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De novo design of transmembrane helix–helix interactions and measurement of stability in a biological membrane

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ABSTRACT

Membrane proteins regulate a large number of cellular functions, and have great potential as tools for manipulation of biological systems. Developing these tools requires a robust and quantitative understanding of membrane protein folding and interactions within the bilayer. With this in mind, we have designed a series of proteins to probe the net thermodynamic contribution of well-known sequence motifs to transmembrane helix-helix association in a biological membrane. The proteins were designed from first principles (*de novo*) using current knowledge about membrane insertion and stabilizing interaction motifs. A simple poly-Leu "scaffold" was decorated with individual helix interaction motifs (G-*XXX*-G, polar residues, heptad repeat) to create transmembrane helix–helix interactions of increasing strength. The GALLEX assay, an *in vivo* assay for measurement of transmembrane helix self-association, was combined with computational methods to characterize the relative strength and mode of interaction for each sequence. In addition, the apparent free energy contribution ($\Delta\Delta G^{app}$) of each motif to transmembrane helix self-association was measured in a biological membrane, results that are the first of their kind for these *de novo* designed sequences, and suggest that the free energy barrier to overcoming weak association is quite small (<1.4 kcal mol^{-1}) in a natural membrane. By quantifying and rationalizing the contribution of key motifs to transmembrane helix association, our work offers a route to direct the design of novel sequences for use in biotechnology or synthetic biology (e.g. molecular switches) and to predict the effects of sequence modification in known transmembrane domains (for control of cellular processes).

Membrane proteins control the flow of critical information across membranes, and thus regulate a large number of cellular functions. Understanding the mechanisms that govern the folding and interactions of membrane proteins, including their ability to interact with lipids, is the key to unlocking their huge potential as tools for manipulation of biological systems. Many investigators have used a rational protein design approach to reveal these mechanisms while making non-natural membrane proteins with unique functions. For example, Lear and coworkers designed a model ion channel, which was selectively permeable to proton ions, using model amphipathic peptides containing only Leu and Ser residues [1]. Similar approaches have been applied to design of electron-transfer systems that bind novel cofactors [2] and cell-penetrating peptides that target tumour cells [3].

Artificial protein systems are typically developed in one of two ways, either by *protein engineering* or by *de novo protein design* [4]. The majority of membrane protein design studies in the literature fall under the heading of protein engineering, where investigators made small changes (mutations) to a native protein sequence in order to improve or alter the properties of the protein (e.g. lipophilicity, oligomeric state [5], fusogenicity [6]). These studies can reveal regions of structural importance in a protein sequence and may impart new properties to the protein, but results are often difficult to interpret in the context of the native sequence (which has been shown to exert significant influence on interactions [7]).

De novo protein design involves the creation of entirely new, non-natural protein sequences. These new sequences can be generated in large numbers using a combinatorial approach, such as a genetic library of thousands of mutants. This approach has been used in the past to select transmembrane (TM) domain sequences that strongly self-associate in a bacterial membrane [8], and has revealed the enrichment

Abbreviations: TM, transmembrane; MHC, major histocompatibility complex; G-XXX-G, Gly-XXX-Gly, where X is any residue; ΔG, Gibbs free energy; MBP, maltose-binding protein; β-gal, β-galactosidase; GpA, glycophorin A; CHI, CNS searching of helix interactions; IPTG, isopropyl β-D-1-thiogalactopyranoside

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of specific sequence motifs and residues, such as Trp [9], Phe [10], Ser and Thr [11], in interacting sequences. Likewise, computational approaches have been used to generate large numbers of new membrane protein sequences and identify those with desirable properties [12,13].

New protein sequences have also been created from first principles, using current understanding of hydrophobicity, membrane insertion, and helix–helix interactions to create model systems that illustrate a sequence/structure/function relationship. These protein sequences are often composed of a very simple "scaffold" or background sequence (poly-Leu [14–19], poly-Ala [20], poly-Met and poly-Val [21] have been used in the past, as have combinations of Leu/Ala [14,15,22–25], Leu/Val [26], Leu/Ser [1] or Ala/Ile [27]) upon which a sequence motif of interest is grafted. Using this approach, investigators have studied the role of amino acid sequence in TM helix interactions [14–21,27], membrane insertion [25], topology [24], membrane fusion [26], membrane anchoring and hydrophobic mismatch [22,23]. Less abundant in the literature are quantitative thermodynamic parameters that describe the interactions of these non-native systems. While some values have been published for model systems using analytical ultracentrifugation [13,28] and Forster resonance energy transfer [25] methods, there is a much richer literature for naturally-occurring TM domains including those in glycophorin A [7,29–33], fibroblast growth factor receptor 3 [34], the rat Neu oncoprotein [35], the bovine papillomavirus E5 protein [36] and class II MHC invariant chain [37]. These values help us to understand the driving forces behind biochemical processes, act as the foundation for new mechanistic models, and have been fundamental in our understanding of protein folding thus far. Therefore, in the case of model proteins, understanding the net thermodynamic contribution of small changes in sequence to overall interaction strength could direct the design of sequences for use in biotechnology or synthetic biology.

With this in mind, we have designed a series of TM domain proteins that allow us to estimate the net thermodynamic contribution of well-known sequence motifs to helix-helix association. TM domain sequences were designed *de novo* (from first principles) using current knowledge about membrane insertion and interaction motifs, and were not derived from a genetic library or a native protein sequence. Because of its propensity to spontaneously insert into bilayers and form a highly α-helical structure [26], the poly-Leu backbone was used as a simple scaffold. Poly-Leu has been reported to exist as a monomer [16,18,19] as well as an oligomer [14]. We then attempted to create TM helical interactions of increasing strength by "decorating" the surface of the low-complexity poly-Leu TM sequence with well-known helix interaction motifs. In a 2006 survey of 445 helical pairs obtained from high-resolution membrane protein structures, investigators found that 75% of helix interactions could be described by simple principles of helix-helix packing and highlighted the importance of two motifs in particular [38], namely the G-*XXX*-G motif and the heptad repeat motif. Extensive manipulation of the heptad repeat motif has been used in the *de novo* design of soluble coiled-coil structures [4,39–41], and similar packing has been observed in membrane proteins [42]. Therefore, both the G-*XXX*-G and heptad repeat motifs were used in this investigation. The contribution of polar residues was also studied, as these have been shown to contribute significantly to oligomerization of TM domains [15,19,43] and are an evolutionarily conserved feature of many native TM proteins [44]. Using an *in vivo* reporter assay called GALLEX, helix interaction strength was measured here both qualitatively and quantitatively for a series of TM domains of increasing sequence complexity. The individual contributions of a range of well-known helix interaction motifs to the overall Gibbs free energy (ΔG) of association were measured in a natural membrane bilayer, the inner membrane of *Escherichia coli,* allowing us to directly compare the efficiency of these motifs for stimulating helix interactions. We supplement this biochemical study with a computational search for favorable dimer conformations over a selected number of *de novo* designs. The results were used to design a bi-functional TM domain that could potentially interact via two competing mechanisms.

1. Materials and methods

1.1. GALLEX assay

The self-association of the rationally-designed TM proteins in the *Escherichia coli* inner membrane was studied using the GALLEX assay, the details of which have been described previously [45]. DNA encoding the designed TM domain of interest was ligated into the pBLM100 plasmid (provided by Prof. D. Schneider) according to the reported protocol [45] to produce a fusion protein containing maltose binding protein (MalE) at the C-terminus and residues 1–87 of the LexA protein from *E. coli* at the N-terminus. During the original validation of the GALLEX protocol [45], Schneider demonstrated that a GpA TM segment of 17 residues in length yielded a ten-fold increase in association compared with a TM segment of 19 residues. On a similar note, the establishment of the ToxR *in vivo* TM reporter assays was optimized to yield significant signal strength using only 13 residues from the GpA TM segment [46]. The vast majority of left-handed dimers are thought to pack with a crossing-angle at around + 20° whereas the crossing-angle of right-handed dimer is around - 50° [47–49]. In order for a TM segment to span the hydrophobic region of the bilayer, short sequences may refrain from adopting an otherwise favorable right-handed orientation. However, it is clear from previous work [45] that a 17 residue long GpA TM segment results in a stable right-handed helical dimer. In light of this, all of our constructs contained 17 amino acid TM domains.

The resulting GALLEX chimeras were expressed in *E. coli* strain SU101 (provided by Prof. D. Schneider) after induction with 0.01 mM IPTG at 37 °C. Self-association of the TM domains leads to dimerization of LexA domains, which then bind to the wild-type *lacZ* promoter in SU101 and repress expression of β-galactosidase (β-gal). Therefore, the degree of repression of β-gal is proportional to the strength of TM domain homo-oligomerisation. Self-association of the LexA-TM-MalE fusion proteins was assessed via measurement of β-gal activity as described previously [50]. The β-gal Miller units were calculated using Eq. (1):

$$\frac{1000(OD_{420}-1.75\times OD_{550})}{t\times v\times OD_{600}} \quad (1)$$

where t is time (min) and v is volume (mL). Prior to performing the GALLEX assay, membrane insertion of all constructs was confirmed using the *malE* complementation assay [45], where cells were grown on M9 agar plates containing 0.4% maltose. A sodium hydroxide wash was also performed to give a qualitative indication of membrane insertion according to the reported protocol [51]. Expression levels for all constructs were confirmed via Western analysis against the MBP domain. The resulting band intensities were determined using the ImageJ software [52], and used to normalize all β-gal activities to total fusion protein expression levels. For all GALLEX measurements described above, a minimum of three independent outgrowths were prepared and measured in order to assess the magnitude of error. The final result was taken from the average of the three samples and the error was given by the standard error of the mean. Statistical analysis of all data was carried out using ANOVA with a probability (P) of 0.05 (95% confidence interval) to establish whether a given change in the "scaffold" sequence resulted in a significant change in the strength of interaction.

In order to estimate the thermodynamic contributions of selected motifs on the low complexity background sequence, quantitative measurements of TM interactions in the *E. coli* inner membrane were also performed using the GALLEX assay according to the reported protocol [30]. Briefly, β-gal activity measurements were collected from cell samples induced with a range of IPTG concentrations ($1\times10^{-4}-1$ mM). Expression of the chimera at each IPTG concentration was assessed by Western blotting against MBP and quantification of the band intensities using ImageJ [52], and these values were plotted against [IPTG]. The plot

of β-Gal activity versus [IPTG] was fit using a non-linear Hill function according to Eq. (2):

$$y = A1 + (A2 - A1)\frac{x^n}{K_d^n + x^n} \quad (2)$$

where A1 and A2 are the minimum and maximum asymptotes of the curve, K_d is the apparent dissociation constant (K_d^{app}), and n = 1 (non-cooperative binding). Fitting to the Hill function and extracting the IPTG concentration that yielded half-maximal signal was performed, and approximates the analysis that was performed previously by Finger et al. [30]. In addition to this analysis, we also fit the data to an explicit monomer-dimer equilibrium model according to Eq. (3).

$$y = \frac{8}{4x + K_d + \sqrt{8xK_d + K_d^2}} \quad (3)$$

All curve fitting was performed using OriginPro 8.5 software.

1.2. Computational searches using CHI

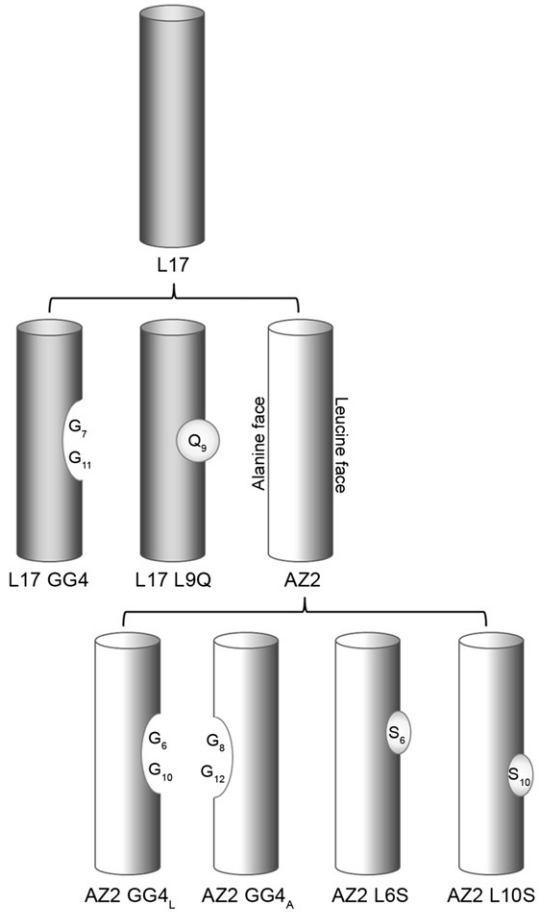
To identify likely dimer packing configurations, structural calculations were performed using the CNS searching of helix interactions (CHI) software, which has been previously described [53], on a Linux cluster made available by the Warwick Centre for Scientific Computing. CHI was used to create homodimer models from two parallel α-helices containing the *de novo*-designed sequences given in Fig. 1. Initial values for the centre-of-mass interhelical distance were set between 1.05 nm to 1.25 nm. Right and left-handed crossing angles, as defined by the angle between the principle axes of each helix, were initially set to −25° and 25°, respectively. These parameters were allowed to vary throughout the simulation. The helices were rotated about their principle axes from 0° to 360° in increments of 20°. After each rotation, four trial MD simulations of 5000 time steps were carried out to relax the conformation of the dimer; for each trial the atomic velocities were assigned at random from a Maxwell-Boltzmann distribution. Groups of a minimum of 6 structures with a backbone RMSD of ≤1 Å were clustered and the average structure for each cluster was calculated.

2. Results

2.1. De novo design of TM helix interactions

To examine the ability of well-known interaction motifs to facilitate TM helix interactions in a low sequence-complexity background, a series of TM helices were designed and are shown schematically (along with their TM sequences) in Fig. 1. At the top of Fig. 1 is the simplest sequence investigated, which we call the "scaffold", consisting of a seventeen amino acid stretch of poly-Leu (L17). Poly-Leu has been studied several times in the past, although there is some disagreement as to whether this sequence self-associates in the membrane. There have been reports that poly-Leu forms an α-helical homodimer with a left-handed crossing angle (i.e. left-handed coiled-coil) via a leucine–zipper interaction [14,42]. Indeed, the flexible leucine side chain is ideal for the heptad repeat's signature 'knobs-into-holes' packing helix–helix arrangement. However, a number of other studies demonstrate that poly-Leu is predominantly monomeric in both membranes and detergent micelles [16,18,19].

To the L17 sequence, we then added either (i) a G-*XXX*-G motif at the center of the helix (L17 GG4), (ii) a single polar residue (in this case Gln) at the center of the helix (L17 L9Q), or (iii) a series of seven alanine residues located at the *b*, *c*, and *f* positions of a heptad repeat sequence (*X*-AA-*XX*-A-*X*) to create what is called an alanine zipper (AZ2). Gln was selected in this first round of design as it has been shown to play a critical role in TM helix–helix interactions in native protein systems



	fgabcdefgabcdefga
L17	LLLLLLLLLLLLLLLL
L17 GG4	LLLLLLGLLLGLLLLL
L17 L9Q	LLLLLLLLQLLLLLLL
AZ2	ALLAALLALLAALLALL
AZ2 GG4$_L$	ALLAAGLALGAALLALL
AZ2 GG4$_A$	ALLAALLGLLAGLLALL
AZ2 L6S	ALLAASLALLAALLALL
AZ2 L10S	ALLAALLALSAALLALL
E5	LLFLGLVAAMQLLLLLFLLLF
Cadherin-I	LGILGGILALLILILLLLLFL

Fig. 1. *De novo* designed TM helices used in this study. A series of TM domain sequences were designed to investigate the ability of well-known interaction motifs to stabilize TM helix interactions in a low-sequence complexity background. In the first round of design, a simple poly-Leu sequence (which we call our scaffold) was decorated with each of three well-known sequence motifs (G-XXX-G, polar residue, and a motif of Ala residues to create an alanine-zipper (AZ2)). A second generation of peptides was then designed, combining the motif that yielded the largest increase in interaction strength in the first round (the AZ2 sequence) with an additional motif. A G-*XXX*-G motif was added to the AZ2 helix on either the Leu-containing face of the helix (AZ2 GG4$_L$) or on the Ala-containing face (AZ2 GG4$_A$). A polar residue (Serine) was also added to the Leu-containing face of the AZ2 helix at two different positions, one at the center of the TM helix (AZ2 L10S) and one which was one helical turn from the center of the TM domain (AZ2 L6S).

[37,54,55], and it has been speculated that the extra length and flexibility of the glutamine side-chain enables hydrogen bond formation in a wide variety of sequence contexts [56]. The AZ2 sequence was designed by Whitley and co-workers in 1994 to contain one helical face composed entirely of Leu residues, and one helical face composed of Ala residues, and was shown to form a stable integral membrane

α-helix [24]. Later, using extensive mutagenesis coupled with ToxR activity assays, Gurezka et al. [14] proposed that AZ2 self-associates via interactions of Leu residues at the heptad repeat positions *a* and *d* since mutation of either site to alanine resulted in significant disruption of oligomers.

A second generation of peptides was then designed, combining the motif that yielded the largest increase in interaction strength in the first round (the AZ2 sequence) with an additional motif. As shown in Fig. 1, a G-*XXX*-G motif was added to the AZ2 helix on the Leu-containing face of the helix (AZ2 $GG4_L$), previously reported to be the primary helix–helix interaction site [14]. Additionally, a polar residue (Ser was used in this round due to the marginal stabilization provided by Gln in the first round as well as the fact that Ser is the most frequently occurring non-charged polar residue in TM α-helices [57]) was added to the Leu-containing face of the AZ2 helix at two different positions, one at the center of the TM helix (AZ2 L10S) and one which was one helical turn from the center of the TM domain (AZ2 L6S). Finally, in an attempt to create a bifunctional helix, a G-*XXX*-G motif was added to the AZ2 helix on the Ala-containing face (AZ2 $GG4_A$).

2.2. Theoretical insertion of designed TM domains

The web based tool ΔG Predictor [58] was used to predict the theoretical Gibbs free energy of insertion into a biological membrane (ΔG_{pred}) for all of the TM sequences investigated in this study. The values are given in Table 1 and, in all cases, the net ΔG_{pred} was negative suggesting that all sequences studied here should spontaneously insert across the bilayer. The most favorable insertion was reported for the L17 sequence, with a $\Delta G_{pred} = -6.33$ kcal mol^{-1}. Increasing the Gly or Ala content of the poly-Leu TM sequence was predicted to make insertion less favorable (ΔG_{pred} less negative). Glycine plays a crucial role in TM helix packing, although it is also considered to be a helix breaker due to the flexibility the side chain provides the immediate peptide backbone [57]. This would expose the hydrophilic peptide backbone to the nonpolar lipid alkyl tails. The addition of a glutamine to the poly-Leu sequence was predicted to be the least favourable mutation given its strong polar nature. Second to cysteine, glutamine has the lowest frequency of occurrence in α-helical TM domains [57].

The ΔG_{pred} of AZ2 predicts the favorable insertion of the TM peptide across the membrane. Even though alanine contributes to an increase in ΔG_{pred} due its borderline apolar nature [59], it is the second most common amino acid in TM α-helices and plays a significant role in helix-helix packing [60]. Incorporating a G-*XXX*-G motif on either the Leu or Ala-containing faces of AZ2 makes insertion slightly less favorable, yet the combined hydrophobic contribution from the leucine residues still produces a net negative ΔG_{pred}. The inclusion of serine towards the center of the TM domain increases the net ΔG of insertion yet the combined ΔG_{pred} still remains in favor of insertion. Serine may satisfy the need to hydrogen bond by forming an intrahelical hydrogen bond with the backbone carbonyl oxygen atom [11].

Table 1
ΔG_{pred} values, obtained using DG Predictor, for constructs investigated in this study.

	ΔG_{pred} (kcal mol^{-1})
L17	−6.33
L17 GG4	−4.07
L17 L9Q	−4.35
AZ2	−2.09
AZ2 $GG4_L$	−0.74
AZ2 $GG4_A$	−1.22
AZ2 L6S	−1.29
AZ2 L10S	−1.22

2.3. Qualitative measurement of self-association of designed proteins using the GALLEX assay

The propensity of the 17-residue poly-Leu scaffold sequence (L17), and several rationally-designed variants, to self-associate in bacterial membranes was assessed using the GALLEX assay [45]. In this assay, TM helix interactions are evaluated in the *E. coli* inner membrane relative to a positive control (the strongly dimerizing GpA TM domain) and a negative control (the dimerization-compromised G83I mutant of GpA). The TM domain of interest is expressed as part of a fusion protein, between residues 1–87 of the LexA protein from *E. coli* and maltose-binding protein (MBP). Self-association of the TM domains is observed via the repression of β-gal expression. GALLEX can be used to study both homo- and hetero-oligomerization of TM helices, and in this work we have exclusively used the homo-GALLEX format. GALLEX signals are given in Miller units (see Materials and Methods), which can be related to the concentration of β-gal in each sample. The magnitude of the signal decreases as the strength of helix interactions increases, due to the repression of β-Gal expression. The results obtained in GALLEX for the L17 construct are shown in Fig. 2A, alongside the positive (GpA) and negative (G83I) controls. All GALLEX data shown in this work were normalized to expression level as evaluated from anti-MBP Western blots for each full-length construct, and also normalized to G83I. The correct insertion and orientation of the chimeric proteins in the *E. coli* inner membrane was confirmed using the *malE* complementation assay (see Fig. S1).

In our hands, the L17 sequence associates very weakly if at all, yielding weaker helix interactions than the negative control (as evaluated using a one-way ANOVA analysis with a significance cutoff of 0.05). Addition of a G-*XXX*-G motif to the center of the poly-Leu TM domain (see L17 GG4 in Fig. 2A) shows similarly weak self-association, and is in agreement with previous results for a similar sequence studied as part of a fusion protein in detergent micelles [16]. Subsequent mutation of the G-*XXX*-G motif with bulkier Ile residues that would disrupt close-packing (L17 GG4 G11I) also yielded no change in oligomerization, as ANOVA analysis suggests there is no difference between the L17, L17 GG4, and L17 GG4 G11I signals. Introduction of a single Gln residue at the center of the helix (L17 L9Q) increases the strength of interaction significantly compared to L17 or L17 GG4, but the interaction is weak compared to the positive control GpA (and is approximately equivalent in strength to that of the weakly-associating negative control, G83I). Nonetheless, to better understand what structural features stabilize helix interactions in the L17 L9Q sequence, computational models were produced using the program CHI [61]. A representative structure is given in Fig. 3A, where Gln packs at the helix-helix interface. As expected from previous work, the conformation of the Gln side-chains in each of the monomers is consistent with interhelical hydrogen bond formation (assigned here when a donor and acceptor are within 1.8 Å of one another). More extensive changes were then made to the L17 scaffold, via addition of seven Ala residues to create the AZ2 construct. As shown in Fig. 2A, these changes resulted in a 50% increase in the strength of helix interactions as compared to L17 alone, yielding a GALLEX signal intermediate between GpA and G83I and suggesting moderate but significant interaction (in agreement with previous studies [9,14]). CHI searches were again performed to determine which residues in AZ2 lie at the interface, however our searches returned structures that packed on both the Ala- and Leu-containing helical faces. Therefore, CHI did not distinguish with any certainty which residues are likely to stabilize interactions. The NACCESS program [62] was used to estimate the lipid accessible surface area of the ambiguous AZ2 CHI dimers, and thus suggest the more likely arrangement of the two based on the largest accessible non-polar surface area, however very similar values were returned for both dimers (see Fig. S2). The fact that the L17 construct showed such poor self-association would suggest that the Ala residues are interacting in AZ2. However, this is at odds with the previous study by Gurezka et al. [14], who clearly

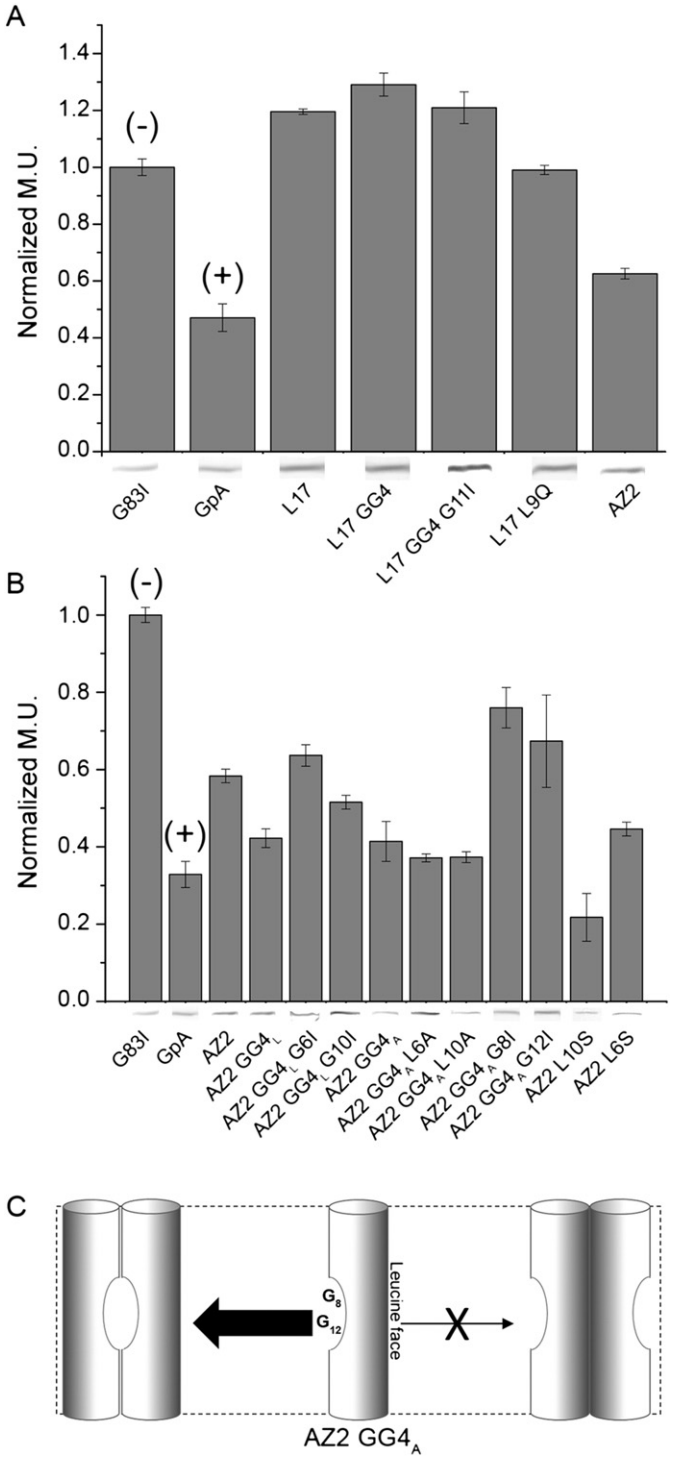


Fig. 2. A. The effect of individual motifs on self-association of poly-Leu "scaffold" evaluated using β-galactosidase activities obtained in semi-quantitative GALLEX assay, induced using 0.01 mM IPTG. All constructs are compared to a positive control (GpA, (+)) and a negative control (GpA G83I (−)), and all data are normalized to the values obtained from G83I. Values are means (+/− standard error of the mean) for three or more biological repeats. All β-Gal activities were also normalized to relative expression levels. **B.** Effect of individual motifs on self-association of AZ2 sequence measured using the semi-quantitative GALLEX assay, as described in part A. **C.** Schematic of bifunctional helix, containing two putative helix–helix interactions sites (the Leu-based heptad repeat and the G-XXX-G motifs). Mutagenesis and GALLEX measurements revealed that the G-XXX-G motif dominates helix–helix interactions (large arrow) while little evidence of interaction on the Leu-containing helical face was found (small arrow and cross).

demonstrated (via mutagenesis) that AZ2 interacted via the Leu residues. We speculate that this enhanced ability (in our hands) for Leu residues to interact within the framework of the AZ2 sequence could be due to the higher helical propensity of Ala vs. Leu [63], leading to a more stable helical fold, as well as the smaller Ala sidechain facilitating enhanced flexibility of the polypeptide chain and thus allowing Leu residues to access optimal packing arrangements.

Regardless, from the above analyses, it was clear that introduction of the AZ2 motif yielded the largest increase in interaction strength for the L17 scaffold. Therefore, an additional motif was added to this sequence with the aim of facilitating further increased self-association. The GALLEX results for this second generation of sequences are shown in Fig. 2B. Because it has been reported that the AZ2 sequence associates on its Leu-containing helical face [14], the substitutions L6G and L10G were made to introduce a G-*XXX*-G motif at the center of the TM domain on the Leu-containing face (at the *d* and *a* positions of the heptad repeat as shown in Fig. 1) and thus create the AZ2 $GG4_L$ construct. In this context, a significant difference is observed upon introduction of the G-*XXX*-G motif, strengthening interactions by 29% relative to the AZ2 signal. The interaction of this TM helix is returned to its original levels (i.e. those observed for AZ2) if either Gly in the G-*XXX*-G motif is mutated to an Ile (see AZ2 $GG4_L$ G6I and G10I mutants), suggesting that helices interact on the Leucine-containing face and utilize the G-*XXX*-G motif to strengthen interactions. CHI searches yielded a structure consistent with this data in which the Gly residues of one helix pack against the Ala face of an adjacent helix (Fig. 3B), however no symmetrical structures were obtained in which two G-*XXX*-G motifs packed together. This asymmetrical result was unexpected and may simply be due to the fact that CHI searches are not performed in a lipid bilayer, but are performed in vacuum with a user defined dielectric constant. This system may require more detailed molecular dynamics simulations in a well-defined bilayer to resolve any packing/mismatch issues that lead to asymmetry. It is also possible that the asymmetrical packing of AZ2 $GG4_L$ is due to contention between a preference to form a left-handed (as would be expected for a Leu-zipper) vs. a right-handed (as would be expected for G-*XXX*-G) homodimer, or the preference to bury alanaine residues at the homodimer interface given their borderline hydrophobicity.

A similar, but more pronounced, effect is observed upon incorporation of a single serine residue at the center of the helix on the Leu-containing face (AZ2 L10S), where a 61% increase in interaction propensity was observed (compared to the AZ2 signal) yielding a signal equivalent to GpA (the positive control). Calculations by Sal-Man et al. [64] estimated that motifs of multiple serine residues could contribute up to 30% to the free energy of association, however we were surprised at the impact a single serine residue had in the context of the AZ2 sequence. When this serine was moved one turn of the helix from the center, as in the AZ2 L6S mutant, the effect was less pronounced. This is in keeping with previous work demonstrating that another polar residue, Asn, has the most stabilizing impact on self-association when it is located near the center of the TM helix [17,28]. CHI searches for both Ser-containing sequences returned structures that suggest interhelical hydrogen bonding of the hydroxyl group on the Ser side chain (Fig. 3C and 3D for AZ2 L6S and L10S, respectively).

All motifs discussed above were added to the Leu-containing face of the AZ2 helix, previously reported to be the primary helix–helix interaction site, in order to further enhance helix interactions. We then asked the question of whether a viable secondary interaction site could be added to create a "bifunctional" helix (Fig. 2C). A second interaction site would facilitate a larger network of potential helix–helix interactions, necessary to create larger and more complex rationally-designed helical structures in the membrane (e.g. channels and pores). To create a second interaction site, we added a G-*XXX*-G motif to the opposite, or Ala-containing, face of the AZ2 helix (see Fig. 1, AZ2 $GG4_A$) and sought to demonstrate whether interactions could also take place through this motif. The GALLEX results for this construct are shown in Fig. 2B. The

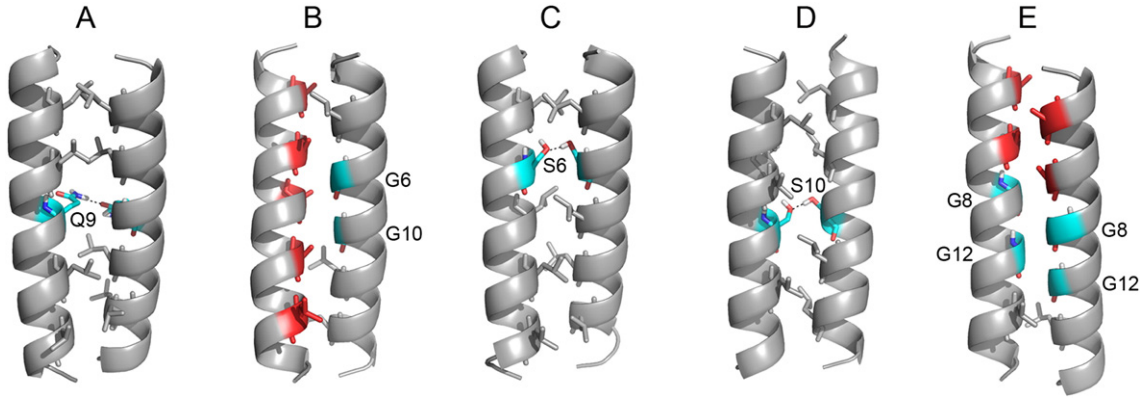


Fig. 3. Molecular models of *de novo* designed TM domains that show stable self-association in this study. Molecular models of A. L17 L9Q, B. AZ2 $GG4_L$, C. AZ2 L6S, D. AZ2 L10S, and E. AZ2 $GG4_A$ obtained using CHI searches of helix interactions (see Materials and Methods). Interactions involving motifs of interest are colored by atom type and labeled. Leu residues are shown in light gray and Ala residues are shown in red. Predicted hydrogen bonding interactions are shown by dashed lines.

AZ2 $GG4_A$ sequence yielded a signal in GALLEX that was intermediate between the positive and negative controls, and statistically equivalent to the signal obtained when the G-*XXX*-G motif was placed on the Leu-containing face of the helix. These results suggest that the G-*XXX*-G motif dominates the AZ2 interaction regardless of whether it is on the Ala- or the Leu-containing faces of the helix. This is confirmed by mutagenesis – mutation of L6 and L10 to Ala have no impact on oligomer formation, while mutation of either Gly residue in the G-*XXX*-G motif to Ile (G8I, G12I) weakens the oligomer significantly as shown in Fig. 2B. CHI searches also returned structures consistent with packing of Gly residues at the helix-helix interface, and a typical structure is given in Fig. 3E.

2.4. Free energy contribution of interaction motifs to L17 association in a biological membrane

Detailed information about the free energies of association of *de novo*-designed membrane proteins in a biological membrane is highly desirable but quite rare thus far. Although there is a wealth of biophysical data on the free energies of TM helix-helix association (as reviewed by Mackenzie and Fleming [31]), there have been few measurements of these parameters obtained in a biological membrane. Detergent micelles are often the membrane mimetic of choice for thermodynamic studies as they are amenable to a wider range of biophysical techniques. However, it has been suggested that studies conducted in detergent micelles may overestimate changes in the free energy of TM helix association as compared to studies performed in membranes [31]. GALLEX is one of few methods which can provide quantitative values for the changes in interaction free energy of TM helices in a biological membrane (the related TOXCAT assay has also been used to obtain similar information upon mutagenesis of GpA [29]). In the GALLEX assay, the investigator can systematically adjust the level of protein expression by varying the IPTG concentration in this inducible system and record the resulting changes in β-gal activity. This feature of the assay was exploited by Finger and co-workers [30] in order to estimate a first approximation of an apparent dissociation constant (K_d) for GpA and several mutants, and calculate the changes in interaction free energy upon mutation ($\Delta\Delta G^{app}$). This method of extracting an apparent K_d assumes a linear relationship between [IPTG] and [GpA], and is used because the GpA concentration cannot itself be directly measured.

We have used this approach to estimate the free energy contributions of selected motifs to the association of the L17 scaffold and the AZ2 variant. The β-gal activity was measured for cultures induced with IPTG at concentrations ranging from 0.1 μM to 1 mM and representative curves are shown in Fig. 4A–D (with the remaining curves given in Fig. S3). A plot of the band intensity from an anti-MBP western blot (obtained using Image J software [52]) vs. IPTG concentration is inset into each panel to demonstrate that an increase in [IPTG] leads to increased expression of each chimera thus allowing us to relate the concentration of IPTG to that of our chimeric protein. This behavior is roughly linear between 0.001 and 0.1 mM IPTG, and deviates from linearity at high and low IPTG concentrations as described previously [30]. The data shown in Fig. 4 were then fit as described in Materials and Methods. Analogous to the method employed previously [30], the apparent dissociation constant (K_d^{app}) was estimated from the IPTG concentration that resulted in half-maximal signal intensity in the assay across the range of concentrations tested (obtained here from a Hill fit of the data, see dashed curves in Fig. 4). The K_d^{app} (μM) values were used to calculate the apparent free energy of dimerization, ΔG^{app}, using the relationship $\Delta G^{app} = -RT\ln(K_d^{app})$, where the temperature was set to the temperature at which the cells were cultured and protein expressed (37 °C).

The resulting values for K_d^{app} and ΔG^{app} for both controls as well as six separate *de novo* designed TM domains are shown in Table 2. The change in ΔG^{app} upon mutation/addition of a motif relative to the "parent" sequence ($\Delta\Delta G^{app}$), where the parent sequences are either wild-type GpA, L17 or AZ2 as appropriate, is also given in Table 2. Negative values of $\Delta\Delta G^{app}$ suggest that a given motif or modification stabilizes interactions of the parent sequence, while positive values suggest destabilization. Broadly speaking, our controls are in good agreement with previous results for identical constructs. Our results for the positive control GpA ($K_d^{app} = 4.8$ μM and $\Delta G^{app} = 7.54$ kcal mol^{-1}) are very near those obtained previously in another laboratory [30] ($K_d^{app} = 3.1$ μM and $\Delta G^{app} = 7.51$ kcal mol^{-1}), which is an endorsement of the reproducibility of this method. In our hands, the dimer-destabilizing G83I mutation in GpA yielded a more modest change in apparent free energy of dimerization than previously observed ($\Delta\Delta G^{app} = 1.32$ vs. 2.15 kcal mol^{-1}, respectively), and more closely resembled the effects of the destabilizing G79I mutation in Finger et al. [30].

GALLEX was then used to estimate the energetic contribution of each interaction motif to stabilization of poly-Leu helix interactions, and these values are also given in Table 2. The poly-Leu helix displayed weak self-association in the assay, as expected, but did yield values for K_d^{app} (34.1 μM) and ΔG^{app} (6.34 kcal mol^{-1}) that suggest this sequence associates more strongly than the negative control, G83I. Addition of a single G-*XXX*-G motif at the center of the L17 TM helix did not result in a statistically significant change in stability with respect to L17 ($\Delta\Delta G^{app} = -0.05$ kcal mol^{-1}). Placing a glutamine residue at the center of the L17 TM domain has a more significant impact on self-association, stabilizing the interaction by $\Delta\Delta G^{app} = -0.31$ kcal mol^{-1} when compared to L17. Similarly, adding the motif of Ala residues on one face of the helix to create the AZ2 construct increased the stability of L17 self-association by a further −0.35 kcal mol^{-1}. Placing a G-*XXX*-G motif at the center of the AZ2 TM domain further stabilized helix–helix

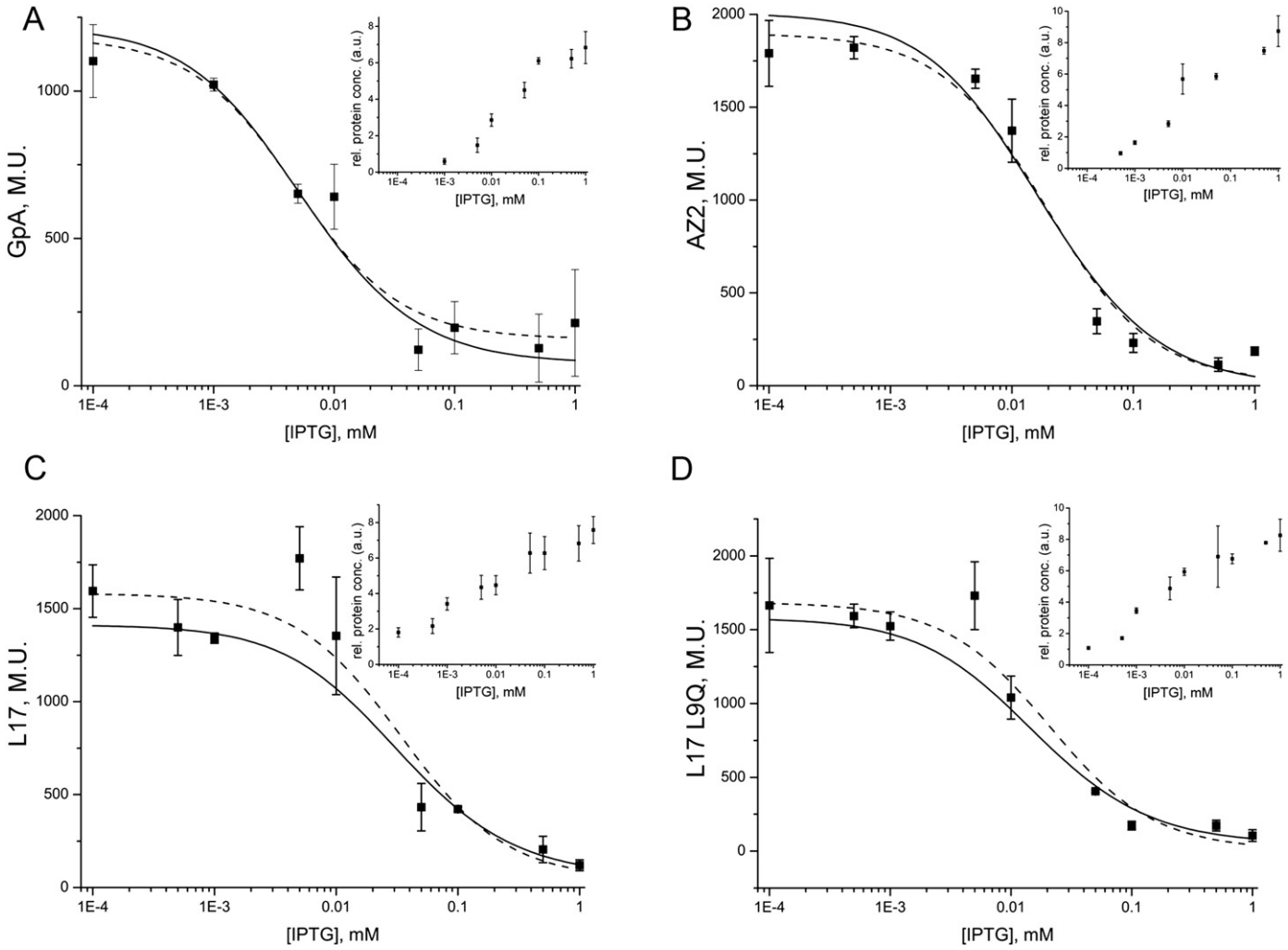


Fig. 4. Estimation of free energy changes in a biological membrane upon introduction of a motif using the GALLEX assay. β-Galactosidase activities (GALLEX signals) were measured after induction of protein expression using a range of IPTG concentrations from 1×10^{-4} – 1.0 mM (log scale). Values are means (+/− standard error of the mean) for three or more biological repeats. Representative curves are shown here for A. GpA, B. AZ2, C. L17 and D. L17 L9Q constructs, with similar behavior observed for all constructs studied. Relative protein expression was resolved by immunoblotting against MBP and quantified through densitometric analysis (see inset in each plot). Data were fit to the Hill equation (dashed line) to obtain the [IPTG] at half maximal signal intensity. Data were also fit to an explicit monomer-dimer equilibrium equation (solid line) as described in Materials and Methods.

Table 2
Self-association free energy of designed TM domains in the *E. Coli* inner membrane. The K_d^{app} is an apparent disassociation constant of helix–helix interactions obtained either from (i) a Hill fit (n = 1) of the GALLEX data to yield the [IPTG] at 50% signal intensity (first value listed) or (ii) a fit of the data to a monomer-dimer equilibrium derived from first principles (values in parentheses). The apparent free energy of association, ΔG^{app}, was calculated from the relationship $\Delta G^{app} = -RT\ln(K_d^{app})$, at a temperature of 37 °C. Free energy changes upon addition of a motif ($\Delta\Delta G^{app}$) were also calculated, relative to the appropriate "parent" sequence (i.e. GpA, L17, or AZ2), with negative values suggesting stabilization of interactions and positive values suggesting destabilization. * denotes values that were not measured directly, but were instead estimated from data obtained in Fig. 2 and the relationship given in Fig. 5.

TM construct	K_d^{app} (μM)	ΔG^{app} (kcal mol^{-1})	$\Delta\Delta G^{app}$ (kcal mol^{-1})
GpA	4.83 (18.1)	7.54 (6.73)	0.00
G83I	41.2 (168.5)	6.22 (5.35)	1.32 (1.37)
L17	34.1 (109.0)	6.34 (5.62)	0.00
L17 GG4	31.6 (93.9)	6.38 (5.71)	−0.05 (−0.09)
L17 L9Q	20.8 (52.0)	6.64 (6.08)	−0.31 (−0.46)
AZ2	19.2 (58.2)	6.69 (6.01)	0.00
AZ2 $GG4_L$	8.84 (23.1)	7.17 (6.58)	−0.48 (−0.57)
AZ2 $GG4_A$	9.27 (29.9)	7.14 (6.42)	−0.45 (−0.41)
AZ2 L6S	*10.8**	*7.05**	*−0.36**
AZ2 L10S	*3.68**	*7.71**	*−1.02**

interactions. Similar to our observations at a single IPTG concentration, the relative change in free energy of association is roughly the same regardless of whether this motif is placed on the Ala-containing face of the helix or the Leu-containing face ($\Delta\Delta G^{app} = -0.45$ and -0.48 kcal mol^{-1}, respectively).

The results summarized above are largely in agreement with the data acquired at a single IPTG concentration ([IPTG] = 0.01 mM, Fig. 2). One possible exception is the AZ2 helix, where the increased stabilization afforded by addition of Ala residues to the L17 helix (to yield the AZ2 helix) appears more dramatic when measured at a single IPTG concentration (compare L17 and AZ2 in Fig. 2) than it does after analysis of data acquired at multiple IPTG concentrations (see Table 2). However, in either case the trend is the same. Only one construct, L17, yielded results in which opposite trends were observed. L17 displayed stronger self-association than G83I when data were acquired at multiple IPTG concentrations (Table 2), whereas L17 showed weaker self-association than G83I when measurements were made at a single IPTG concentration (Fig. 2). Whilst the source of this discrepancy is unknown, and since the errors in the L17 and G83I measurements are not out of line with the other measurements made, we suggest that it might relate to the weakly-interacting nature of these two sequences. This discrepancy is the only one observed in the study, which speaks to the robustness and reproducibility of the assay. Indeed, the two datasets show a strong

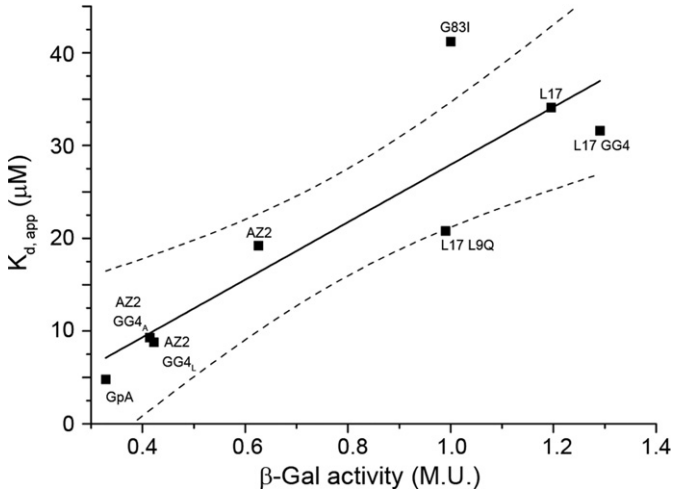


Fig. 5. Correlation of GALLEX data obtained at a single and multiple IPTG concentrations. β-Galactosidase activity obtained at a single IPTG concentration (0.01 mM, see Fig. 2 and 4) is plotted against $K_{d,app}$ obtained from GALLEX data acquired at multiple IPTG concentrations (1×10^{-4} – 1.0 mM) for a selection of TM sequences studied here. A linear fit of these data is shown by the solid line, and yields a Pearson's R = 0.89, a slope of 31.009 and an intercept of −3.07. The 95% confidence intervals for the fit are shown by dotted lines.

positive correlation, as plotted in Fig. 5. A linear fit of the data yields a Pearson's correlation coefficient R = 0.89, and suggests that all but one data point (that of G83I) lie within the 95% confidence interval of the fit (dotted lines in Fig. 5).

In addition to estimating K_d^{app} from the IPTG concentration that resulted in a half-maximal GALLEX signal, we also fit the data to an explicit monomer-dimer equilibrium model (Eq. (3)) in which K_d^{app} was extracted from the fit (see solid curves in Fig. 4). These values and the corresponding values for ΔG^{app} and $\Delta\Delta G^{app}$ are also given in Table 2 (in parentheses). Only a simple monomer-dimer model was explored in this work, although we fully acknowledge that this is likely an over simplification which will require refinement in the future. Nevertheless, a monomer-dimer equilibrium appeared to describe the data well and is appropriate for GpA, L17, and AZ2, all of which have been reported to form oligomers consistent with homodimers [14,65]. Fitting of the data to a monomer-dimer equilibrium, as compared to half-maximal IPTG concentration, universally yielded higher values for K_d^{app} and lower values for ΔG^{app}, but the free energy changes ($\Delta\Delta G^{app}$) obtained upon mutation/addition of a motif were similar and followed the same trend with respect to stabilization and destabilization.

3. Discussion

We have used a *de novo* approach to design TM helices based on a poly-Leu background decorated with one or more well-known helix interaction motifs. The first aim was to increase the strength of helix interactions in a small number of design steps. The data demonstrate that rational design of TM helix interactions is feasible using our current understanding of helix interaction motifs. While we acknowledge that membrane protein folding is complicated by restrictions imposed by the membrane bilayer, and one should avoid overly simplistic models of membrane protein folding, the use of motifs as predictors of helix–helix interactions has been remarkably successful thus far and has led to many examples (which have been discussed extensively in the literature and are not summarized here) that support their role in stabilizing key interactions. We were able to achieve > 80% increase in association strength (based on β-Gal activities and changes in K_d^{app}) and create a strongly interacting TM domain by making very few modifications to the weakly-associating poly-Leu TM sequence. If we look to natural TM domain sequences, we see that strongly interacting, leucine-rich TM domains such as those found in BPV E5 [36] and cadherin-I [66] (see sequences in Fig. 1) may have evolved from stretches of poly-Leu stably inserted in the membrane.

The second aim was to estimate the energetic contribution of various helix interaction motifs to self-association of a low sequence-complexity scaffold. By doing this, we hoped to remove (as much as possible) the effects of sequence context in order to focus purely on the contribution of each motif. Of course, there is no way to completely remove sequence context, but the poly-Leu sequence is about as near as one can come. Using the GALLEX assay, the poly-Leu scaffold (L17) appeared predominantly monomeric or, at best, "weakly-associating". Incorporation of a G-*XXX*-G motif in the center of the L17 sequence had no impact on self-association in the *E. coli* inner membrane, thus it is evident that this motif does not promote self-association of a poly-Leu helix. This is not surprising given the previous findings of Doura and Fleming [7], who reported that G-*XXX*-G-mediated dimerization was sequence context dependent, and that without long range interactions (those over 12 Å) G-*XXX*-G alone is not sufficient for dimerization. In another study, Brosig and Langosch found that several additional residues were required to assist G-*XXX*-G-mediated oligomerization [21].

Addition of a polar residue (Gln) to the center of the L17 helix did stabilize helix–helix interactions by − 0.31 and −0.46 kcal mol^{-1} (depending on the fit). This result was predicted from previous studies of model peptides that clearly demonstrate stabilizing polar interactions (especially for Asn and Gln, but also for Asp, Glu and His) for residues placed at the center of the TM helix [19,28]. From these studies we know that if a single polar residue is not affected by the native van der Waals packing then it should be at the helix-helix interface, shielded from the low dielectric bilayer core. In the context of native TM domains, Gln has been shown to play a critical role in TM helix–helix interactions of Tumour Necrosis Factor 5 [54], Class II MHC Invariant chain [37], and the E5 protein from bovine papillomavirus [55]. The magnitude of the stabilization, however, was lower than expected. Although thermodynamic parameters have not, to our knowledge, been reported previously for our TM domain sequences, Lear and co-workers reported that a centrally-located Asn residue stabilized helix–helix interactions of a model TM peptide (based on a coiled-coil in the protein GCN4) in detergent micelles by $\Delta\Delta G = -2.0$ kcal mol^{-1}. This value is six times larger than the value we obtain for L17 L9Q in the *E. coli* inner membrane using GALLEX, and lends support to the suggestion that studies conducted in detergent micelles may overestimate changes in the free energy of TM helix association as compared to studies performed in membranes [31]. Indeed, our data suggest that only ~ 1.3 kcal mol^{-1} lies between a protein considered to be weakly associated in the membrane and one considered to be a strong dimer. It has been suggested previously that free energy differences of less than 2 kcal mol^{-1} can lead to significant separation of protein and lipid components in a biological membrane and thus stabilize TM helix–helix interactions whilst destabilizing TM helix–lipid interactions [67]. Of course, the small magnitude of stabilization afforded by Gln in this context may also be due to (i) side-chain/side-chain hydrogen bonds competing with alternative hydrogen bonds between side-chain and backbone [68], or (ii) the packing of neighbouring leucine side-chains preventing an ideal angle and distance between the two glutamine side-chains [69,70]. Once the hydrogen bond moves beyond 2 Å it becomes a weak electrostatic interaction [54]. However, results from molecular modeling using CHI suggest that formation of interhelical hydrogen bonds for L17 L9Q homodimers is chemically feasible (Fig. 3A).

Incorporation of Ala residues into the L17 sequence to create the AZ2 sequence stabilized self-association by approximately − 0.4 kcal mol^{-1}. The increased stability of AZ2 vs. poly-Leu is in close agreement with a POSSYCCAT study by Ridder et al. [9], however there are no reported values for free energy that we can use for comparison. Regardless, it was clear that of the three constructs designed from a simple poly-lLeu scaffold, AZ2 was the most stable oligomer and was taken forward to the next stage of the work.

In the next stage, four new sequences were designed by adding an additional motif to the AZ2 sequence. Addition of a single Ser residue into the AZ2 sequence increased the interaction propensity significantly, depending on where the serine was placed. If Ser was placed at position 10 (i.e. the center) of the helix, the AZ2 construct displayed interactions equivalent to those observed for GpA. If Ser was placed at position 6, one turn of the helix from the center, the effect was less pronounced but still significant. This result was slightly unexpected, given past reports that a single serine residue was incapable of promoting significant helix-helix association [71,72] and that the frequently occurring motifs S-*XXX*-SS-*XX*-T and S-*XX*-SS-*XX*-T were the minimum requirements for any significant serine-based helix-helix association [11]. However, a recent report by Schneider and coworkers demonstrated that serine can stabilize helix–helix interactions, most likely via formation of a hydrogen bond between the side chain — OH and a backbone carbonyl on the adjacent helix, and that this ability is sequence-context dependent [73]. Using the same GALLEX approach as used here, Finger et al. [30] estimated that a Ser hydrogen bond stabilized a GpA mutant dimer by −0.4 kcal mol^{-1} per monomer. The free energies of the AZ2 L10S and AZ2 L6S constructs were not measured here, however using the data shown in Fig. 2 acquired at a single IPTG concentration and the linear relationship given in Fig. 5 we can make a rough approximation of the values for K_d^{app}, ΔG^{app}, and $\Delta\Delta G^{app}$ upon addition of Ser. Thus we estimate that addition of Ser stabilizes self-association of AZ2 by a further − 0.36 kcal mol^{-1} at position 6 and − 1.02 kcal mol^{-1} at position 10. This agrees well with the results of Finger et al., and suggests that either one (AZ2 L6S) or two (AZ2 L10S) new interhelical hydrogen bonds are formed by Ser in these constructs. It should be mentioned that no more than one interhelical H-bond was observed in our CHI models of these sequences, however these searches are not performed in a lipid bilayer (they are performed in vacuum) and are thus used here purely for illustrative purposes. Regardless, a single Ser residue is one of the most stabilizing mutations investigated here.

Addition of a central G-*XXX*-G motif on the Leu-containing face of the helix at positions *d* and *a* of the heptad repeat (AZ2 $GG4_L$) stabilized helix interactions by approximately −0.5 kcal mol^{-1}. Mutagenesis confirmed that the Gly residues pack at the helix-helix interface, suggesting that the G-*XXX*-G motif stabilizes a left-handed coiled-coil. This has been reported previously by Senes and co-workers [74] for parallel and anti-parallel left-handed coiled-coils, and has been observed in other model [75] and naturally occurring TM domains, such as the class II MHC α- and β-chains [76]. However, the G-*XXX*-G motif has been found much more frequently to stabilize right-handed coiled-coils [77,78], and it has been suggested that the affinity of this motif is higher in right-handed vs. left-handed coiled-coils [75].

A similar enhancement in stability (−0.45 kcal mol^{-1}) was observed when the G-*XXX*-G motif was placed on the Ala-containing face of the AZ2 helix. This helix was designed to create a "bifunctional" helix that could associate via two different motifs, (i) a G-*XXX*-G motif and (ii) a Leu-zipper motif, located on opposite helical faces. However, mutagenesis suggests that the G-*XXX*-G motif dominates the interaction (depicted in Fig. 2C) since mutation of Leu residues has no impact on self-association. This does not bode well for the ability of our helix to interact via two motifs, but it does suggest a hierarchy of stability within the context of this helix (i.e. G-*XXX*-G > Leu-zipper).

4. Conclusions

The standalone GALLEX results and calculation of apparent free energy changes in a biological membrane are the first of their kind for *de novo* designed sequences. These data suggest that very few single changes to a simple polyleucine sequence are required to encourage significant self-association, and that the free energy barrier to overcoming weak association is actually quite small (<1.3 kcal mol^{-1}) in a natural membrane. The results shown here quantify and rationalize the contribution of key motifs to transmembrane helix association, offering a route to direct the design of novel sequences for use in biotechnology or synthetic biology (such as molecular switches and artificial channels) and to predict the effects of sequence modification in naturally-occurring transmembrane domains.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2015.02.020.

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