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# Association energetics of membrane spanning α-helices

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Since Popot and Engelman proposed the 'two-stage' thermodynamic framework for dissecting the energetics of helical membrane protein folding, scientists have endeavored to measure the free energies of helix–helix associations to better understand how interactions between helices stabilize and specify native membrane protein folds. Chief among the biophysical tools used to probe these energies are sedimentation equilibrium analytical ultracentrifugation, fluorescence resonance energy transfer, and thiol disulfide interchange experiments. Direct and indirect comparisons of thermodynamic results suggest that differences in helix–helix stabilities between micelles and bilayers may not be as large as previously anticipated. Genetic approaches continue to become more quantitative, and the propensities for helices to interact in bacterial membranes generally correlate well with *in vitro* measurements.

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## Introduction

Helical membrane proteins represent nearly a quarter of open reading frames in genomes [1], yet little is known about the dynamical nature of their folding and stability. In 1990 the Popot and Engelman 'two-stage' model proposed a simplification of the membrane protein-folding problem for helical membrane proteins [2]. It suggested that membrane protein stability could be divided into two independent thermodynamic events: (i) initial establishment of a helix in a membrane environment and (ii) subsequent side-to-side interactions of helices within the membrane. While the crystal structures of some membrane proteins show that this thermodynamic model may be somewhat oversimplified, the parallel alignment of a remarkable number of membrane protein helices provides strong support for this thermodynamic approach to understanding the physical origins of membrane protein folds. Interactions between independently stable helices, this second step of membrane protein assembly, have been intensively investigated using biophysical tools in recent years. We highlight the most useful methods as well the insights they have revealed for this challenging biological problem.

## Biophysical methods used to measure transmembrane helix–helix interactions

Three principal methods for measuring the energetics of transmembrane helix–helix interactions have taken prominent positions in the field: sedimentation equilibrium analytical ultracentrifugation (SE), fluorescence resonance energy transfer (FRET), and thiol disulfide interchange (TDI). SE provides a direct measure of mass, unlike any of the other methods. The 'density matching' SE protocol pioneered by Reynolds and Tanford [3,4] eliminates the mass contributions of bound lipids or detergents and therefore enables the molecular weight of solely the protein to be measured. Reversibility of membrane protein self-associations in detergent micelle environments can be experimentally verified, which means that both the stoichiometry and equilibrium constants can be rigorously determined [5••]. The main disadvantage of SE experiments in measuring transmembrane domain (TMD) association free energies is that SE is limited to membrane proteins solubilized in detergent micelles, which means that micelles must be chosen that support native protein properties. SE is therefore not useful for experiments in lipid vesicles, since the measured mass in vesicles would be the sum of the masses of all membrane proteins residing within any one vesicle, whether or not there existed preferential interactions between them.

Some of the limitations of SE can be addressed using FRET [6–9,10•] or TDI [11••]. Both these methods rely on proximity as a measure of interactions between TM helices, and both can be applied in either detergents or bilayers. FRET reports on dipolar overlap between donor and acceptor fluorophores on TM domains, while TDI relies on the formation of disulfide bonds between cysteines on different subunits at redox potentials set by varying the ratio of reduced to oxidized glutathione. FRET returns molar ratios for self-association reactions, which means that the reaction stoichiometry must be the prior information assumed in the data analysis. In cases where suspected interactions cannot be observed and the molecules of interest are monomeric, FRET will result in a lack of signal, a negative result, whereas SE experiments will return the monomer molecular weight.

TDI returns mole fractions of disulfide crosslinked dimers; for oligomeric orders other than dimer the fraction oligomer must be inferred from fraction dimer. Both FRET and TDI require the introduction of a label: if a natural cysteine is unavailable TDI requires the incorporation of one; FRET experiments generally involve the introduction of both donor and acceptor fluorophores on two different samples. Since fluorophores are typically large hydrophobic groups, there are a number of controls to quantify labeling efficiency and ensure that the labels do not influence the reaction in undesired ways [10•,12]. Despite these many caveats, all three methods work remarkably well and are complementary, orthogonal tools for addressing the energetics of helix–helix stabilities.

