integral component of the membrane of influenza B virus, J. Gen. Virol. 77 (Pt 11) (1996) 2689–2694.
[91] F.A. Kovacs, T.A. Cross, Transmembrane four-helix bundle of influenza A M2 protein channel: structural implications from helix tilt and orientation, Biophys. J. 73 (5) (1997) 2511–2517.
[92] F.A. Kovacs, J.K. Denny, Z. Song, J.R. Quine, T.A. Cross, Helix tilt of the M2 transmembrane peptide from influenza A virus: an intrinsic property, J. Mol. Biol. 295 (1) (2000) 117–125.
[93] I.T. Arkin, P.D. Adams, A.T. Brunger, S.O. Smith, D.M. Engelman, Structural perspectives of phospholamban, a helical transmembrane pentamer, Annu. Rev. Biophys. Biomol. Struct. 26 (1997) 157–179.
[94] M. Tada, M. Yabuki, T. Toyofuku, Molecular regulation of phospholamban function and gene expression, Ann. N.Y. Acad. Sci. 853 (1998) 116–129.
[95] H.K.B. Simmerman, L.R. Jones, Phospholamban: protein structure, mechanism of action, and role in cardiac function, Physiol. Rev. 78 (4) (1998) 921–947.
[96] H.K.B. Simmerman, Y.M. Kobayashi, J.M. Autry, L.R. Jones, A leucine zipper stabilizes the pentameric membrane domain of phospholamban and forms a coiled-coil pore structure, J. Biol. Chem. 271 (10) (1996) 5941–5946.
[97] V.N. Malashkevich, R.A. Kammerer, V.P. Efimov, T. Schulthess, J. Engel, The crystal structure of a five-stranded coiled coil in COMP: a prototype ion channel? Science 274 (5288) (1996) 761–765.
[98] J. Fujii, K. Maruyama, M. Tada, D.H. MacLennan, Expression and site-specific mutagenesis of phospholamban. Studies of residues involved in phosphorylation and pentamer formation, J. Biol. Chem. 264 (22) (1989) 12950–12955.
[99] I.T. Arkin, P.D. Adams, A.T. Brunger, S. Aimoto, D.M. Engelman, S.O. Smith, Structure of the transmembrane cysteine residues in phospholamban, J. Membr. Biol. 155 (3) (1997) 199–206.