Published in final edited form as:

J Mol Biol. 2010 March 5; 396(4): 924–936. doi:10.1016/j.jmb.2009.12.023.

Intermonomer hydrogen bonds enhance GxxxG-driven dimerization of the BNIP3 transmembrane domain: roles for sequence context in helix-helix association in membranes

Charles M. Lawrie^a, Endah S. Sulistijo^{a,1}, and Kevin R. MacKenzie^a

^a Rice University, Department of Biochemistry and Cell Biology MS-140, PO Box 1892, Houston, TX 77251-1892 USA

Abstract

We determined the sequence dependence of human BNIP3 transmembrane domain dimerization using the biological assay TOXCAT. Mutants in which intermonomer hydrogen bonds between Ser 172 and His 173 are abolished show moderate interaction, indicating that side chain hydrogen bonds contribute to dimer stability but are not essential to dimerization. Mutants in which a GxxxG motif composed of Gly 180 and Gly 184 has been abolished show little or no interaction, demonstrating the critical nature of the GxxxG motif to BNIP3 dimerization. These findings show that side chain hydrogen bonds can enhance the intrinsic dimerization of a GxxxG motif and that sequence context can control how hydrogen bonds influence helix-helix interactions in membranes. The dimer interface mapped by TOXCAT mutagenesis agrees closely with the interfaces observed in the NMR structure and inferred from mutational analysis of dimerization on SDS-PAGE, showing that the native dimer structure is retained in detergents. We show that TOXCAT and SDS-PAGE give complementary and consistent information about BNIP3 TMD dimerization: TOXCAT is insensitive to mutations that have modest effects on self-association in detergents but readily discriminates among mutations that completely disrupt detergent-resistant dimerization. The close agreement between conclusions reached from TOXCAT and SDS-PAGE data for BNIP3 suggests that accurate estimates of the relative effects of mutations on native state protein-protein interactions can be obtained even when the detergent environment is strongly disruptive.

Introduction

Lateral interactions between single spanning transmembrane domains (TMDs) direct stable and specific protein-protein contacts in processes as diverse as cell signaling and pore formation 1–4. While such lateral interactions are of functional and medical interest in their own right, they also provide an opportunity for determining the physical basis for membrane protein folding, stability, and specificity 5. Biophysical, biochemical, and biological methods for measuring or estimating the extent of TMD oligomerization have begun to define the roles of individual side chains and sequence context in driving biological TMD oligomerization (reviewed in 6), several sequence elements or motifs that support TMD interactions have been identified, including hydrogen bonding side chains and the GxxxG motif (reviewed in 7), and

Corresponding author: Kevin R. MacKenzie, mev@rice.edu, phone: 713-348-2228, fax: 713-348-5154.

¹present address: Yale University, Department of Molecular Biophysics & Biochemistry, P.O. Box 208114, New Haven, CT 06520-8114 USA

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

the energetic basis for TMD interactions are starting to be understood in some detail (reviewed in 8).

The GxxxG motif occurs widely in nature ⁹, and in the case of the glycophorin A TMD dimer, this motif supports dimerization by allowing close approach of helices and formation of favorable intermonomer side chain/side chain, side chain/backbone, and backbone/backbone contacts ¹⁰. In this and other systems, the importance of small residues that lie on the same face of a helix in supporting both homo- and hetero-oligomerization is well established ^{1, 6, 11}. Because not all GxxxG-containing TMDs self-associate, differences in adjacent residues, or sequence context, are thought to contribute to both specificity and stability. Sequence context strongly influences GxxxG-driven dimerization of glycophorin A ¹²: single apolar substitutions to the residues that flank the GxxxG motif modulate dimerization free energy over a range of 3.7 kcal mole⁻¹. The perturbing effects of single point mutations on the well-characterized glycophorin A dimer are consistent with structure-based steric models ^{12, 13}, but the complex effects of pairs of mutations ¹⁴ show that we are only beginning to scratch the surface of how sequence context modulates GxxxG-driven dimerization⁸.

Canonical hydrogen bonds are likely candidates for directing strong lateral interactions between TMDs embedded in bilayers because of the low dielectric and lack of competing water in an apolar environment. Protein design experiments showed that strongly polar residues in an otherwise apolar TMD with no 'small' residues cause strong oligomerization in the biological assay TOXCAT and in detergent micelles ¹⁵⁻¹⁸, with an energetic contribution of ~2 kcal mole⁻¹ per hydrogen bonding side chain¹⁹. This strong role for inter-helix hydrogen bonding in membrane protein folding contrasts with mutational analyses in bacteriorhodopsin ²⁰, where a set of side chain/side chain hydrogen bonds stabilize the protein by only 0.6 kcal mole⁻¹ on average, although one hydrogen bond stabilizes bacteriorhodopsin by as much as 1.6 kcal mole⁻¹. It may turn out that only a small proportion of side chain/side chain hydrogen bonds between helices strongly stabilize membrane proteins.

We are determining the contributions that side chain hydrogen bonding, GxxxG motifs, and sequence context make to membrane protein folding by analyzing the TMD-mediated lateral interactions of BNIP3, a protein that is tail-anchored to the mitochondrial outer membrane and that plays a role in apoptosis. We have shown that the BNIP3 TMD dimerizes strongly in membranes and in detergents ²¹. Based on an extensive mutational analysis in detergents, we identified three small residues and two strongly polar side chains as critical to this dimerization, and we predicted that Ser 172 and His 173 formed side chain intermonomer hydrogen bonds ²². A recent paper describes disorder in the solution NMR structure of the BNIP3 TMD dimer at Ser 172 and His 173 and downplays the role of the hydrogen bond in the stability of the dimer ²³. However, our structure in a different detergent/lipid mixture indicates that His 173 is well ordered and forms a hydrogen bond with Ser 172 of the opposite monomer ²⁴. The strong modulation of the BNIP3 peptide dimer NMR spectra by titration of different lipids into the mixed micelle solution ²⁴ raises the possibility that the structural and mutational data published for BNIP3 so far, which have mostly been measured using detergent-solubilized samples, may not accurately reflect its behavior in membranes. We have previously shown that the biological assay TOXCAT ²⁵ (Figure 1) provides measures of TMD-TMD interactions in cell membranes that can be used to validate qualitative and quantitative data from experiments in detergents ²⁶. To determine which residues drive BNIP3 TMD dimerization in membranes, we undertook a mutational analysis using TOXCAT.

Results

Mutations that strongly disrupt dimerization of the BNIP3 TMD in detergents have modest effects in membranes

Using saturation mutagenesis and SDS-PAGE we identified a set of interacting residues²² that map to the BNIP3 TMD dimer interface²⁴, but mutations appear to be less disruptive toward BNIP3 TMD dimerization in TOXCAT than in SDS-PAGE. Figure 2B shows that the wild-type BNIP3 TMD gives more than twice the TOXCAT signal of glycoprotein A (GpA), and that a phenylalanine scan reveals only one position (Gly 180) where the substitution disrupts dimerization to the level of the negative control (GpA mutant G83I). The strongly disruptive effect of G180F confirms that the assay reports on sequence dependent dimerization. However, at four other positions where phenylalanine substitutions significantly disrupt dimerization as assayed in SDS-PAGE (Ser 172, His 173, Ala 176, and Gly 184)²², these mutations have only modest effects on dimerization, with all four of these ‘disruptive’ mutants exhibiting stronger TOXCAT signals than the GpA positive control. The very strong TOXCAT signal of the wild-type BNIP3 TMD and the relatively modest effects of hydrophobic mutations that are significantly disruptive in SDS-PAGE suggest that the BNIP3 TMD causes maximal activation of the reporter gene signal in the TOXCAT assay, and that mutations that modestly disrupt dimerization have little impact on the observed TOXCAT signal. We tested this hypothesis by determining if we could increase the sensitivity of the BNIP3 TMD to disruptive mutations and by performing extensive mutagenesis on an optimized TMD.

A minimal BNIP3 TMD construct is more sensitive to mutations than the full-length construct

To determine if we could modulate the intrinsic dimerization capacity of the BNIP3 TMD so as to facilitate disruption by mutations, we made a series of TMD deletions (Figure 2A) and assessed the effects of phenylalanine scanning mutagenesis on the deletion variants. All constructs complement *malE*, indicating that the ToxR-TMD-MBP fusion is correctly inserted into the *E. coli* inner membrane²¹, and all cells express similar levels of ToxR-TMD-MBP fusion (not shown). C-terminal TMD deletions that eliminate the GxxxG motif strongly attenuate dimerization (not shown), but Figures 2C through 2E show that N-terminal deletions of two or four residues had no effect on ‘wild-type’ TOXCAT signal, whereas deletion of six residues slightly decreased the signal. Although the $\Delta 6$ -BNIP3 construct still gives almost twice the CAT activity of the GpA positive control, the effects of phenylalanine substitutions at positions 172, 173, 176, and 184 are more pronounced than in the full-length construct. We attribute the increased sensitivity to disruptive mutations to a decrease in the intrinsic dimerization propensity of the truncated wild-type BNIP3 TMD, perhaps due to the geometrical constraints placed on the ToxR DNA binding domains of the ToxR-TMD-MBP fusion construct. The decrease in intrinsic dimerization is not readily detected because the assay is driven to maximal activation by wild-type BNIP3, and to near-maximal activation by the $\Delta 6$ construct, but the weaker initial self-association of the deleted construct makes it more sensitive to the effects of mutations. Note that the N-terminal truncations do not displace the positional dependence of disruption along the sequence, only the degree to which the signal is attenuated. We conclude that saturation mutagenesis is likely to identify more disruptive mutations using the $\Delta 6$ -BNIP3 construct rather than the full-length construct.

Mutations to the BNIP3 TMD have a wide range of effects on TOXCAT signal

We generated single point mutants at fifteen transmembrane positions in the $\Delta 6$ -BNIP3 construct, tested each construct for proper membrane insertion by its ability to complement *malE*²⁵, and then analyzed three independent cultures of each of 101 mutants by TOXCAT (see Materials and Methods). CAT activities are reported in Figure 3 as a percentage of wild-type $\Delta 6$ -BNIP3 with error bars corresponding to the standard error of three or more replicate cultures, and place-holders without error bars are used to indicate that the wildtype sequence

already contains the substituting residue at that position. Data are shown for all mutants generated; no constructs had to be excluded due to failure to complement *malE*. Most substitutions have little effect: six positions (171, 174, 175, 177, 179, and 182) never drop below 90% of wild-type CAT activity for any substituting residue, and three positions (170, 178, and 181) drop below 90% of wild-type for only one mutant. Only 26 mutants give CAT activities of less than 50% of wild-type, and the seven mutants that give CAT activities of less than 10% of wild-type all occur at Gly 180 or Gly 184. The low average CAT activities for mutations at Gly 180 and Gly 184 (12% and 26% of wild-type, respectively) clearly identify these positions as important to dimerization, and the moderate average CAT values for Ser 172 and His 173 (69% and 52% of wild-type, respectively) also implicate these residues in dimerization. However, if the wild-type BNIP3 TMD causes near maximal CAT transcription, significant disruption of self-association could result in only small decreases in CAT activity, and averaging the fractional CAT activity at each position might mask small but important differences. We therefore established a non-linear binning scheme for the mutagenesis data set.

The effects of mutations on TOXCAT signal can be binned into six classes

The mean scores and error bars for the most associated constructs in Figure 3 indicate that we should usually be able to distinguish between a wild-type level of CAT activity and 90% of wild-type. We assume that 90% of wild-type CAT activity corresponds to 9:1 active:inactive reporter gene promoter, and we take changes in this ratio by factors of three (9:1, 3:1, 1:1, 1:3, and 1:9 active:inactive) to generate geometrically spaced bin limits of 90%, 75%, 50%, 25%, and 10% of wild-type CAT activity. We bin the 101 mutants into six classes: 61 mutants have 90% (or greater) of wild-type CAT activity (score=0), 8 have 75–90% of wild-type activity (score=1), 6 have 50–75% of wild-type (score=2), 14 have 25–50% of wild-type (score=3), 5 have 10–25% of wild-type (score=4), and 7 have less than 10% of wild-type CAT activity (score=5).

Error bars for 27 mutants extend into an adjacent bin (see Figure 3), and it is therefore likely that some of these mutants are misclassified, but because no mutants have error bars that cross two classification boundaries, mutants are unlikely to be binned more than one class away from their true class. The error bars for only one mutant classified as maximally disruptive extend above the bin cut-off of 10%, and the error bars for only one mutant classified as ‘4’ extend below the cut-off of 10%, indicating that binning is not likely to be significantly affecting identification of the most important sites. Using a smaller geometric factor in constructing the scale (a factor of two instead of three) would have allowed a few error bars to span multiple bins; such a scheme would probably have broken the data set into more bins than would be justified based on the reproducibility of the measured data.

All constructs express similar levels of ToxR-TMD-MBP fusion by western blot (not shown). In our previous TOXCAT work with GpA, many replicate cultures showed two-fold or three-fold differences in CAT activity, and correcting the TOXCAT data using relative ToxR-TMD-MBP fusion expression level as assessed by western blot greatly improved the agreement between replicates, allowing us to extract thermodynamics of dimerization in the membrane²⁶. In this study, no constructs show twofold differences in CAT signal between replicates; in fact, 88% of constructs show small (< 1.4 fold) differences in CAT activity between the highest and lowest replicate, and 66% of constructs show very small (< 1.2 fold) differences. For such small differences between replicates, correction for expression levels is not needed to achieve small standard errors, especially since we are using a binning scheme instead of trying to estimate free energy changes. Furthermore, correcting for fusion protein expression would be more complicated than in the GpA case. For strongly dimerizing constructs such as wild-type BNIP3, small fusion protein concentration changes should not require a correction because all

available CAT promoters are fully occupied, whereas for strongly disruptive mutants a correction would be meaningful, since changes in fusion protein concentration should clearly alter the amount of dimer bound to the CAT promoter when binding is not saturated. Consistent with this expectation, the seven constructs that show the largest variations in CAT activity (1.5 to 1.8 fold differences between highest and lowest replicates) all have average CAT activities of less than 20% of wildtype and TOXCAT scores of 4 or 5. Correcting for ToxR-TMD-MBP fusion protein expression would not affect the binning of these data points.

Some non-interfacial mutants show slight fold increases in mean TOXCAT scores over wild-type, but these do not correlate with ToxR-TMD-MBP fusion expression (not shown). We hypothesize that these reflect small increases in plasmid copy number, which would affect the TOXCAT output by altering the number of reporter gene constructs per cell. Accordingly, we interpret TOXCAT signals that modestly exceed wild-type as wild-type-like; 12 of 44 non-interfacial mutants give TOXCAT scores that are 1.2 fold greater than wild-type. Similar fold decreases in plasmid copy number could cause mutants to give smaller than appropriate TOXCAT scores, but this would not greatly affect the outcome of our geometrically scaled binning scheme.

A TOXCAT-based hierarchy of BNIP3 residue contributions to TMD dimerization in membranes

The binned phenotype scores for the 101 mutants of Figure 3 are presented graphically in Figure 4A. 60% of mutants are classified as non-disruptive, so that even modestly disruptive mutations stand out in this representation. Substitutions to proline are significantly disruptive at nine positions, and these are largely concentrated in the C-terminal half of the TMD, but no proline substitution achieves the most disruptive phenotype score. Based on the average phenotype scores of non-proline substitutions, the importance of BNIP3 TMD positions to dimerization is: Gly 180 > Gly 184 > His 173 > Ser 172 > Ala 176 > Ile 183 > Ile 181. These observations parallel the SDS-PAGE dimerization phenotype scores we previously published for the BNIP3 TMD²², although that study ranked the first four mutants in this list as equally (and completely) disruptive. The SDS-PAGE dimerization phenotypes of the subset of the previously published mutants that correspond to the mutants studied here by TOXCAT are presented in Figure 4B. The previously described phenotype bins for the SDS-PAGE data correspond to approximately threefold changes in the proportions of dimer and monomer on the gel²² (see the legend of Figure 4B), but the mutations are almost invariably more disruptive in SDS-PAGE than in TOXCAT, as can be appreciated by comparing Figures 4A and 4B. 43% of the SDS-PAGE mutants are classified as completely disruptive, and only 27% are classified as non-disruptive. Both methods score mutants using an integer from 0 to 5, with 5 being maximally disruptive, but the distributions of phenotype scores generated by the two methods is quite different (Table 1); a chi-squared statistic gives $P < 1.3 \cdot 10^{-7}$ for randomly selecting the set of TOXCAT scores from a parental distribution with the ratios seen for the SDS-PAGE scores (or vice versa). We conclude that the two assays give very different scores for this set of BNIP3 mutants.

Interfaces identified by TOXCAT and SDS-PAGE match one another and the NMR structure

To determine if the disruption of dimerization by mutations in TOXCAT and SDS-PAGE map the same physical interface, we plot the averaged TOXCAT phenotype bin scores for non-proline substitutions at each position along the TMD sequence in Figure 4C, along with the averaged SDS-PAGE phenotypes for the same substituting residues²². We also plot the number of intermonomer contacts per residue, based on the NMR structure of the BNIP3 dimer (PDB ID: 2KA2), as determined by counting the number of non-hydrogen atom-atom pairs in which one atom lies in the residue of interest and the other atom belongs to the opposite monomer and is no more than 4.5 Å away (as previously described²⁴). Although the amplitudes of the TOXCAT and SDS-PAGE averages do not correspond closely, the positions

at which mutations cause disruption generally align with one another and with the packing index from the NMR structure (Figure 4C). The TOXCAT average deviates substantially from the SDS-PAGE average and packing score at positions 177 and 181, whereas the SDS-PAGE average deviates substantially from the TOXCAT average and the packing score at position 183. In general, mutations at the known dimer interface affect self-association as assayed by either TOXCAT or SDS-PAGE.

Ile 177 and Ile 181 are outliers in the correlation between packing and mutagenesis

A quantitative relationship between intermonomer contacts and the mutagenesis scores can be evaluated by plotting the residue packing score against the TOXCAT or SDS-PAGE position averages (Figure 5) and subjecting the data pairs to rank order correlation and linear least-squares regression analyses (Table 2). The rank order of the fifteen packing scores is similar to the rank orders of either the TOXCAT average ($R_S = 0.70$) or the SDS-PAGE average ($R_S = 0.76$), and the associated P values (Table 2) confirm that the position rankings by mutagenesis average are significantly correlated with the ranking by residue packing. Linear least-squares regression analysis indicates that packing correlates weakly with either the position-averaged TOXCAT score ($R^2 = 0.57$) or the position-averaged SDS-PAGE score ($R^2 = 0.39$). In Figure 5A, the points corresponding to Ile 177 and Ile 181 seem to be outliers: mutations at these positions have little effect on dimerization even though the residues have high intermonomer packing scores. Excluding positions 177 and 181 increases the correlations between the packing score and either the TOXCAT position averages ($R_S = 0.94$, $R^2 = 0.90$) or the SDS-PAGE position averages ($R_S = 0.88$, $R^2 = 0.50$); P values indicate that this is highly significant for TOXCAT (Table 2). Leaving out these residues does not substantially alter the same tests when the TOXCAT and SDS-PAGE scores are compared to one another directly (Table 2), indicating that the mutagenesis scores agree with one another as well at positions 177 and 181 as anywhere else, but that the packing score does not agree well with either type of mutagenesis data at these sites. We previously observed that substitutions at these positions do not introduce intermonomer clashes and only slightly decrease favorable intermonomer packing contacts because the backbone atoms and C β make the majority of such contacts for the wild-type residues²⁴. We conclude that the packing interactions made by the side chain atoms of Ile 177 and Ile 181 are not critical to dimerization in either TOXCAT or SDS-PAGE.

Position-averaged TOXCAT and SDS-PAGE scores are positively correlated

To assess the significance of correlations between the TOXCAT and SDS-PAGE phenotype scores, we first plot the position-averaged binned phenotype scores against one another (Figure 6A). A positive correlation is evident and linear least-squares fitting gives $R^2 = 0.68$ (see Table 2), but the distribution of data points is clearly non-linear. Rank order correlation analysis of the fifteen pairs of position-averaged scores gives a Spearman correlation coefficient $R_S = 0.82$ and a significance $P < 10^{-4}$; we conclude that position-averaged TOXCAT and SDS-PAGE scores are strongly correlated.

TOXCAT is insensitive to mild disruptions but distinguishes between strongly disruptive mutants

To evaluate correlations and rank order relationships of pairs of TOXCAT and SDS-PAGE phenotype scores for individual mutants, we bin each of the 99 mutants common to both data sets in a contingency table according to their TOXCAT and SDS-PAGE phenotype score pairs (Figure 6B). For linearly correlated variables, all data pairs would lie in one of the shaded diagonal elements; random scatter would cause some to lie in off-diagonal elements. But the result is neither a simple correlation nor a scatter plot; rather, 93 of 99 mutants lie in the eleven squares that comprise the left or top edges of the table, and the remaining six mutants are within one square of the edge(s). The central four diagonal elements contain no data pairs at all;

instead, all but two mutants lie on or well above the diagonal. Among the 99 pairs of scores, 66 pairs have greater SDS-PAGE scores than TOXCAT scores, 31 pairs have equal scores for the two tests, and for only two pairs the TOXCAT score is greater than the SDS-PAGE score. We assessed the significance of this correlation using the Wilcoxon signed-rank test; the null hypothesis that the two tests give the same mean score is strongly rejected ($P < 1.8 \cdot 10^{-12}$). We conclude that the TOXCAT score for a mutant is usually significantly lower than the SDS-PAGE score for that same mutant.

Of the 40 mutants that have non-zero TOXCAT scores, 34 have SDS-PAGE scores of '5' and four have SDS-PAGE scores of '4'. Except for two mutants with TOXCAT scores of '1' and SDS-PAGE scores of '0', only mutants that strongly disrupt dimerization in SDS-PAGE have any effect on association as measured by TOXCAT. However, mutations that are very strongly disruptive in SDS-PAGE show the full range of TOXCAT scores from '0' to '5', indicating that the TOXCAT assay can further discriminate between mutants that lie at the end of the SDS-PAGE scoring scale. This observation is consistent with the hypothesis that the wild-type BNIP3 TMD is much more strongly associated under TOXCAT conditions than during SDS-PAGE: the proportion of dimer and monomer in the SDS-PAGE assay is poised such that even slightly disruptive mutations affect the observed bands, whereas the dimerization and binding of the ToxR-BNIP3-MBP fusion protein to the ctx promoter (Figure 1) is driven strongly to the right, and changes that only modestly decrease fusion protein self-association have little effect on transcription. The SDS-PAGE assay does not distinguish between mutations that very strongly disrupt the BNIP3 dimer because such constructs all give essentially zero dimer at the buffer conditions and protein concentrations used, but the significant association of these mutants in the TOXCAT assay allows them to be ranked relative to one another.

A unified score improves discrimination between mutant phenotypes

Given that the two assays are sensitive to differences in dimerization affinity in different ranges, we decided to combine the two scores to yield a single scale with increased ability to discriminate the rank order of mutants. Simply adding the TOXCAT and SDS-PAGE scores of each mutant generates an integer range from zero to 10 that can be used to rank the mutations from least to most disruptive, and the position-averaged unified score identifies the same seven rank-ordered residues as critical to dimerization as the position-averaged TOXCAT data. Note that for the 93 mutants that lie in the leftmost or topmost bins of Figure 6B, the new unified scale simply corresponds to numbering the bins from zero to 5 going up the left column, and then numbering from 6 to 10 along the top row to the right. The original TOXCAT and SDS-PAGE scores can be readily extracted from the unified scale: if the unified score is over 5, then the TOXCAT score is calculated as (unified – 5) and the calculated SDS-PAGE score is 5; if the unified score is 5 or less, the calculated TOXCAT score is 0 and the calculated SDS-PAGE score is just the unified score. For 93 of the 99 mutants, this calculation exactly reproduces the original scores; for six mutants, the extracted scores differ by just 1 from the original experimental scores. As a result, experimental and extracted scores are very highly correlated ($R^2 = 0.98$ for TOXCAT and $R^2 = 0.99$ for SDS-PAGE), indicating that little of the information present in the original scales is lost in creating the unified scale. We therefore use the unified score as a single parameter that incorporates the discriminating power of both experimental scales to describe the impact of mutations on BNIP3 TMD dimerization (Figure 7). We conclude that the TOXCAT and SDS-PAGE methods are reporting on different aspects of the same self-association phenomenon and that the unified scale provides a more comprehensive ranking than either primary scale.

Discussion

Our mutational analysis of BNIP3 TMD dimerization with the biological assay TOXCAT allows us to map the native dimer interface by using the degree to which hydrophobic substitutions disrupt dimerization to infer the importance of those positions to self-association. In rank order of importance, the residues that contribute to dimerization in membranes are: Gly 180 > Gly 184 > His 173 > Ser 172 > Ala 176 > Ile 183 > Ile 181. Both the position-averaged TOXCAT data (Figure 4C) and the effects of individual mutations (Figure 4A) show that Gly 180 and Gly 184 are critical to BNIP3 dimerization, and that polar residues Ser 172 and His 173 contribute significantly to dimerization but are not essential. Eleven non-proline mutants that eliminate either Ser 172 or His 173 have an average TOXCAT score of 2.1 (range: 0 to 4), indicating that the BNIP3 GxxxG motif and apolar sequence context still support significant self-association in the absence of intermonomer side chain hydrogen bonding. Mutants such as G180A or G184L, which completely fail to dimerize in TOXCAT despite retaining both Ser 172 and His 173, show that the ability of the polar side chains to stabilize the BNIP3 dimer depends upon the context of the geometry conferred by the GxxxG motif. In the BNIP3 TMD dimer structure²⁴, Gly 180 is the point of closest approach of the two helix backbones and the Gly 180 H α 2 forms a C α -H · O=C hydrogen bond with Ile 177. Mutations at the GxxxG should sterically prevent the precise helix orientation that supports wild-type Ser 172 - His 173 intermonomer side chain hydrogen bonding geometry, but the ability of strongly polar residues to drive association of hydrophobic helices that lack motifs of small residues 15–18 suggests that alternate favorable interaction modes could be available. The complete loss of TOXCAT dimerization signal for mutants such as G180A shows that no alternative helix-helix interaction geometry supports formation of a stable dimer interface, despite the presence of the strongly polar histidine side chain. By establishing that intermonomer side chain hydrogen bonding is contingent upon the GxxxG motif, these results show that sequence context can confer considerable specificity on hydrogen bonding interactions in membranes.

Biochemical and biophysical approaches that use detergent micelles to solubilize membrane proteins are subject to the caveat that detergent may alter the protein structure compared to what would be found in a membrane. One motivation for our extensive TOXCAT mutational analysis was to obtain data that report on BNIP3 helix-helix interactions under native physiological conditions and to use these findings to assess the validity of our NMR structure in detergent micelles. The excellent agreement between the position-averaged TOXCAT mutational data and the interfacial contacts observed in our BNIP3 TMD dimer NMR structure from dodecylphosphocholine micelles²⁴ (Figure 4C) indicates that the native structure in membranes is retained in our detergent-solubilized NMR samples. Because our previous mutational analysis of BNIP3 dimerization using SDS-PAGE²² identified the same interfacial positions as TOXCAT and the NMR structure, it seems that even the harsh detergent SDS has little effect on the native structure of the BNIP3 TMD dimer. These findings validate our solution NMR structure²⁴ and show that biochemical and biophysical experiments on the BNIP3 TMD that use detergent micelles are likely to report on biologically relevant conformations of the protein.

Although mutational analysis in either TOXCAT or SDS-PAGE yields similar conclusions about the identities of BNIP3 interfacial residues, the degree to which mutations affect dimerization as assessed by the two methods differs sharply. We analyzed our previous mutagenesis data in the context of our NMR structure of the wild-type BNIP3 dimer and showed that almost all mutations that disrupt dimerization on SDS-PAGE would cause steric clashes, loss of favorable packing, or loss of intermonomer hydrogen bonds²⁴. Despite the demonstrated physical basis for the effects of these mutations, only those mutations that are most disruptive in SDS-PAGE have any measurable effect upon the TOXCAT dimerization signal (Figure 4). The lack of any effect on TOXCAT signals for 33 hydrophobic interfacial

mutants that decrease dimerization in SDS-PAGE leads us to conclude that the wild-type BNIP3 TMD drives the TOXCAT assay to saturation and makes the system insensitive to all but the most severely disruptive mutations. The methods therefore provide complementary information: TOXCAT scores extend the SDS-PAGE scale at its more disruptive end by distinguishing among the 43 mutants that show no dimer on gels, and the SDS-PAGE scores extend the TOXCAT scale at its more associated end by distinguishing among the 58 mutants that do not impact CAT activity (see Figure 6). The unified scale that results from adding the two scores together captures the information in each original score and allows us to rank order wild-type BNIP3 and its mutants into an experimental scale of dimerization propensity with eleven classes (Figure 7). We note that the ability of TOXCAT to discriminate among the ‘completely’ disruptive mutants identified by SDS-PAGE is critical to dissecting the relative importance of the GxxxG motif and side chain intermonomer hydrogen bonding to dimerization; in our previous analysis using SDS-PAGE ²², essentially all mutations at Ser 172, His 173, Gly 180, or Gly 184 completely abolished dimerization (Figure 4).

In our TOXCAT work, we use the well characterized glycophorin A (GpA) TMD ^{25–30} as a positive control for dimerization. Wild-type BNIP3 gives about twice the TOXCAT signal of wild-type GpA, but the compression of the TOXCAT scale for strongly associating sequences suggests that BNIP3 dimerizes much more strongly than GpA in *E. coli* membranes. This can be appreciated by comparing the TOXCAT scores of BNIP3 mutants and GpA wild-type. The average wild-type GpA TOXCAT signal lies at the disruptive end of BNIP3 mutant class ‘2’, with individual GpA cultures occasionally scoring as class ‘3’. Only His 173, Gly 180, and Gly 184 have average TOXCAT scores that are more disruptive than ‘2’ (Figure 4C), and the four non-proline BNIP3 mutants that have TOXCAT scores of ‘2’ are all at key interfacial residues (S172I, S172V, H173F, and G184V). Because even BNIP3 TMDs with disruptive mutations at critical interfacial positions still associate about as tightly as GpA in TOXCAT, we conclude that wild-type BNIP3 must dimerize much more strongly than GpA in membranes. On the other hand, each of these BNIP3 class ‘2’ TOXCAT mutants is completely monomeric on SDS-PAGE ²², whereas the wild-type GpA TMD dimerizes strongly on SDS-PAGE ^{27–31}. What is the source of this disparity?

The relative ease with which mutations abolish dimerization of the BNIP3 TMD under conditions of SDS-PAGE can be explained by unfolding of the BNIP3 TMD in SDS. We previously showed that the tendency of non-interfacial mutations to disrupt BNIP3 TMD dimerization in SDS-PAGE correlates with loss of hydrophobicity ²², and we used FTIR and hydrogen/deuterium exchange experiments in SDS micelles to support our contention that the predominantly helical species in our samples are in exchange with a partially unfolded monomer that partitions to the surface of the SDS micelle ²². Unfolding according to equilibrium constant K_U (Figure 8) disfavors dimerization by depleting the folded monomer available to assemble into folded dimers. The simple dimer-monomer equilibrium is described by the dissociation constant K_D , but when monomer unfolding is included the apparent dimerization dissociation constant becomes $K_D(1 + K_U)^2$; for sequences with $K_U > 1$, the apparent K_D is greatly increased. We propose that the disparity in the observed dimerization tendencies of BNIP3 and GpA on SDS-PAGE (compared to TOXCAT) arises from differences in monomer unfolding at the surface of SDS micelles caused by the strongly polar residue His 173 in the BNIP3 TMD. An internal histidine strongly opposes translocon-mediated integration of otherwise hydrophobic helices into membranes ³², disfavors transbilayer topography when located in the middle of a hydrophobic sequence ³³, and alters the transverse position of a hydrophobic sequence when located near one end of a membrane span ³⁴. Transferring a charged histidine into a hydrophobic environment is highly unfavorable ³⁵, and the net negative charge of the SDS micelle should stabilize a protonated His side chain at the micelle surface, making it very energetically costly to deprotonate the histidine and bury it in the apolar interior of the micelle. We conclude that the much stronger TOXCAT dimerization signal for BNIP3

compared to GpA reflects the true relative dimerization propensities of the TMDs, and that whereas SDS significantly decreases BNIP3 TMD dimerization by monomer unfolding, SDS minimally affects GpA TMD dimerization, resulting in similar degrees of self-association for these two systems on SDS-PAGE despite very different intrinsic dimerization propensities.

The idea that detergent-resistant dimerization of a largely hydrophobic transmembrane helix can be influenced by unfolding is also supported by mutational data for both GpA and BNIP3. Replacing non-interfacial GpA hydrophobic residues with strongly polar residues is invariably disruptive to dimerization on SDS-PAGE ³¹, perhaps because the side chain hydrogen bond donors and acceptors would be unsatisfied in the interior of the micelle and therefore induce unfolding. Such mutants often exhibit altered migration of the monomer band ³¹, suggesting a substantially altered interaction between the TMD and the SDS micelle that is consistent with local unfolding or kinking and exposure of the strongly polar residue and its exposure at the micelle surface. Non-interfacial GpA mutations to hydrophobic but polar tyrosine have no effect on dimerization in TOXCAT but are disruptive on SDS-PAGE, whereas mutations to completely apolar phenylalanine at the same non-interfacial positions are non-disruptive in both assays ²⁵. Proline mutations at any position from Ser 172 to Gly 184 completely disrupt BNIP3 dimerization on SDS-PAGE ²², and proline substitutions alter the migration of the BNIP3 monomer, in some cases giving rise to two distinct monomer bands (ESS, Ph.D. thesis). Proline mutations almost always disrupt GpA dimerization on SDS-PAGE and cause altered monomer migration ³¹, although one interfacial and one non-interfacial proline substitution within the GpA TMD do support near-wild-type dimerization in SDS-PAGE ³⁶. A role for proline in unfolding is readily explained: like histidine, proline strongly opposes both TMD integration by the translocon ³² and transverse topography of hydrophobic peptides in membranes ³⁴, and partitioning a proline-containing TMD into a micelle interior would force at least one unsatisfied backbone hydrogen bond acceptor into a hydrophobic environment.

Physiologically important TMD oligomerization occurs within membranes, but many analyses of biological TMD interactions are made using detergent-solubilized proteins, and the relevance of these studies to behavior in membranes can be difficult to establish experimentally. We previously used TOXCAT to show that the effects of hydrophobic interfacial mutations on GpA TMD dimerization in membranes are consistent with qualitative mutational data from SDS-PAGE and with quantitative data from analytical ultracentrifugation ²⁶. Here, we show that the interface identified by TOXCAT agrees with our NMR structure from dodecylphosphocholine micelles and that the rank order of mutations that disrupt dimerization in biological membranes agrees with the rank order from SDS-PAGE, even though the SDS-PAGE data are strongly affected by monomer unfolding. Our findings suggest that detergent-based studies of TMD-TMD interactions that include strongly polar residues will need to account for unfolding to explain quantitative differences between association in detergents and in membranes, and that increasing the number of prolines or strongly polar residues in a TMD will likely cause artifactually enhanced disruption in detergents. If the effects on the unfolded state do not vary strongly with sequence (and they probably do not for BNIP3 given the similarities between the conclusions based on hydrophobic mutations in SDS-PAGE and TOXCAT), comparisons of interaction propensities of wild-type and most mutant TMD sequences in detergents should accurately reflect relative stabilities in membranes. However, comparing the self-association propensities of two entirely different sequences in detergents may be misleading, since differences in the extent of detergent-induced unfolding for two unrelated sequences may be quite large.

Materials and Methods

Cloning

BNIP3 TMD deletion mutants were generated by PCR to contain appropriate Xba I and Bam HI ends, cut, and inserted in-frame into the Nhe I and Bam HI sites of the pccKAN vector²⁵. DNA for most BNIP3 point mutants was generated by PCR amplification of previously published point mutants in the SNase fusion protein system²², cut with Xba I and Bam HI, and inserted in-frame into the Nhe I and Bam HI sites of the pccKAN vector²⁵. For sites near the ends of the TMD, mutations were generated using the QuickChange mutagenesis kit (Stratagene). All sequences were determined using automated dideoxynucleotide sequencing.

TOXCAT methods

Cultures and controls were performed as previously described^{11, 21}, using 3.0 OD₄₂₀ of NT326 cells in late log phase. All constructs conferred the ability to grow on maltose plates to the *malE* strain NT326, which indicates that proper membrane insertion of the ToxR-TMD-MBP fusion protein has occurred²⁵, and all constructs show similar expression levels of ToxR-TMD-MBP fusion protein as determined by western blot using an anti-MBP antibody. On a typical day, six to twelve constructs carrying mutant BNIP3 TMDs were grown, harvested, and processed, with wild-type BNIP3 (or wild-type NΔ6-BNIP3), wild-type GpA, and GpA G83I controls processed in parallel. Data in Figure 2 are presented as raw CAT activity scores, and error bars represent the standard deviation from three independent activity assays of the same culture. Data for each mutant in the first 8 panels of Figure 3 represent at least three independent cultures; CAT activities are scaled (relative to wild-type NΔ6-BNIP3 processed on the same day) prior to being averaged, and error bars reflect the culture-to-culture reproducibility.

Acknowledgments

The authors thank members of the MacKenzie and Shamoo laboratories for helpful suggestions, and Drs. R.B. Hill and J.S. Olson for critical comments on the manuscript. This work was supported by NIH grant R01 GM067850.

Abbreviations

SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
TMD	transmembrane domain
GpA	glycophorin A
CAT	chloramphenicol acetyltransferase
MBP	maltose-binding protein

References

1. Moore DT, Berger BW, Degrado WF. Protein-protein interactions in the membrane: sequence, structural, and biological motifs. *Structure* (London, England) 2008;16:991–1001.
2. Li E, Hristova K. Role of receptor tyrosine kinase transmembrane domains in cell signaling and human pathologies. *Biochemistry* 2006;45:6241–51. [PubMed: 16700535]
3. Yi JY, Han I, Oh ES. Transmembrane domain-dependent functional oligomerization of syndecans. *The Scientific World Journal* 2006;6:457–9.
4. Li R, Bennett JS, Degrado WF. Structural basis for integrin alphaIIb beta3 clustering. *Biochem Soc Trans* 2004;32:412–5. [PubMed: 15157149]
5. Yin H, Slusky JS, Berger BW, Walters RS, Vilaire G, Litvinov RI, Lear JD, Caputo GA, Bennett JS, DeGrado WF. