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The Safety Dance: Biophysics of Membrane Protein Folding and Misfolding in a Cellular Context

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Abstract

Most biological processes require the production and degradation of proteins, a task that weighs heavily on the cell. Mutations that compromise the conformational stability of proteins place both specific and general burdens on cellular protein homeostasis (proteostasis) in ways that contribute to numerous diseases. Efforts to elucidate the chain of molecular events responsible for diseases of protein folding address one of the foremost challenges in biomedical science. However, relatively little is known about the processes by which mutations prompt the misfolding of α -helical membrane proteins, which rely on an intricate network of cellular machinery to acquire and maintain their functional structures within cellular membranes. In this review, we summarize the current understanding of the physical principles that guide membrane protein biogenesis and folding in the context of mammalian cells. Additionally, we explore how pathogenic mutations that influence biogenesis may differ from those that disrupt folding and assembly, as well as how this may relate to disease mechanisms and therapeutic intervention. These perspectives indicate an imperative for the use of information from structural, cellular, and biochemical studies of membrane proteins in the design of novel therapeutics and in personalized medicine.

Keywords

Proteins; Membrane; Membrane Proteins; Folding; Misfolding; Proteostasis; Translocon; Cellular Trafficking; Endoplasmic Reticulum; Quality Control; ERAD; Disease; Peripheral Myelin Protein 22; Steric Wedge

1. Introduction

A breakdown in the capacity of cells to produce and distribute functionally folded proteins and to dispose of misfolded proteins has been implicated in many diseases. This dysfunction, often spurred by mutations, causes a variety of biochemical outcomes including enhanced protein degradation, retention of proteins within the secretory pathway (Wiseman et al., 2007a), formation of intracellular protein aggregates (Kopito, 2000), and deposition of amyloid fibrils in tissues (Selkoe, 2003). These biological outcomes provide clues about the cellular components involved in pathogenesis and the biochemical nature of the dysfunction. However, the mechanisms by which these mutations trigger pathogenesis is often not

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obvious (Kelly & Balch, 2006; Powers et al., 2009). This is especially true for mutations occurring in α -helical membrane proteins, which must fold, assemble, and maintain functional structures within the chemically diverse membranes of the endoplasmic reticulum (ER), the Golgi complex, the plasma membrane (PM), and other organelles (Kelly & Balch, 2006; Sanders & Mittendorf, 2011). It is clear that the production of many wild-type membrane proteins in the cell is marginally efficient, which suggests that the energetics governing competing assembly and misassembly pathways may often be comparable (Sanders & Nagy, 2000). This may account for the fact that, in many cases, a multitude of diverse mutations are capable of prompting pathogenic misfolding of α -helical membrane proteins. For example, there are ca. 2000 mutations in the cystic fibrosis transmembrane regulator (CFTR) chloride channel that are known to cause cystic fibrosis, a majority of which are likely to induce misfolding as the primary cause of channel loss of function (cystic fibrosis mutation database: www.genet.sickkids.on.ca/cftr).

The precise origins of the cellular misfolding of α -helical membrane proteins remain elusive for several reasons. First, much less is known about the structure and conformational stability of α -helical membrane proteins compared to soluble proteins (White, 2009). Indeed, technical limitations have long impeded the investigation of the structure and folding of membrane proteins in their native membrane solvent (Booth & Curnow, 2009; Stanley & Fleming, 2008). Second, the synthesis, folding, and assembly of membrane proteins are elaborate cellular processes (Figure 1A), which could potentially be disrupted in any one of a variety of ways (Figure 1B–D) (Ng et al., 2012). For these reasons, efforts to rationalize the mechanisms of the pathogenic misfolding of α -helical membrane proteins often encounter technical and conceptual challenges.

Misfolding diseases are typically described as loss- or gain-of-function disorders; pathogenesis may arise as a result of the loss of functional protein due to misassembly or from the accumulation of cytotoxic protein aggregates (Cohen & Kelly, 2003). The inherent linkage between events that lead to the loss of functional protein and those leading to the emergence of toxic protein aggregates allows related disease phenotypes to arise from distinct biochemical mechanisms. Indeed, characteristic phenotypes of misfolding diseases often stem from diverse genetic mechanisms, even within a single disorder. For example, the majority of cystic fibrosis (CF) patients carry the F508 mutation in CFTR, which compromises its folding and biogenesis (Welsh & Smith, 1993). However, a number of other CFTR mutations are known to result in a loss of CFTR function through mechanisms other than misfolding (Ramsey et al., 2011; Sheppard et al., 1993; Van Goor et al., 2009). Rapid identification of new disease-linked mutations resulting from the implementation of personal genome sequencing will provide new challenges and opportunities in mechanistic biology. Assessments of the effects of newly identified pathogenic mutations on the cellular processing, biochemical activity, folding, and structure of these proteins represents a laborious undertaking. Nevertheless, delineation of the effects of pathogenic mutations on α -helical membrane proteins may be critical for optimal design of therapeutics and, eventually, for the tailoring of therapeutic regimens. With regard to personalized medicine it should be emphasized that strategies to treat or avoid disease by correcting or avoiding misfolding of a protein are likely to be distinct from strategies to correct defects in other pathogenic variants of the very same protein with compromised function.

In this review, we discuss the physical principles governing the biogenesis and folding of α -helical membrane proteins and the potential influence of pathogenic mutations on these processes in the context of mammalian cells. Additionally, we highlight current progress and demonstrate potential applications of existing tools to rationalize the influence of pathogenic mutations on the biogenesis of α -helical membrane proteins.

2. Cotranslational Folding and Misfolding of α -Helical Membrane Proteins

2.1 Translocon-mediated membrane integration of α -helical membrane proteins

In eukaryotic cells, translation of most α -helical membrane proteins occurs at the ER membrane and is mediated by the Sec61 translocon complex (Figure 1A, step I). In addition to the Sec61 translocon itself, which consists of the integral membrane proteins Sec61 α , β , and γ (Denks et al., 2014; Egea & Stroud, 2010; Van den Berg et al., 2004), the translocation process involves a number of accessory proteins that tune the function of the translocon complex and process the nascent chain (Johnson & van Waes, 1999; Schnell & Hebert, 2003). During the early stages of membrane protein translation, the ribosome is docked to the translocon by the signal recognition particle (SRP), which effectively extends the ribosomal exit tunnel through the ER membrane and into the lumen. As translation continues at the ER membrane, the nascent polypeptide chain can access the ER membrane through the lateral gate of the translocon (Figure 2) (Heinrich et al., 2000). Transmembrane (TM) helices pass through the lateral gate and into the ER membrane during translation, which establishes the initial topology of the α -helical membrane protein (topogenesis). Tertiary interactions between TM helices begin to form during translation (Cymer & von Heijne, 2013; Khushoo et al., 2011; Meindl-Beinker et al., 2006; Sadlish et al., 2005), which represent the earliest steps of α -helical membrane protein folding (Figure 1A, step I). The structural properties of cotranslational folding intermediates remain somewhat unclear. Thus, additional insights into the structure and function of the Sec61 translocon complex will ultimately be needed to enhance our understanding of the initial steps of α -helical membrane protein folding.

The structural and physical details of the events leading to the integration of individual nascent α -helices into the ER-membrane provide insights into the topogenic process. In many cases the structure, dynamics, and functional mode of the translocon complex, which is regulated by a host of protein-protein interactions (Johnson & van Waes, 1999; Schnell & Hebert, 2003; Snapp et al., 2004), may directly influence the manner by which individual helices are integrated into the membrane (Devaraneni et al., 2011). Nevertheless, a sizeable body of knowledge on the translocon-mediated membrane integration mechanism has indicated that the selection of TM helices by the translocon is principally guided by the physiochemical properties of the nascent chain itself (White & von Heijne, 2008). Portions of the emerging polypeptide chain transiently sample both the hydrated interior pore of the translocon and a cross section of the ER membrane in a manner that is well-described by equilibrium partitioning models (Hessa et al., 2005; Öjemalm et al., 2011; White & von Heijne, 2008). Moreover, the elucidation of an empirical code for the energetics of translocon-mediated insertion has enabled reasonably accurate topogenic predictions from protein sequence (*G* prediction server, www.dgpred.cbr.su.se) (Hessa et al., 2005; Kauko

et al., 2010; Virkki et al., 2014). Translocon-bilayer partitioning energetics of amino acid side chains is generally consistent with both water-octanol and water-bilayer partitioning (Fleming, 2014; Moon & Fleming, 2011; White, 2003; White & von Heijne, 2008; Wimley & White, 1996). Notably, the influence of a given amino acid on partitioning is strongly dependent on its position relative to the membrane (Hessa et al., 2005; Hessa et al., 2007; Moon & Fleming, 2011), which reflects the position-dependent polarity of the bilayer solvent (White, 2003; White & von Heijne, 2008). Partitioning of TM helices into the bilayer is dominated by the energetics associated with the burial of apolar surface area within the membrane core as well as by the positioning of positively charged residues among anionic phospholipid lipid head groups (positive-inside rule) (von Heijne, 1986; Öjemalm et al., 2011; von Heijne, 1992). These revelations provide a framework for understanding the sequence determinants of the early phase of α -helical membrane protein biogenesis and folding as well as the potential influence of pathogenic mutations on these processes.

2.2 Topogenesis of α -helical membrane proteins

The logic of the translocon suggests that a topogenic code should be written into the amino acid sequence of each α -helical membrane protein. Interestingly, a genomic survey of the predicted partitioning energetics of TM helices has revealed stark differences between single-pass and multi-pass α -helical membrane proteins (Hessa et al., 2007; White & von Heijne, 2008). In most cases, translocon-mediated insertion of the TM helices of single-pass α -helical membrane proteins appears to be highly favorable. This implies that membrane integration of single-pass TM helices is usually robust and may be insensitive to the influence of most single point mutations. Nevertheless, there are likely to be some exceptions, as recent work by Feige and Hendershot has demonstrated that topogenesis of less hydrophobic single-pass TM helices can require the formation of complimentary interactions with the TM helices of its native interaction partners (Feige & Hendershot, 2013).

Unlike single-pass membrane proteins, translocon-mediated membrane integration is predicted to be unfavorable for about one quarter of the TM helices in multi-pass α -helical membrane proteins (Hessa et al., 2007; White & von Heijne, 2008). These TM helices sometimes feature polar or charged residues positioned deep within the membrane, which are often critical for protein function or conformational stability (Adamian & Liang, 2002; Cao & Bowie, 2012; Gratkowski et al., 2001; Illergard et al., 2011; Popot & Engelman, 2000). The energetic penalty for burial of polar residues within the membrane may be partially offset by the formation of tertiary interactions between neighboring TM helices during translation (Meindl-Beinker et al., 2006; White & von Heijne, 2008) or perhaps in some cases by the formation of transient hydrogen bonds with buried water molecules, which are often found in the crystal structures of α -helical membrane proteins (Miyano et al., 2010). However, pathogenic mutations that introduce non-native polar side-chains within TM helices, which constitutes the most common class of amino acid substitution for disease-linked point mutations in α -helical membrane proteins (Partridge et al., 2004), bear the potential to disrupt these critical interactions. Both the influence of pathogenic mutations

on topogenesis and the ramifications of cotranslational misfolding on cellular proteostasis merit further consideration.

The molecular details of multi-pass α -helical membrane protein topogenesis has been intensely studied for a number of proteins. In particular, the topogenesis of CFTR has served as a key model system (Kim & Skach, 2012; Sadlish & Skach, 2004). One interesting property revealed by these studies is the heterogeneous nature of its biosynthetic pathway. For instance, two charged residues within the first TM segment of CFTR prevent its efficient recognition as a TM helix during the early steps of biogenesis (Lu et al., 1998). Because the initial topology of the N-terminal TM domains influences that of the subsequently synthesized TM helices (Chen & Zhang, 1999; Kanki et al., 2002), inefficient recognition of the first TM helices may ultimately cause topological heterogeneity in the nascent structural ensemble. Characterization of the topology of various truncated CFTR constructs suggests that ca. 70% of the nascent proteins eventually acquire the correct topology in the first two TM helices (Lu et al., 1998; Xiong et al., 1997). It is tempting to speculate that this topological heterogeneity may contribute to the poor efficiency with which even wild-type CFTR is known to correctly fold and traffic to the cell surface (Ward & Kopito, 1994). Topological heterogeneity appears to be a feature of the nascent forms of a number of multi-pass α -helical membrane proteins including P-glycoprotein (Pgp, MDR1) (Moss et al., 1998; Skach et al., 1993), sarcoplasmic/endoplasmic reticulum Calcium ATPase 2 (SERCA2) (Bayle et al., 1995), anion exchanger-1 (AE1, band 3) (Kanki et al., 2002), and aquaporin-1 (AQP1) (Buck & Skach, 2005; Lu et al., 2000; Skach et al., 1994). In some cases, the misincorporation of entire TM helices can occur during the biosynthesis of topologically “frustrated” membrane proteins (Gafvelin & von Heijne, 1994), which feature sequences with ambiguous topogenic codes (von Heijne, 2006). Studies of AE1 (Kanki et al., 2002), rhodopsin (Kanner et al., 2002), and AQP1 (Lu et al., 2000; Virkki et al., 2014) biosynthesis have shown that aberrant topomers of nascent proteins may be corrected post-translationally. However, to our knowledge, the mechanisms and molecular players involved in correcting aberrant topomers are currently unclear. Regardless of the mechanism, the reorientation of TM helices can sometimes require hours (Lu et al., 2000) and may often be outpaced by the rapid degradation of misassembled topological intermediates by the proteasome (Buck & Skach, 2005).

Non-ideal topogenesis of multi-pass α -helical membrane proteins is consistent with the prediction that the translocon-bilayer partitioning equilibrium of many TM helices within these proteins is predicted to be close to 0 kcal/mol (Hessa et al., 2007; White & von Heijne, 2008), suggesting that only a fractional population of the nascent TM helices should spontaneously assume the correct topology during translation. These findings highlight the inherent plasticity of the translocon-mediated membrane integration process and again suggest the potential for facile distortion of this process by pathogenic mutations.

2.3 Influence of pathogenic mutations on the translocon-mediated insertion of TM helices

The apparent biochemical inefficiency of topogenesis suggests a potentially disruptive role for pathogenic mutations at the translocon. To our knowledge, it is not clear whether this is a common effect of pathogenic mutations. We utilized the ΔG prediction server to survey the

effects of 470 non-synonymous mutations known to be associated with misfolding diseases occurring within or near the TM helices of five multi-pass α -helical membrane proteins including rhodopsin, vasopressin V2 receptor (V2R), CFTR, peripheral myelin protein 22 (PMP22), and the voltage-gated potassium channel KCNQ1 (manuscript in preparation). In the absence of tertiary contacts, 13 of the 36 total TM helices are predicted to insert with moderate efficiency ($-1 \text{ kcal/mol} < G_{\text{app}} < 1 \text{ kcal/mol}$) and 7 are predicted to insert with poor efficiency ($G_{\text{app}} > 1 \text{ kcal/mol}$). Of the surveyed pathogenic mutations implicated in misfolding diseases, 63 mutations (ca. 10%) were predicted to increase the predicted free energy for the insertion of their respective TM helices by more than 1 kcal/mol. Furthermore, 31 of these 63 mutations occur within TM helices predicted to have moderate or poor insertion efficiency in the wild-type protein. The true partitioning behavior of these TM helices and the magnitude of the energetic effects of these mutations are likely to be different in the context of the full length protein due to the formation of helical hairpins within the translocon (Engelman & Steitz, 1981; Heinrich & Rapoport, 2003; Hermansson & von Heijne, 2003; Meindl-Beinker et al., 2006). Nevertheless, it seems feasible that some of these pathogenic mutations could interfere with topogenesis. Furthermore, the rapid degradation of aberrant topological intermediates (Buck & Skach, 2005) suggests a mechanism by which pathogenic mutations that influence topogenic efficiency may decrease the yield of mature protein. The considerations outlined in this section suggest that the induction of misfolding by mutations that interfere with topogenesis may not be rare, but may be much less common than mutations that disrupt or alter later stages of folding (discussed below). Testing this hypothesis is an avenue for future research.

3. Energetics of Folding and Misfolding of α -Helical Membrane Proteins

3.1 Physical principles of post-translational α -helical membrane protein folding

As is true for soluble proteins (Anfinsen, 1973), the conformational trajectories of membrane proteins seek free energy minima (Fleming, 2014; Huang et al., 1981; Kim et al., 2014; Popot et al., 1986; Stanley & Fleming, 2008; White, 2003). Furthermore, the conformational energy landscapes of α -helical membrane proteins may dictate the nature of their post-translational interactions with the cellular quality control machinery (Roth & Balch, 2011; Sanders & Myers, 2004). After TM helices are inserted into the bilayer by the translocon, the helices associate in order to establish their native tertiary structure (Figure 1A, step II) (White, 2003); a process often rationalized by the two-stage model (Engelman et al., 2003; Popot & Engelman, 1990). Despite long-standing interest in this phenomenon, practical limitations have long hampered the characterization of the tertiary folding of α -helical membrane proteins under equilibrium conditions (Booth & Curnow, 2009; Hong et al., 2009). As a result, our current understanding of the conformational energy landscapes of α -helical membrane proteins remains rudimentary (Bowie, 2005; Kim et al., 2014).

Surveying the energy landscapes of α -helical membrane proteins ideally involves measurement of the kinetic and thermodynamic barriers separating native and non-native states occurring within biological membranes; a daunting challenge. Nevertheless, the characterization of experimentally tractable conformational equilibria has revealed a number of fundamental principles. Despite the low dielectric environment within the membrane, the

energetic contribution of hydrogen bonds to membrane protein conformational equilibria appears similar to that of soluble proteins (Bowie, 2011; Faham et al., 2004; Joh et al., 2008; Li et al., 2006). The energetic contribution of van der Waals packing interactions also appears to be similar for soluble proteins and α -helical membrane proteins (Doura et al., 2004; Faham et al., 2004; Fleming et al., 1997; Joh et al., 2009). However, unlike soluble proteins, membrane proteins are subject to forces imposed by cellular membranes. Various lines of evidence involving both α -helical and β -barrel membrane protein folding in lipid bilayers have suggested the width and curvature of the membrane can significantly influence folding reactions (Allen et al., 2004a; Booth & Curnow, 2009; Brown, 2012; Burgess et al., 2008; Hong & Tamm, 2004). Determination of the means by which these forces combine to shape the conformational equilibria of α -helical membrane proteins in membranes represents a frontier in protein science (Dill & MacCallum, 2012).

3.2 Conformational energetics of multi-pass α -helical membrane proteins

Efforts to probe the basic features of the conformational energy landscapes of multi-pass α -helical membrane proteins have proven challenging due, in part, to the fact that commonly used denaturing agents such as urea are rarely capable of sufficiently disrupting their conformational equilibria (Fleming, 2014; Stanley & Fleming, 2008). For this reason, such studies have relied heavily on the use of mild detergent micelles or detergent-lipid mixed micelles that energetically favor the native ensemble, which can be titrated with a charged denaturing detergent (i.e. SDS) that promotes the formation of a denatured ensemble. Perturbation of the conformational equilibrium can be accomplished in mixed micelle systems by modulating the mole fraction of the denaturing detergent. Providing that reversibility can be achieved (Fleming, 2014; Moon et al., 2011), observations of the conformational ensemble upon the addition or dilution of the denaturing detergent can provide quantitative insights into the kinetic and thermodynamic properties of the conformational equilibrium. Such experiments facilitate the measurement of an equilibrium between the native ensemble and a non-native ensemble that typically retains some α -helical secondary structure but lacks tertiary or quaternary structure (Dutta et al., 2010; Krishnamani et al., 2012; Lau & Bowie, 1997; London & Khorana, 1982; Riley et al., 1997; Schlebach et al., 2011; Stanley & Fleming, 2008). Though mixed micelle solvents are certainly not a perfect proxy for biological membranes (Matthews et al., 2006; Warschawski et al., 2011; Zhou & Cross, 2013), the loss of tertiary structure and partial loss of secondary structure that accompanies denaturation of α -helical membrane proteins with a charged detergent is generally consistent with the documented structural defects in the misfolded forms of pathogenic rhodopsin variants responsible for retinitis pigmentosa (Liu et al., 1996). Thus, assessment of the conformational equilibria of α -helical membrane proteins in mixed micelles may provide insight into the nature of the energetic transitions relevant to the misfolding process. Nevertheless, strategies to measure conformational stability of proteins within bilayers and even native membranes are on the horizon. For instance, the recent advent of the “steric trap” approach by Hong and Bowie (Figure 3), has enabled quantitative assessments of conformational equilibria under bilayered membrane conditions (Chang & Bowie, 2014; Hong et al., 2010; Hong & Bowie, 2011). Application of the steric trap has already demonstrated, quite strikingly, that dimerization of glycoporphin A seems to be weaker in membranes than in micelles (Hong & Bowie, 2011). Thus, many aspects of the

nature of the interplay between the conformational equilibrium of membrane proteins with biological membranes remain to be explored.

The conformational stability of a handful of purified α -helical membrane proteins have been quantitatively assessed in various membrane mimetics. Interestingly, conformational stability measurements of diacylglycerol kinase (DAGK) (Lau & Bowie, 1997), bacteriorhodopsin (bR) (Curnow & Booth, 2007), the KcsA potassium channel (Barrera et al., 2008), and aquaglyceroporin GlpF (Veerappan et al., 2011) have suggested large thermodynamic separation of the native and denatured reference states ($G_{\text{unf}} = 16$ to 31 kcal/mol). On the other hand, studies of disulfide bond reducing protein B (DsbB) (Otzen, 2003), galactose transporter GalP (Findlay et al., 2010), lactose permease LacY (Harris et al., 2014), human peripheral myelin protein 22 (PMP22) (Schlebach et al., 2013), and the rhomboid protease (Baker & Urban, 2012) have suggested a more modest thermodynamic preference for their native conformations ($G_{\text{unf}} = 0$ to 4.5 kcal/mol). Can it be that the range of thermodynamic stabilities of single domain wild-type α -helical membrane proteins varies by more than an order of magnitude? This remains to be resolved. However, in addition to technical issues involving empirical stability extrapolations (Chang & Bowie, 2014; Schlebach et al., 2012; Sehgal et al., 2005), the seemingly large dynamic range of stability measurements may arise, in part, from the distinct structural and energetic properties of the *unfolded* states generated by the different denaturants employed in these studies (Stanley & Fleming, 2008). Indeed, recent work by Chang and Bowie has revealed that the proposed free energy difference between native bR and SDS-denatured bR is about twice as large as that separating native bR from a sterically trapped unfolded bR ensemble under identical phospholipid bicelle conditions in the absence of SDS (Chang & Bowie, 2014; Curnow & Booth, 2007). Comparisons of the thermodynamic stabilities is also complicated by the fact that unfolding is coupled to changes in oligomeric state for some of these proteins (DAGK, KcsA, and GlpF), the energetics of which are dependent on the protein-to-detergent (and/or lipid) ratio utilized in the chosen reaction conditions (Fleming, 2002). Despite such difficulties, apples-to-apples comparisons of stability measurements for wild-type and mutant variants in mixed micelle systems have proven widely useful (Baker & Urban, 2012; Cao & Bowie, 2012; Curnow & Booth, 2009; Curnow et al., 2011; Joh et al., 2009; Otzen, 2011). Nevertheless, the steric trap method (Chang & Bowie, 2014; Hong et al., 2010; Hong & Bowie, 2011), alternative applications of current methods (reviewed in (Hong et al., 2009)), and possibly new approaches will be needed to characterize the thermodynamic preference for the native ensemble within bilayered vesicles and actual biological membranes.

Conformational energy landscapes also dictate the *rates* of α -helical membrane protein folding. The rates of soluble protein folding reactions vary greatly but are generally rapid (Plaxco et al., 2000); folding of soluble proteins generally requires anywhere from microseconds to minutes *in vitro*. Similar to soluble proteins (Brockwell & Radford, 2007), kinetic intermediates of helical membrane proteins can form within milliseconds of the initiation of folding from detergent-denatured states (Allen et al., 2004b; Booth et al., 1995; Krishnamani & Lanyi, 2011; Lu & Booth, 2000; Otzen, 2003). However, complete refolding and/or oligomerization from can require anywhere from minutes to days *in vitro* (Allen et

al., 2004b; Cao et al., 2011; Jefferson et al., 2013; Krishnamani & Lanyi, 2011; Riley et al., 1997; Schlebach et al., 2012; Schlebach et al., 2013). The folding of the soluble denatured forms of β -barrel membrane proteins into membranes also appears to be quite slow (Burgess et al., 2008; Gessmann et al., 2014; Huysmans et al., 2010; Huysmans et al., 2012), though in this case the rate-limiting step seems to involve the transfer of the unfolded protein from the aqueous phase to the membrane phase (Gessmann et al., 2014; Huysmans et al., 2010; Huysmans et al., 2012). Slow folding relative to biological time scales suggests an essential biological role for chaperones and folding enzymes, a possibility consistent with the observed differences in the chaperone binding of wild type CFTR relative to the slow-folding pathogenic F508 variant (Coppinger et al., 2012; Qu et al., 1997; Wang et al., 2006). Thus, characterization of folding intermediates and their reactivity with the components of cellular quality control may be central to understanding the folding and misfolding mechanisms of pathogenic variants (Roth & Balch, 2011).

The cellular turnover of α -helical membrane proteins is also likely to depend on their rates of unfolding (kinetic stability). For many soluble proteins (Park et al., 2007; Xia et al., 2007), conformational stability is effectively achieved through a high kinetic barrier to unfolding (Jaswal et al., 2002). Several lines of evidence indicate that some α -helical membrane proteins may also be kinetically isolated from non-native states in some cases. For instance, the extrapolated unfolding rate of bR in mixed micelles suggests that accessing the detergent-denatured ensemble under native conditions would require a very, very long time (Curnow & Booth, 2007; Curnow & Booth, 2010). Furthermore, the dissociation and/or unfolding of trimeric DAGK in lipid bicelles has been found to require weeks (Jefferson et al., 2013). High kinetic barriers to unfolding and/or dissociation of oligomers would be consistent with the slow subunit exchange observed for both DAGK and AcrB in membranes (Jefferson et al., 2013; Lu et al., 2012). Thus, in contrast to rapidly folding and unfolding single-domain soluble proteins, it is unclear whether most membrane proteins effectively achieve an equilibrium conformational ensemble *in vivo*. One intriguing possibility is that nascent membrane proteins freely interconvert between native and non-native conformations in the endoplasmic reticulum in the presence of the chaperones of quality control and low cholesterol concentrations, but become kinetically trapped in their native states after export to the Golgi and beyond.

Collective elucidation of the interplay between the conformational stability, folding rates, and unfolding rates of α -helical membrane proteins may ultimately be necessary to rationalize the cellular proteostasis of α -helical membrane proteins.

3.3 Influence of pathogenic mutations on α -helical membrane protein folding and assembly

Numerous diseases are linked to the defective folding and/or trafficking of membrane proteins. The cellular biosynthesis and assembly of membrane proteins is an inherently inefficient process (Ellgaard & Helenius, 2003; Sanders & Nagy, 2000) and pathogenic variants are typically assembled with even lower efficiencies, which suggests these mutations cause structural defects that are detected by cellular quality control machinery (Kaushal & Khorana, 1994; Naef & Suter, 1999; Sanders & Myers, 2004; Sung et al., 1991).

However, little is known about the influence of pathogenic mutations on the conformational equilibria of such proteins. This impasse primarily stems from the restricted number of α -helical membrane proteins that have thus far been amenable to *in vitro* investigations of folding and assembly. Nevertheless, a survey of the nature of pathogenic side chain substitutions and their distributions throughout the topological domains of α -helical membrane proteins suggests that these mutations are likely to disrupt their conformational stability (Sanders & Myers, 2004). For example, the currently identified pathogenic mutations in PMP22 are distributed throughout its sequence, with many involving the introduction of charged or helix-breaking residues within its TM helices (Figure 4). In the case of rhodopsin, a majority of pathogenic mutations seem to fall within a cluster of residues predicted to be essential for folding (Rader et al., 2004). Regardless of their position within the protein or the nature of the substitution, a majority of tested membrane protein mutations have been seen to destabilize tertiary or quaternary structure (Baker & Urban, 2012; Cao et al., 2011; Curnow & Booth, 2009; Curnow et al., 2011; Faham et al., 2004; Fleming & Engelman, 2001; Nagy & Sanders, 2004; Otzen, 2011), which may potentially explain why pathogenic mutations in α -helical membrane proteins are often well-distributed throughout the sequence. The magnitude of the effects of mutations on the conformational equilibria of α -helical membrane proteins seem to be on par with that of soluble proteins. Furthermore, a semi-quantitative investigation of DAGK folding has suggested that the degree of destabilization imparted by point mutations is inversely correlated with kinetic stability (Nagy & Sanders, 2004), which suggests that an increase in the free energy of the native ensemble may be a common effect of such point mutations. Mutations that specifically destabilize the rate-limiting transition state for folding may be relatively rare, but have been documented (Curnow et al., 2011; Nagy & Sanders, 2002; Otzen, 2011). Alternatively, a number of pathogenic mutations have been found to promote the formation of alternative structures via non-native contacts and/or disulfide bonds (Figure 1D) (Dhaunchak & Nave, 2007; Goldberg et al., 1998; Hwa et al., 1999; Li et al., 2006; Ng & Deber, 2010; Therien et al., 2001; You et al., 2007). Thus, it seems that cellular misfolding of α -helical membrane proteins may arise as a result of disruption of native contacts, formation of non-native contacts, or some combination of both.

New insights into the influence of pathogenic mutations on the tertiary folding of α -helical membrane proteins have been afforded by recent characterizations of the conformational stability and the structural dynamics of human PMP22, the cellular misfolding of which causes a spectrum of peripheral neuropathies (Jetten & Suter, 2000; Li et al., 2012b; Sanders et al., 2001; Suter & Snipes, 1995). The Human Gene Mutation Database currently lists 44 point mutations in PMP22 that are known to cause Charcot-Marie-Tooth disease (CMTD), Dejerine-Sottas syndrome (DSS), or hereditary neuropathy with liability to pressure palsies (HNPP) (hgmd.cf.ad.uk) (Figure 4). The α -G prediction server (www.dgpred.cbr.su.se) suggests favorable translocon-mediated membrane integration for each of the 4 TM helices of PMP22 and few of these mutations are predicted to significantly influence topogenesis (manuscript in preparation). Thus, the primary sequence suggests that the TM helices should spontaneously partition into the membrane even in the absence of favorable tertiary contacts. For this reason, it seems likely that pathogenic mutations in PMP22 primarily influence folding and assembly processes that occur *after* translocon-mediated membrane integration,

which is consistent with the observation that the cellular trafficking of pathogenic PMP22 variants is stalled at the so-called “intermediate compartment” between the ER and the Golgi (Tobler et al., 1999).

Emerging evidence suggests that the tertiary structure of wild-type PMP22 is only marginally stable and that most pathogenic mutations further reduce conformational stability. Reversible unfolding measurements of wild-type PMP22 have revealed a minimal free energy difference between folded and unfolded conformations in *n*-dodecylphosphocholine (DPC) micelles ($G_{\text{unf}} \approx 0$ kcal/mol) (Schlebach et al., 2013) (Figure 5A). It is, of course, likely that PMP22 possesses a somewhat greater degree of conformational stability in biological membranes. Nevertheless, slow folding and marginal stability of wild-type PMP22 in cellular membranes could potentially account for its poor cellular trafficking efficiency (only ~20% of cellular WT PMP22 reaches the PM) and rapid cellular degradation ($T_{1/2} \approx 30$ min) (Pareek et al., 1997). Characterization of the effects of the quintessential pathogenic mutations L16P and G150D, which cause CMTD in mice and humans (Suter et al., 1992), have suggested that these mutations both decrease conformational stability and enhance the aggregation propensity of PMP22 (Myers et al., 2008; Tobler et al., 2002). Furthermore, a comparison of the dynamics of WT and L16P PMP22 by NMR has revealed that this mutation enhances transient dissociation of the first TM helix and the transition of other three TM segments into a molten globular domain (Figure 5B) (Sakakura et al., 2011), which may suggest that the nature of the unfolding reactions responsible for cellular misfolding of PMP22 may be relatively minor. We have recently expanded our analysis of pathogenic PMP22 variants and found that most pathogenic mutations do indeed destabilize its tertiary structure and decrease its cellular trafficking efficiency (manuscript in preparation). These findings will provide much needed insight as to how the conformational stability of pathogenic α -helical membrane proteins variants is tied to their cellular fates.

Though mutations in PMP22 appear to influence the native tertiary structure, many other pathogenic mutations are known to perturb the oligomerization of α -helical membrane proteins. Dimerization and activation of receptor tyrosine kinases (RTK) is a critical step in a number of cellular signaling pathways and mutations within the TM domains of these proteins are known to cause a various forms of cancer and developmental disorders. Investigation of pathogenic RTK variants *in vitro* has provided strong evidence that these mutations can promote unregulated RTK dimerization through the formation of non-native hydrogen bonds or perhaps non-native disulfide bonds (Li et al., 2006; You et al., 2007). Interestingly, quantitative studies of such mutations *in vivo* have suggested that energetic effects of mutations as little as 0.5 kcal/mol can have profound consequences on biochemical activity and cellular turnover under certain circumstances (Chen et al., 2011; He & Hristova, 2008; Placone & Hristova, 2012). Despite these advances, exhaustive efforts to identify consensus sequence motifs specifying the interaction of TM helices or to predict the effects of mutations on dimerization from structure have been somewhat unsuccessful (Li et al., 2012a; MacKenzie & Fleming, 2008). Thus, the rationalization of the effects of pathogenic mutations on the folding and assembly of α -helical membrane proteins in the cell presently remains a pressing challenge in membrane protein biophysics.

4. Proteostasis and the Cellular Trafficking of α -Helical Membrane Proteins

4.1 α -Helical membrane protein misfolding in the context of endoplasmic reticulum quality control

Both in concert with and following translocon-mediated integration of transmembrane segments into the membrane of the endoplasmic reticulum, eukaryotic membrane proteins form tertiary and quaternary structure to attain their native structural state (Figure 1A). During this process, membrane proteins interact with a host of proteins that are dedicated to facilitate the folding process and also to police the misfolding of nascent proteins. These proteins include chaperones, prolyl isomerases, disulfide bond isomerases, glycosyltransferases, glycosidases, ubiquitin ligases, and proteases. In this section we explore later stages of membrane protein folding and misfolding in the context of the complex environment of mammalian cells. Elucidation of the molecular basis of misfolding diseases will ultimately require interpretation of the effects of these conformational defects on the native (and non-native) interactions of the protein with other proteins as well as lipids and metabolites (Ellgaard & Helenius, 2003; Kelly & Balch, 2006; Vembar & Brodsky, 2008). Elucidation of pathogenic mechanisms will also hinge on our understanding of the response of cells to dramatic changes in the flux of misfolded proteins caused by these mutations (Brodsky & Skach, 2011).

In many cases, only a fraction of nascent membrane proteins achieve maturity (Pareek et al., 1997; Sanders & Myers, 2004; Sanders & Nagy, 2000; Ward & Kopito, 1994), and eukaryotic cells have evolved elaborate systems to cope with the hazards of error-prone membrane protein synthesis and assembly. Nascent membrane proteins are subject to the scrutiny of a variety of folding-sensor proteins within the ER associated folding (ERAF) and ER associated degradation (ERAD) pathways (comprehensively reviewed in (Vembar & Brodsky, 2008)). Collectively, these processes are referred to as ER folding quality control (Figure 6). As a consequence of their residence in the ER membrane, the cytosolic, luminal, and transmembrane portions of nascent membrane proteins are interrogated by distinct sets of quality control machinery in each of these compartments (Brodsky & Skach, 2011; Buchberger et al., 2010). Gross conformational defects in the soluble domains of membrane proteins (or perhaps the misincorporation of TM helices) may result in the inappropriate exposure of hydrophobic patches, which are likely recognized by soluble molecular chaperones such as Hsp70 in the cytosol and the Hsp70 homolog BiP in the lumen (Buchberger et al., 2010; Meacham et al., 1999; Otero et al., 2010). ER quality control machinery also uses N-linked glycosylation markers to monitor the folding process, wherein nascent membrane proteins enter the kinetically controlled calnexin/calreticulin chaperone cycle (Ellgaard & Helenius, 2003; Hebert & Molinari, 2012; Sanders & Myers, 2004; Vembar & Brodsky, 2008). It is less clear how defectively folded transmembrane domains are recognized (Houck & Cyr, 2012). However, a number of proteins potentially capable of recognizing defectively assembled TM domains have been identified including the E3 ubiquitin ligase HRD1 (Sato et al., 2009), the rhomboid homolog Derlin-1 (Sun et al., 2006), UDP-glucose: glycoprotein glucosyltransferase (Dedola et al., 2014; Taylor et al., 2004), and the translocating-chain associated membrane protein (TRAM) (Tamborero et al., 2011). Calnexin itself has also been reported to directly sense defective TM domains in membrane

proteins in a manner that is independent of its ability to recognize glycans (Cannon & Cresswell, 2001; Fontanini et al., 2005; Swanton et al., 2003). Recognition of incompletely folded or misfolded proteins in which natively-buried trafficking sequence motifs are exposed also plays a key role in ER retention or Golgi-to-ER retrieval (Geva & Schuldiner, 2014; Lee et al., 2004; Michelsen et al., 2005; Teasdale & Jackson, 1996; Yamamoto et al., 2001). The biosynthesis of most membrane proteins is typically governed by interactions with some combination of the folding sensors described above. Protein quality control pathways are not typically “one set fits all” (Buchberger et al., 2010). Rather, different client proteins are engaged by different components and subsystems of quality control based on their physiochemical properties and the cellular physiological state (Eletto et al., 2012; Jung et al., 2011; Kanehara et al., 2010; Maattanen et al., 2006; Molinari et al., 2004). For instance, the conformational equilibrium of CFTR is known to be surveyed and adjusted by at least three different components of protein folding quality control: (1) its large cytosolic domain interacts with cytosolic heat shock protein chaperones (Hsp40, Hsp70, Hsp90), (2) the luminal/extracellular and transmembrane domains of the protein interact with the components of the calnexin cycle, and (3) small sequence motifs are recognized by the gatekeepers of the ER-to-Golgi transport pathway, leading to ER-retention (Farinha et al., 2013).

The collective interrogation of nascent membrane proteins by folding sensors leads to one of two fates for each client protein (Brodsky, 2012; Merulla et al., 2013; Olzmann et al., 2013). Proteins deemed to be correctly and fully assembled by quality control are exported to their intended cellular compartment by way of the secretory pathway. Alternatively, proteins that fail to achieve a native fold in a timely fashion are retrotranslocated into the cytoplasm and polyubiquitinated, leading either to degradation by the proteasome or to formation of intracellular inclusions, as discussed below. The precise identity of the retrotranslocon (also referred to as the disolocon) has long been debated. It is quite possible that there is more than one protein or protein complex, including the Sec61 translocon itself, capable of serving as a retrotranslocon for various client proteins (Brodsky, 2012; Merulla et al., 2013; Olzmann et al., 2013). The genetic, cellular, and structural details of the ERAF and ERAD protein networks remains an active and fascinating area of research that can be expected to yield key insights into the molecular basis of a number of misfolding diseases. Closely aligned with ERAF and ERAD is the cellular “unfolded protein response” (UPR) system that activates to relieve the burden of misfolding and/or excess nascent protein on QC pathways and the ubiquitin-proteasome systems (Bence et al., 2001; Buchberger et al., 2010; Fortun et al., 2005).

Perhaps the most obvious outcome of the misfolding of α -helical membrane proteins is the loss of native protein function associated with a reduction in the efficiency of trafficking to the intended cellular compartment (Cheng et al., 1990; Naef & Suter, 1999; Sanders et al., 2001; Sung et al., 1991; Wiseman et al., 2007a). In some cases the pathogenesis arising as a result of misfolding seems to be due almost exclusively to the absence of functional protein in its native cellular compartment. For instance, the loss of CFTR channel function and related pathophysiology seems to be the most common outcome of the many genetic aberrations that cause CF. However, the toxic accumulation of excess misfolded membrane proteins may also represent a pathogenic stressor in some cases. Failure by the cell to fold or

dispose of immature protein results in accumulation of the misfolded protein as aggregates (Kopito, 2000), usually following retrotranslocation of the misfolded protein into the cytosol. Though chaperones and proteasome components often colocalize with protein inclusions (Garcia-Mata et al., 1999; Wigley et al., 1999), the aggregates themselves tend to be relatively homogenous in composition (Rajan et al., 2001). Nevertheless, intracellular aggregation of one protein can sometimes trigger the precipitation of other aggregation-prone proteins (Gidalevitz et al., 2006) as a result of competition between multiple proteins for shared components of the ERAD and ERAF pathways (Garcia-Mata et al., 2002; Gidalevitz et al., 2010). This principle has been elegantly demonstrated through a series of experiments involving genetically modified *Caenorhabditis elegans*, where the expression of temperature-sensitive variants of various soluble proteins was found to enhance the aggregation of fluorescent polyglutamine repeat proteins under ambient conditions (Figure 7) (Gidalevitz et al., 2006).

The mechanisms by which eukaryotic cells cope with the burden of accumulated misfolded membrane proteins are currently the subject of intense investigation. Such mechanisms principally include the sequestration of misfolded and extruded membrane proteins into specialized subcellular compartments (Sontag et al., 2014). Depending on the identity and ultimate fate of the misfolded protein, it is targeted to one or more subcellular quality control compartments which include Q-bodies (Escusa-Toret et al., 2013), the juxtanuclear quality control (JUNQ) (Kaganovich et al., 2008), the insoluble protein deposit (IPOD) (Kaganovich et al., 2008), aggresome-like induced structures (ALIS) (Szeto et al., 2006), or aggresomes (Johnston et al., 1998). While both soluble proteins and α -helical membrane proteins are capable of forming these structures under certain conditions (Garcia-Mata et al., 1999; Garcia-Mata et al., 2002), aggresome formation has been characterized in considerable detail for a number of misfolding-prone α -helical membrane proteins, in particular for CFTR (Johnston et al., 1998), PMP22 (Notterpek et al., 1999; Ryan et al., 2002), and rhodopsin (Saliba et al., 2002). Targeting of extruded and/or aggregated membrane proteins to aggresomes is achieved through an intricate network of cytosolic protein-protein interactions. First, poly-ubiquitinated aggregates are recognized by HDAC6 (Kawaguchi et al., 2003) and HSP70-bound proteins are detected by BAG3 (Gamerding et al., 2011). These sensors then facilitate dynein-mediated retrograde transport of small diffusive aggregates to the microtubule-organizing center (MTOC) (Garcia-Mata et al., 1999; Johnston et al., 2002; Kopito, 2000). A collapse of intermediate filaments around clusters of aggregates then leads to the formation of stable micron-scale aggresomes (Garcia-Mata et al., 2002). These aggresomes are then gradually degraded by proteasomes and/or in lysosomes via autophagy (Fortun et al., 2003; Johnston et al., 2002; Wigley et al., 1999). Importantly, it has been found that such sequestration and disposal mechanisms preserve the function of the secretory pathway (Garcia-Mata et al., 1999) and enhances cellular fitness (Escusa-Toret et al., 2013), suggesting aggresomes may serve in a protective capacity (Kawaguchi et al., 2003; Kopito, 2000; Sontag et al., 2014). Nevertheless, as these mechanisms appear to be saturable (Gidalevitz et al., 2006), an increase in the flux of nascent α -helical membrane proteins through these degradation pathways or failure to properly complete aggregate disposal may have catastrophic consequences for the cell. It should also be noted that some of the critical degradation pathways appear to decline in

efficacy with aging, a factor that may contribute to many aging-related disorders (Taylor & Dillin, 2011).

4.2 α -Helical membrane proteins at the proteostasis boundary

Efforts to understand the linkage between protein stability and cellular proteostasis represent a frontier bridging protein biophysics with systems biology. Simplistic models are beginning to reveal the mechanisms by which the physical chemistry of protein folding gives rise to the collective functions and dysfunctions associated with cellular proteomes. Recent studies have provided reasonable predictions of the folding energetics of soluble proteins directly from the amino acid sequence (Ghosh & Dill, 2009). Genomic surveys using this model predict that the cooperative unfolding of a few unstable proteins can give rise to a “proteostasis catastrophe” at elevated temperatures, which coincides with the thermal cell death temperatures of bacteria, yeast, and nematodes (Ghosh & Dill, 2010). Moreover, physical limitations imposed on cells by the collective instability of the proteome, the rates of biochemical reactions, and the diffusion rates of soluble proteins within the cell also seem to account for growth rates and cellular protein concentrations (Dill et al., 2011). These observations suggest a prominent role for protein stability in organismal fitness.

Bridging the knowledge gap between the energetics of protein (mis)folding and the functionality of cellular protein-protein interaction networks represents a considerable challenge. Nevertheless, a promising formalism has emerged from characterizations of transthyretin (TTR), a secreted soluble tetramer that causes systemic amyloid disease when mutated. Exhaustive folding measurements revealed an empirical relationship between the effects of pathogenic mutations on the kinetic and thermodynamic stability of TTR and both the efficiency with which the proteins are secreted by mammalian cells and the inherent amyloidogenicity of the variants (Sekijima et al., 2005). Furthermore, it was also found that secretion efficiency is highly dependent on the functionality of cell-specific chaperone machinery as well as the concentration of native TTR ligands, which act as a chemical chaperones in the secretory pathway. Wiseman et al. later constructed a minimal model describing the linkage between folding and export (FoldEx) by simplifying the relevant interactions in the ERAD and ERAF pathways using Michealis-Menten formalism and estimated rate constants for the relevant protein-protein interactions (Wiseman et al., 2007b) (Figure 8A). Strikingly, this model was able to account for the observed relationships between the conformational stability and observed secretion efficiency of both TTR and bovine pancreatic trypsin inhibitor (BPTI) (Kowalski et al., 1998a; Kowalski et al., 1998b), which suggests the principles of physical chemistry may offer utility in efforts to rationalize the elaborate cellular processes encompassed by cellular quality control. Among the concepts emerging from this model is that of a “proteostasis boundary” (Powers et al., 2009; Roth & Balch, 2011) (Figure 8B). This concept suggests the concentrations and activities of chemical and molecular chaperones within the cell define a “minimal export threshold” that corresponds to a minimal degree of conformational stability required for proteins to pass cellular quality control. According to this model, pathological misfolding may occur when a mutation increases the misfolding rate or decreases the folding rate and/or decreases thermodynamic stability to an extent that falls below the minimal export threshold. Such variants with non-permissive conformational energetics may be targeted to ERAD, which

may decrease the yield of functional protein, destabilize interaction partners, and potentially leads to the formation of cytotoxic aggregates. Together, these advances provide a general framework that may ultimately provide a means to quantitatively assess the linkage between the conformational equilibrium of a protein and its cellular fate.

The generality of these models suggests they may eventually provide clarity to the physical limitations governing the cellular trafficking and misfolding of α -helical membrane proteins. However, rationalizing the proteostasis of membrane proteins may prove more difficult than TTR and BPTI due to the fact that significantly less is known about the conformational energetics of proteins in cellular membranes or the physical nature of their interactions with the quality control machinery. Moreover, it may be necessary to factor in the physiochemical properties of the membrane to achieve a useful proteostatic model for membrane proteins. For instance, if curvature is indeed a significant factor that influences the conformational stability of membrane proteins (Brown, 2012), then the negative membrane curvature experienced by some proteins as they traffic through some organelles may represent a destabilizing influence on proteins that have evolved to maintain functional conformations in a destination membrane with positive curvature (Figure 9A–B). Additionally, given that the lipid composition of cellular organelles can vary dramatically (van Meer et al., 2008), the conformational energetics of α -helical membrane proteins and their capacity for export may be highly dependent on the compatibility of the native conformation with the lipid compositions of the membranes that comprise each cellular compartment (Figure 9C–D) (Lundbaek et al., 2003). For instance, the structural integrity of the β_2 adrenergic receptor depends on the cholesterol concentration (Zocher et al., 2012), yet the cholesterol concentration is known to be low in the ER where quality control takes place. The degree to which these factors impact the partitioning of nascent α -helical membrane proteins between the ERAF and ERAD pathways is a largely unexplored frontier. Additional biophysical studies of the effects of membranes, metabolites, and chaperones on misfolding-prone α -helical membrane proteins will be needed to formulate semi-quantitative models of the proteostasis boundary for α -helical membrane proteins.

4.3 Pharmacological rescue of membrane proteins from misfolding and mistrafficking

Current perspectives on misfolding and proteostasis suggest several avenues for the therapeutic rescue of destabilized mutant proteins (Ong & Kelly, 2011; Powers et al., 2009). The most obvious strategies involve increasing the conformational stability or suppressing misfolding and aggregation of the mutant protein. Indeed, it has long been appreciated that decreasing growth temperatures, which may increase conformational stability and suppress protein aggregation in the cell, can afford partial rescue of pathogenically misfolded membrane protein variants (Denning et al., 1992). Stabilization-based approaches have been validated by the rescue of the cellular trafficking of a number of misfolded α -helical membrane proteins in the presence of non-specific stabilizing osmolytes including trimethylamine N-oxide (TMAO), glycerol, and dimethyl sulfoxide (DMSO) (Brown et al., 1997; Robben et al., 2006; Sato et al., 1996; Tamarappoo & Verkman, 1998). However, the non-specific effects of these compounds on cellular proteostasis as well as the high concentration of osmolytes required to effectively stabilize proteins undermines their potential therapeutic utility. On the other hand, small molecules that bind with high affinity

and specificity to native conformations are also capable of rescuing misfolded proteins, typically at much lower concentrations (Yu et al., 2007). Thus, for loss of function mutations that cause misfolding, low concentrations of a ligand, an agonist, or even an antagonist can facilitate a partial rescue of activity (Fan et al., 1999; Ficker et al., 2002; Morello et al., 2000; Petaja-Repo et al., 2002; Sawkar et al., 2002). Intriguingly, small molecule mediated rescue of proteins targeted for degradation suggests that conformers recognized as non-native by cellular quality control must be capable of sampling near-native, binding-competent conformations early in the secretory pathway (Figure 10A). These near-native states likely compete with the non-native states that feed kinetically irreversible misfolding pathways (Figure 10A–C). Thus, the influence of these compounds can be attributed to their preferential binding to and/or stabilization of the native and near-native states relative to those recognized by quality control (Bolen & Rose, 2008; Brown et al., 1997; Perlmutter, 2002; Welch & Brown, 1996), which enhances both the population and the lifetime of native conformers and facilitates their escape from the ER.

Compounds capable of restoring the cellular trafficking and/or native function to pathogenic variants are often referred to as “pharmacological chaperones”. A number of pharmacological chaperones capable of preventing the misfolding of soluble proteins have progressed through clinical trials for the treatment of misfolding diseases including familial amyloid polyneuropathy (Berk et al., 2013), Gaucher disease (Yu et al., 2007), and Fabry disease (Fan et al., 1999). With regard to diseases of membrane protein misfolding, pharmacological rescue of pathogenic CFTR variants represents a long-standing goal. Several CFTR “correctors,” which mend defective CFTR trafficking, and “potentiators,” which increase the open-state probability of the mature channel, have been identified and entered into clinical trials (Eckford et al., 2014; Odolczyk et al., 2013; Rowe & Verkman, 2013; Sampson et al., 2011; Van Goor et al., 2009; Van Goor et al., 2011). However, identification of a compound capable of mediating sufficient and robust rescue of the common F508 CF variant remains an ongoing challenge (Rowe & Verkman, 2013). Efforts to develop novel pharmacological chaperones have also targeted a number of other pathogenic membrane protein variants believed to promote misfolding including the E90K variant of gonadotropin releasing hormone receptor (GhRHR), the cellular mistrafficking and the associated disease phenotype of which have recently been corrected in mice using both a native ligand and an agonist (Janovick et al., 2013). Numerous other misfolding-prone membrane proteins are known to be competent for rescue by pharmacological chaperones include some 18 different GPCRs including rhodopsin (retinitis pigmentosa), the vasopressin V2 receptor (diabetes insipidus) (Tao & Conn, 2014), nicotinic acetylcholine receptors (Srinivasan et al., 2014), K_{ATP} channels (Martin et al., 2013), and the HERG potassium channel (Gong et al., 2006). These advances highlight the growing interest in pharmacological chaperones as a therapeutic strategy for diseases of protein folding.

Proteostasis regulators (PR), which alter the general capacity of cellular ERAD and ERAF pathways, represent an emerging alternative in cases where the development of protein-specific pharmacological chaperones may be impractical or ineffective (Balch et al., 2008; Mu et al., 2008; Powers et al., 2009). This approach has recently shown great promise. Screening efforts have yielded a number of compounds that may offer general therapeutic utility for misfolding diseases (Calamini et al., 2012; Carlile et al., 2012; Mu et al., 2008;

Tardiff et al., 2013). Interestingly, the rescue afforded by PRs seems to arise through diverse biochemical mechanisms including the modulation of ER stress pathways (Ozcan et al., 2006; Ryno et al., 2013; Wiseman & Balch, 2005), the tuning of heat shock protein (HSP) expression and activity (Calamini et al., 2012), inhibition of the proteasome (Mu et al., 2008), and inhibition of ubiquitin ligases (Tardiff et al., 2013). Therefore, these compounds may represent a toolbox that can be used to address the potentially distinct cellular stressors that arise as a result of various misfolding mechanisms. However, due to the lack of specificity of such compounds, it is currently unclear what kinds of off-target effects they may elicit in the context of the human body. Nevertheless, considering the systemic nature of certain misfolding disorders, it may very well be that the therapeutic merits of these compounds outweigh potential side effects.

It has been observed that certain pathogenically misfolded variants of α -helical membrane proteins appear to be incompetent for ligand binding (Ficker et al., 2002; Kaushal & Khorana, 1994; Sung et al., 1991), which may indicate that the misfolded ensembles achieved by these variants are kinetically isolated from binding-competent, near-native states. In the context of α -helical membrane proteins, one possible cause of this class of misfolding are pathogenic variants that prompt cotranslational misfolding and reside in binding-incompetent topologies, which may not readily interconvert with native topologies (Figure 10D). In this case, pharmacological chaperones are unlikely to be of use and modulation of the proteostasis boundary may instead represent a more effective strategy to manage the consequences of misfolding. The effects of pathogenic mutations on the biochemical function of the rescued protein must also be factored into rational therapeutic design. A pointed example was provided by a recent study demonstrating that a mutation in a sodium channel that prompts ER-retention also causes aberrant constitutive activation upon correction of folding defects (Cestèle et al., 2013). One can imagine that, in such a case, a channel blocker-mediated pharmacological rescue might be more desirable than rescue mediated by an activating ligand. Such considerations provide the impetus for experimentally examining effects of a given pathogenic mutation on the biogenesis, folding, and function of a target α -helical membrane protein, suggesting important roles for biochemistry and structural biology in personalized medicine.

5. Conclusions and Outlook

Fundamental questions involving the principles of membrane protein folding and misfolding have long remained elusive. Nevertheless, significant advances in recent years are beginning to shed light on the relationships between the biophysics of α -helical membrane protein misfolding, the role of the translocon in their biosynthesis, the nature of their interactions with components of ER quality control, the biochemical nature of the resulting cellular pathology, and the molecular basis of misfolding disease. Delineation of the effects of pathogenic mutations on membrane proteins may offer a new degree of clarity for therapeutic design. Moreover, future efforts to unite structural biology with the tools and perspectives of cellular biology, biochemistry, and chemical biology offer the potential to comprehensively elucidate the effects of pathogenic mutations at the level of systems biology. These advances and perspectives offer hope for the development of novel therapeutics that may be of use in a multitude of diseases, potentially in a genotype-specific

(personalized) manner. These diseases will include not only inherited (Mendelian) disorders with simple mutation-disease relationships, but also “complex” disorders such as sporadic Alzheimer’s disease, various cardiovascular problems, and type 2 diabetes, where risk factors that promote membrane protein misfolding may be common contributors to disease etiology and pathology.

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Figure 1.

Folding and misfolding of α -helical membrane proteins. Membrane protein biosynthesis involves several coupled processes, which are vulnerable to the influence of pathogenic mutations. **A)** A cartoon depicts a typical biosynthetic pathway for an α -helical membrane protein. Biosynthesis begins with cotranslational integration of nascent α -helices (red) into the membrane (gray bar) by the translocon (gray donut), which is accompanied by early folding events (I). The nascent membrane protein (yellow) is released into the ER membrane following synthesis, where folding may continue with assistance of chaperones and folding enzymes (II). Once the protein achieves its native fold (green), it may form oligomeric complexes with potential interaction partners (blue) (III) prior to export from the ER. **B)** Pathogenic mutations (red circle) may cause misincorporation of TM helices by the translocon, which establishes an incorrect topology for the nascent protein. **C)** Pathogenic mutations may disfavor the formation of native tertiary or quaternary interactions. **D)**

Mutations may favor the formation of non-native contacts and/or aggregate formation. Here, we illustrate one of many possibilities, where the mutation both destabilizes the monomer structure and promotes formation of a non-native heterodimer.

Figure 2.

Structure of the translocon. **A)** A surface representation of the structure of the *Pyrococcus furiosus* SecYE β translocon in an open conformation (PDB code 3MP7) is shown. A yellow circle indicates the position of the protein conducting channel. The membrane is indicated in blue. **B)** A cross section of the structure reveals that the protein conducting pore is lined with both apolar and polar side chains. **C)** A top down view depicts the opening of the lateral gate, through which nascent transmembrane helices may access the ER membrane. (Figure from Öjemalm et al. 2011)

Figure 3.

Steric trap method originally developed by Heedeok Hong and James Bowie to assess the conformational equilibria of a membrane protein by measuring interactions between biotinylated membrane proteins and engineered monovalent streptavidins (mSA). Biotin labels are introduced at two proximal sites in the native conformation of the protein of interest (black circles). Binding of a single mSA to one of the biotins attached to the folded membrane protein occurs with high affinity. Binding of a second mSA to the other biotin can take place only after the membrane protein spontaneously unfolds. Unfolding and binding of this second mSA are therefore thermodynamically coupled. The position of the equilibrium between the single mSA-bound folded protein and the mSA-double bound unfolded form is assessed as a function of mSA concentration using any one of a variety of possible methods (unfolding-induced dissociation of a fluorescent excimer pair is implied in this example). Changes in the observed signal as a function of [mSA] can be fit for the equilibrium constant for unfolding (K_{unfold}) by accounting for the known binding affinity of mSA for avidin. This method relies on use of an engineered series of mSA with $K_{\text{d,avidin}}$ spanning several orders of magnitude. Choice of the optimal engineered mSA to use is based on the need to tune the balance between the binding and unfolding equilibria.

Figure 4.

Topology of human peripheral myelin protein 22 (PMP22) and distribution of pathogenic missense-encoded single amino acid mutations within its sequence. Residues for which pathogenic variants have been identified are indicated in red, and the identities of the pathogenic side chain substitutions are indicated. Mutations in PMP22 result in Charcot-Marie Tooth disease and related disorders.

Figure 5.

Folding and conformational dynamics of human PMP22. **A)** WT PMP22 was equilibrated in mixed micelles containing dodecylphosphocholine (DPC) in the absence (diamonds) and presence (circles) of 15% glycerol and varying levels of the denaturing detergent lauroyl sarcosine (LS). For each point tertiary structural content was monitored using the near-UV circular dichroism signal at 299 nm ($[\theta]_{299}$). Closed symbols are for a forward titration (unfolding) with LS. Open symbols are for a reverse (refolding) titration. The match between the forward and reverse titration data indicates the complete reversibility of the unfolding/refolding transitions, as confirmed by complementary kinetic studies (Schlebach et al., 2013). Kinetic and thermodynamic analyses suggest that the fraction of folded PMP22 in the absence of 15% glycerol (diamonds) is close to 0.5 in DPC micelles in the absence of LS ($G_{\text{unf}} \approx 0$ kcal/mol). PMP22 is stabilized in the presence of 15% glycerol, which shifts the unfolding transition to higher mole fractions of denaturing detergent under this condition (circles). However, a fit of the unfolding transition to a two-state equilibrium model (black line) suggests the folded conformation is still only marginally favored in the presence of glycerol ($G_{\text{unf}} = 1.5 \pm 0.1$ kcal/mol). (Figure from Schlebach et al. 2013) **B)** The structural

dynamics of WT and L16P PMP22 were assessed in tetradecylphosphocholine (TDPC) micelles using solution NMR and other methods. The results indicate that the initial state of PMP22 unfolding involves dissociation of its first transmembrane segment from the 4-helix bundle, with the remaining 3-helix bundle adopting a molten globule-like state in the membrane. The pathogenic L16P mutation breaks the first transmembrane segment and shifts the folding equilibrium towards the partially unfolded state. (Modified figure from Sakakura et al. 2011)

Figure 6.

ER quality control in eukaryotic cells. This diagram depicts the interplay between the ER associated folding (ERAF) and ER-associated degradation (ERAD) pathways within the pathway of membrane protein biosynthesis, folding, and trafficking. Nascent α -helical membrane proteins are cotranslationally inserted into the ER membrane as they come off the ribosome. The proteins then engage in quality control through numerous interactions with chaperones and folding sensors in the ERAF pathway. Nascent proteins that pass quality control are eventually exported to their destination membranes by way of the Golgi apparatus. Proteins that fail to achieve their native fold are eventually extruded from the ER membrane into the cytoplasm and polyubiquitinated. These proteins are ultimately disposed of by proteasomes or form aggregates that may eventually be degraded or might ultimately elicit cellular toxicity.

Figure 7.

A finite capacity of cellular quality control. The *trans*-effects of protein misfolding were examined using genetically modified *Caenorhabditis Elegans*. Fluorescence images are shown of larvae carrying one (Q40m het, **A**) or two (Q40m, **B**) copies of a fluorescently-labelled, aggregation-prone polyglutamine repeat protein at permissive growth temperatures. Images are also shown of Q40m larvae carrying temperature-sensitive mutations in the ras (**C**) and paramyosin (**D**) proteins grown at permissive temperatures. **E**) Quantification of visible polyglutamine aggregates in these organisms demonstrates that the expression of other unstable proteins (i.e. mutant ras or paramyosin) can prompt the aggregation of an otherwise soluble protein (Q40m), presumably due to competition between the two proteins for shared components of the ERAF pathway. (Figure from Gidalevitz et al. 2006)

Figure 8.

Physical chemistry of folding and export. **A**) A schematic illustration of a simplified model for folding and export (FoldEx) of nascent proteins from the ER. Secreted proteins are synthesized by the translocon (T), and the nascent protein (U) either productively forms the native conformation (F) (pathway 8), or is entered into a chaperone (C) binding cycle (pathway 3). Hydrolysis of ATP is coupled to conformational changes that release the nascent protein (pathways 5 and 6) in order to enable productive folding (pathway 8) or misfolding (pathway 13). Proteins that achieve the native fold are recognized by the export machinery (E) and exported from the ER (pathways 9 and 10). However, proteins that misfold or fail to fold quickly are either reengaged by chaperones (pathways 7 and 14) or are recognized by the retrotranslocon machinery (pathways 11 and 15) and targeted for degradation. Simplification of the relevant interactions using Michaelis-Menten formalism and estimation of relevant rate constants (summarized in box) recapitulates the observed relationships between the conformational stability and export of transthyretin (TTR) and bovine pancreatic trypsin inhibitor (BPTI). (Figure from Wiseman et al. 2007) **B**)

Simplification of the FoldEx model suggests cell-specific expression and activities associated with ERAD and ERAF components are capable of handling proteins (different proteins represented by green) with permissible combinations of thermodynamic stability, folding rates, and misfolding rates as indicated by the cellular proteostasis boundary (purple). Proteins that fall within the proteostasis boundary are produced and degraded normally. However, destabilized variants (red) may breach this boundary (left), which can lead to the saturation of quality control and pathogenic misfolding. Because folding and export hinge on protein-protein interactions (connecting lines), destabilized variants may either directly destabilize interaction partners or indirectly destabilize competing quality control substrates (right). The destabilized variant and its interaction partners may then saturate quality control, prompt pathogenic misfolding, and induce cellular stress (Figure from Powers et al. 2009)

Figure 9. Potential influence of membrane curvature and lipid composition on the conformational equilibrium of α -helical membrane proteins. A given membrane protein may experience a variety of different membrane curvatures and lipid compositions as it traffics through the membranes of various organelles and transport vesicles. Cartoons depict the potential influences of membrane curvature and cholesterol content on protein stability. **A)** For this hypothetical membrane protein the native conformation (green) is favored over unfolded conformations (yellow) in membranes with positive curvature. (Other membrane proteins may have the opposite preference.) **B)** For this same hypothetical protein, negative membrane curvature destabilizes the native conformation relative to unfolded conformations. **C)** For this hypothetical protein the native conformation is favored in membranes containing high concentrations of cholesterol and is less stable in membranes

than contain lower cholesterol (**D**). (Other membrane proteins may exhibit an opposite trend).

Figure 10.

Influence of pharmacological chaperones on the conformational equilibrium of multi-pass α -helical membrane proteins. **A)** Scheme depicting the influence of pharmacological chaperones on the folding of nascent multi-pass α -helical membrane proteins. Nascent proteins are initially inserted into the ER membrane, where they assume a partially folded intermediate ensemble. Funneling of these intermediate ensembles through the biological folding pathway results in the formation of the correctly folded protein, which is competent for export from the ER. However, failure to do so in an efficient or timely manner may lead to a loss of the nascent protein through the ERAD pathway. The kinetics and thermodynamic of the folding transition are sensitive to the influence of pharmacological chaperones, which selectively stabilize the native conformation and/or the rate limiting transition state for folding. **B)** The partitioning of the nascent protein between folding and ERAD is illustrated with an energy diagram. Unfolded conformations of nascent wild-type membrane protein (yellow) kinetically partition between the folding pathway and the ERAD pathway. Nascent proteins must overcome a rate-limiting energy barrier for folding ($\ddagger$) to achieve the native conformation (green) prior to recognition by components of the ERAD pathway. **C)** The incorporation of a pathogenic mutation within a TM helix (red circle) destabilizes the native conformation and/or the rate-limiting transition state for folding, which decreases the rate and efficiency of folding of the mutant protein. The effects of the mutation (red) may be partially offset in the presence of a pharmacological chaperone (blue) that preferentially binds and stabilizes the native conformation and/or the rate-limiting

transition state for folding. **D)** Pathogenic variants with incorrect topologies may be kinetically isolated from the native folding pathway. Reorientation of the TM helices with respect to the membrane may be outpaced by the association of the nascent protein with ERAD components. Thus, the influence of pharmacological chaperones on the conformational energy landscapes may be insufficient to rescue nascent membrane proteins with aberrant topologies.