There are two molecular issues related to energetic measurements. The first is 'adventitious' interactions between TM helices, which are a concern for both FRET and SE experiments. This phenomenon is especially problematic for studying the interactions between weakly interacting TMDs (such as growth factor receptors TMDs). To populate associated states, relatively high protein concentrations are required. In SE experiments, micelles become limiting when the protein:micelle mole ratio approaches unity; under these conditions there is a nonzero probability that two proteins will occupy the same micelle even if there are no preferential interactions between them [13]. FRET or TDI measurements made in micelles suffer from this same problem. For FRET measurements in bilayers, long Forster distances for donor/acceptor pairs can allow energy transfer to occur even when the TM helices are not 'binding' to each other. There are analytical solutions to correct for both types of these adventitious interactions [13,14].

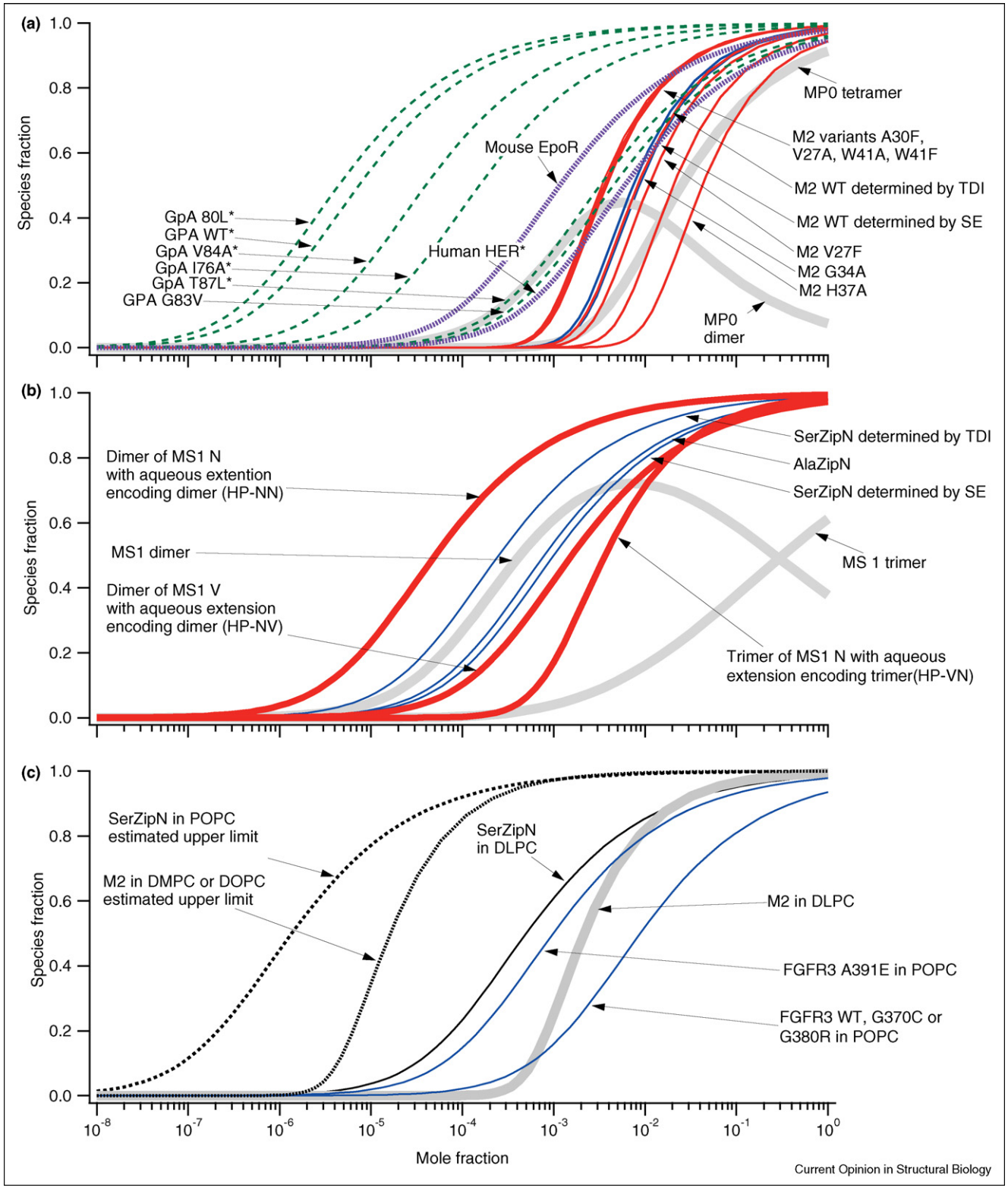
A second issue with stability measurements of TMDs is the choice of units in which to express the thermodynamic parameters of interest. Since integral membrane proteins are not soluble in water, they do not partition out of lipid vesicles (or micelles) into the aqueous phase, and lipid vesicles do not mix their contents with other vesicles on the time scale of typical experiments. Therefore, bulk aqueous concentrations of protein and lipids are not appropriate: the relevant concentration scale for thermodynamic parameters is the one that relates the amount of protein to the amount of its available solvent. A mole fraction scale has been generally adopted, although it has been justifiably argued that a Molar scale in which the volume was that of the hydrophobic phase would also be appropriate [15]. The size of the hydrophobic phase in vesicle experiments is set by the vesicle dimensions and would not normally be adjustable during an experiment. By contrast, the size of the hydrophobic volume can be more easily modulated in detergent micelle experiments since increasing the bulk detergent concentration increases the number of micelles. Over 20 years ago, Tanford postulated that micellar solutions could be thermodynamically considered as one single micellar phase as long as there is dynamic exchange of micelle contents [16]; Fleming has shown how to normalize the apparent equilibrium constant for the size of this phase [5••]. The ability to vary the size of the hydrophobic phase means that the experimentalist can deliberately tune the oligomeric population for a particular membrane protein by changing either the protein or the micelle concentration.

## How stable are interactions between transmembrane helices?

Thermodynamic free energy values provide a numerical index of population distributions that leads to enormous insight into how biological activities are encoded and regulated by protein self-association. Figure 1a shows the oligomeric distributions for representative helix–helix interactions from the literature, including the WT glycophorin A (GpA) TMD dimer, and sequence variants [17,18•,19,20], the M2 TMD tetramer and sequence variants [21•,22•,23,24], the HER [25] and erythropoietin receptor TMD dimers [26], and the myelin protein zero dimer (MP0) and tetramer species [27] all as a function of mole fraction protein. Population distribution plots facilitate comparisons of stabilities between proteins of different stoichiometries and show that the GpA WT dimer interaction is quite stable relative to other helical membrane proteins studied to date in micelles. Figure 1c shows that POPC lipid bilayers strongly stabilize both the M2 TM tetramer [11••] and designed 'serine zipper' peptide dimers [28].

Thermodynamic measurements can be used to determine the energetic consequences of mutations. The dimer stabilities of over 50 sequence variants of GpA have been determined [17,18•,19,20]; a representative few are shown in Figure 1a. Despite the limited diversity of amino acids available to a transmembrane segment, the GpA sequence shows remarkable plasticity in its ability to tune its energetics, which can vary almost continuously over a range of nearly 4 kcal $mol^{-1}$ [17,18•]. Using structural modeling, a correlation between GpA TMD dimer structural features and energetics suggests that van der Waals interactions explain most of the thermodynamic consequences for the sequence variants; remarkably, a common set of structure-based parameters explains the free energy consequences of amino acid substitutions in helix B of bacteriorhodopsin determined by protein unfolding experiments [29]. By contrast, sequence changes in the M2 TMD tetramer from influenza A virus modulate its tetramer stability over a much narrower concentration range [22•]. Unlike GpA, in which all sequence substitutions (except one) destabilized its dimer, several substitutions in the M2 TMD stabilize its tetramer in micelles [22•] (Figure 1a).

**Figure 1**




Oligomeric species distributions. The labels indicate the identity of each protein. Most proteins form only dimers, and this is the implied species unless otherwise marked, with the exception of M2, for which the implied species is tetramer. All data in this panel were collected at 25 °C, which means that each 10-fold decrease in mole fraction protein is equivalent to a free energy change of −1.36 kcal mol$^{-1}$. Panel **(a)** shows the species distributions for naturally occurring TMD sequences and some engineered sequence variants in micelles. Panel **(b)** shows species distributions for designed peptides

## GxxxG motifs, polar groups, and designer TMD interactions in micelles

Analyses of membrane protein structures and of predicted TM helical segments from sequenced genes have identified a plethora of motifs that may be important for stabilizing helix–helix interactions in membranes, and thermodynamic measurements are uniquely suited to test these ideas. A well-known motif, GxxxG, is overrepresented in open reading frames of TMD regions, and it was hypothesized that it may represent a dimerization code [30]. Energetic measurements of the role of GxxxG motifs in natural TMDs have returned a mixed verdict. Mutations to large aliphatic groups at either site in the GxxxG motif in GpA are strongly destabilizing, indicating the importance of these glycines for the most stable GpA TMD dimer, but similar changes at other interfacial sites, particularly at position T87, cost about as much association free energy. By contrast, the human HER TMDs, which also contain GxxxG motifs, showed only a marginal propensity for dimerization in two independent studies [25,31], and the GxxxG containing CCK-4 TMD shows no preferential free energy of association at all [13]. Even the myelin protein zero TMD, which contains a GxxxGxxxG 'glycine zipper' motif [32], shows a dimerization interaction energy only slightly better than the GpA G84V sequence variant [27]. The wide range of interaction energies exhibited by GxxxG-containing TMDs is illustrated by the population distributions in Figure 1, where an asterisk identifies proteins containing the motif. Note that this range is consistent with the observation that sequence context of the GpA TMD GxxxG motif can strongly modulate dimerization free energy [17,18•].

Roles for polar residues in driving helix–helix interactions have also been vetted using thermodynamic measurements. In the low dielectric environment of a membrane, polar interactions and hydrogen bonding may be expected to have significant energetic contributions. Ser is the most frequently occurring polar amino acid in TM helix sequences [30], and it occurs at TMD interaction surfaces [33,34], yet North *et al.* found that 'serine zippers' in designed TM peptides promote dimerization no better than the equivalent sequences containing alanine substitutions [28] (Figure 1b), suggesting that van der Waals interactions were more probably the mechanism of stability encoded by these small side chains. On the contrary, Asn and other side chains that can make two interhelical hydrogen bonds drive helix assembly in a designed membrane peptide, MS1 [7,35]. While the dimer populations of both MS1 and serine zippers populate at about the same mole fraction protein (Figure 1b), showing they are energetically equivalent mechanisms, replacing Asn with Val in MS1 collapses both its dimer and trimer species to monomers [7,36].

The interplay between soluble and membrane regions was further probed using MS1 [37•], which was extended to include an aqueous segment from the parent GCN4-P1 sequence to form a hybrid peptide (HP). While the aqueous region was short enough to have no interaction energy when studied in isolation, it could direct the resulting stoichiometry of the HP interaction: when the aqueous region contained signature sequences for dimers (HP-Nx), the resulting hybrid peptide was dimeric. By contrast, dimer formation was suppressed and only trimeric interactions were observed when the aqueous region encoded trimeric interactions (HP-Vx). The membrane-embedded Asn in the TMD of MS1 simply stabilized the overall structure: when substituted with Val, the resulting oligomeric states were destabilized, but their stoichiometries were still set by the aqueous region. Despite the different stoichiometries, the energetic effects of the membrane regions were not small, as can be observed by the population distributions for these peptides in Figure 1b.

## How do stability measurements in micelles translate to those in lipid bilayers or cell membranes?

Helix–helix interactions should be enhanced in lipid bilayers as compared with detergent micelles simply because of the reduced dimensionality of the bilayer as compared with a micellar phase. Grasberger *et al.* estimated that this effect, combined with lateral localization and crowding, could result in a million-fold increase in the association constant for a monomer–dimer reaction and considerably more for higher order assemblies [38]. Experiments addressing this question, however, suggest that pre-orientation effects in a bilayer may not be the main enhancement mechanism. North *et al.* found that the association propensities of serine zippers in dilauroylphosphatidylcholine (DLPC) vesicles were comparable to those in DPC micelles [28] (compare Figure 1b and c), whereas the same serine zipper dimers did not dissociate at mole fractions as low as 1:1000 palmitoyloleoyl phosphatidylcholine (POPC) lipids (upper limit

in micelles. Panel **(c)** shows species distributions of both designed and naturally occurring sequences that were measured in lipid bilayers. The three panels are vertically aligned with the same abscissa scale to enable direct comparisons. Names with an asterisk indicate sequences containing a GxxxG motif. The species fraction for each protein was calculated using the binding partition function [64] from the reported mole fraction $\Delta G_x$ or $K_x$ values (where $\Delta G_x = -RT \ln K_x$) as referenced in the main text with the following exceptions: (1) $\Delta G_{x,12}$ for HER TMD was reported as no greater than 1 kcal mol$^{-1}$ [25], and a value of 0.5 kcal mol$^{-1}$ was used in this graph; (2) $\Delta G_{x,12} = -3.38$ kcal mol$^{-1}$ and $\Delta G_{x,14} = -9.79$ were calculated for MP0 using the standard state equation [5••] and the reported equilibrium constants and detergent concentration [27]; (3) The serine zipper POPC dimer distribution in panel (c) was calculated using $\Delta G_{x,12} = -8$ kcal mol$^{-1}$ that represents an upper distribution limit based on the reported observation that only dimers were observed at mole fractions as low as 1:1000 and assuming 3% dimer at MF = .001 is not detectable [28]. For clarity, fraction monomer is not shown, but equals 1 − (sum of all species fractions) for each protein.

estimated as the dotted curve in Figure 1c). A similar theme resulted from studies of the M2 TMD tetramer: while DLPC vesicles stabilized the tetramer as compared with DPC micelles by a modest 2.25 kcal $mol^{-1}$, only tetramers were observed in longer chain dimyristoylphosphatidylcholine (DMPC) or POPC vesicles at experimentally accessible concentrations [11••].

The association propensities of several TMDs derived from growth factor receptors have been studied in both detergent micelles and lipids. Direct comparisons of stabilities between the two environments are not possible because different protein constructs were used in these experiments. Nevertheless, studies in both lipids and micelles suggest that relatively weak interactions are characteristic for these TM segments. The human HER TMDs have a slight propensity for dimerization in micelles [25], whereas the both the mouse and human EpoR TMDs are more stable than HER TMD dimers by as much 1 kcal $mol^{-1}$ [26], consistent with a model for pre-dimerization of the EpoR before ligand binding. Peptides corresponding to the TMD of fibroblast growth factor receptor 3 (FGFR3) were also shown to dimerize relatively weakly in POPC vesicles using FRET [39]. In the same study the pathogenic mutation A391E was found to stabilize FGFR3 dimers, whereas neither the thanatophoric dysplasia associated G370C [40] nor the achondroplasia mutation G380R [41] dimerize any stronger than WT in POPC vesicles. By comparison, the TMD interactions of FGFR3 are less stable than those of both the M2 TM tetramer and serine zipper peptides in DLPC or POPC vesicles (Figure 1c) and are consistent with the weak interactions of growth factor TMDs studied in micelles.

From both direct and indirect comparisons, it may therefore be speculated that the dimensionality of the lipid bilayer plays a modest energetic role in helix–helix interactions, which suggests that TMD helices may have more conformational freedom in a bilayer than previously appreciated. The direct comparisons between the FGFR3, M2, and serine zipper peptides in POPC vesicles suggest that conformational freedom may also depend on the TMD sequence. As methodologies continue to improve it will be of interest to determine the energetic consequences of bilayer thickness for a larger number of helix–helix interactions as well as how membrane protein sequestration by lateral lipid domains may influence the reaction.

## How do *in vitro* and *in vivo* stabilities of helix–helix interactions compare?

Biological assays of helix–helix interactions offer a complementary approach for addressing TMD interactions *in vivo* and have contributed significantly to our understanding of how TMDs associate in bilayers (reviewed in reference [42]). In these assays, reporter gene expression in *E. coli* is controlled by TMD-mediated interactions between fusion proteins. The ToxR [43] and related TOXCAT [44] assays give increased reporter gene activity with increasing self-association of a fusion protein, whereas the GALLEX assay gives increased repression of reporter gene activity with increased association of two heterologous fusion proteins [45]. Two groups have recently reported using dominant negative effects in either the ToxR [46] or the TOXCAT [47] assays to detect heteromeric interactions between TMDs, and the ToxR assay has been extended to allow positive or negative selection schemes using a suite of promoters and reporter genes [48].

The strong dependence of assay signals on the identities of the fusion protein TMDs permits rank ordering of TMD interaction strengths, with the strongly self-associating TMD of glycophorin A usually serving as a positive control. For GpA and sequence variants, results from ToxR, TOXCAT, and SE show similar rank orders [20,43,49••]. An Asn side chain drives tight association of TMDs in TOXCAT [50,51] in agreement with thermodynamic data in micelles [35]. The HER TMDs show moderately strong interactions in TOXCAT (especially HER4) [52]. However, FRET and SE data in detergent micelles indicate that self-association of the HER TMDs is quite weak [25,31], although the SE study did find that the HER4 TMD dimerized more strongly than the TMDs of HER1, 2, or 3 [25], consistent with the TOXCAT rank order. Roles for GxxxG motifs, single strongly polar residues, sets of small polar residues, and leucine zippers in driving association of TMDs have been identified or confirmed using biological assays (reviewed in reference [42]). More recently, selection from a randomized library and mutations of selected and designed sequences using the POSSYCAAT variant of the ToxR assay led to the conclusions that tryptophan can support helix–helix interactions in membranes [53] and that interaction of TMDs bearing a GxxxG motif can be enhanced by an appropriately positioned phenylalanine [54]. Roles for aromatic or cation–pi interactions in driving TMD association have been inferred using the TOXCAT assay [55].

*In vivo* assays are now routinely used to determine if the TMDs of biologically important molecules interact in isolation (reviewed in reference [42]). Weakly self-associating TMDs were recently identified in this manner in the anthrax toxin receptor, whose TMD contains no previously identified interaction motif and probably forms higher order oligomers [56] and in the receptor tyrosine kinase RET, which contains an SxxxS motif [57]. The TMD of the class II MHC receptor Ii protein self-associates strongly using a polar glutamine [58], and the TMD of neuropilin-1 (the semaphorin receptor ligand-binding subunit) self-associates strongly by virtue of a GxxxGxxxG 'glycine zipper' motif [59]. The four human syndecan TMDs exhibit a wide range of self-association

tendencies despite strong sequence similarity and a conserved GxxxG motif, again affirming the importance of sequence context for this dimerization motif [60]. Determining the biological significance of interactions observed in these assays is not straightforward: although the SARS coronavirus spike protein TMD interacts in TOXCAT and a GxxxG motif is implicated in this interaction [61], mutations to the GxxxG motif do not affect spike protein trimerization *in vivo* or spike protein mediated cell–cell fusion, and only one G to L substitution significantly affects spike protein mediated cell entry [62].

Two recent studies have attempted to develop quantitative formalisms for interpreting TMD interaction data from biological assays; such efforts could identify differences between *in vitro* and *in vivo* measurements. Finger *et al.* report apparent *in vivo* free energies of association in the GALLEX assay for wild type (and five mutant) GpA fusion proteins obtained by determining the expression level for each construct at which the reporter gene is half repressed and using this concentration to calculate apparent $K_d$ and $\Delta G_{app}$ values [63•]. Duong *et al.* report changes in apparent association free energy due to mutations in the glycophorin A TMD ($\Delta\Delta G_{mut}$) without direct measures of $\Delta G_{app}$ [49••]; in this approach, TOXCAT reporter gene activity for each mutant glycophorin A fusion protein is scaled to wild type, corrected for fusion protein expression levels (again relative to wild type), and $\Delta\Delta G_{app}$ is calculated from an expression that assumes a dilute limit [49••]. The two methods each achieve some level of agreement with physical data for the GpA TMD and mutants in detergents, suggesting that biological assays may be able to provide quantitative measures of helix–helix interaction energies *in vivo*.

The $\Delta G_{app}$ values measured by Finger *et al.* for wild type and mutant GpA TMDs provide $\Delta\Delta G_{mut}$ values [63•] that can be compared with the measurements from SE [18•,20]. The rank order of the effects is largely the same, but mutations have a more modest impact on the free energy of association in membranes than they do in detergents. The apparent $\Delta\Delta G_{mut}$ values measured by Duong *et al.* using TOXCAT [49••] also give largely the same rank order as the SE (and the GALLEX) data, but the TOXCAT data show roughly the same extent of disruption as the SE data: a regression analysis of the biological against the physical $\Delta\Delta G_{mut}$ data gives a slope of essentially 1.

The number of caveats associated with either of these calculations makes it remarkable that any agreement at all can be achieved with experimental data from purified proteins in detergents. Both methods rely on only a handful of data points and are thus very sensitive to errors, and both require assumptions about the order of oligomerization. The behavior of a culture of *E. coli* from one day to the next is not highly reproducible, and although testing fusion protein expression levels in every sample helps control for such variations [49••], it must be noted that stochastic differences between cells within populations can affect the biological measurements, which treat whole extracts and not individual cells. Small amounts of misfolded or aggregated fusion protein could also make varying different contributions to reporter gene expression, and if these amounts were below the detection limits of the controls they could not be accounted for quantitatively. Indeed, this last point must be considered crucial to both qualitative and quantitative analyses of biological TMD interaction data. The wide range of apparent $\Delta\Delta G_{mut}$ values reported by Duong *et al.* for GpA derives from over a 100-fold difference in reporter gene activity [49••]; most other studies of TMD interactions using ToxR, GALLEX, or TOXCAT rarely show even a 10-fold change in signal caused by single point mutations. For some TMDs the extent of association may be so high (e.g. the systems are at the right of the population distribution curves in Figure 1) that mutations lower the fraction of oligomeric fusion protein by only a small amount. However, given the wide range of effects that can be achieved in SE by point mutations in the strongly associating GpA TMD [17,18•,20], it seems prudent to exercise caution when making even qualitative interpretations of signals from biological TMD interaction assays that cannot be significantly lowered by point mutations.

## Conclusions

Biological assays and biophysical free energy measurements of TMD interactions provide experimental tests of bioinformatics-derived motifs and quantitatively probe the physical basis of helix–helix interactions. Despite the many differences between detergent micelle and *in vivo* hydrophobic environments, the tendencies for transmembrane domains to self-associate agree remarkably well. Comparisons of measurements in multiple hydrophobic environments suggest that the dimensionality of the bilayer may play a more modest role in promoting helix–helix interactions than previously anticipated.

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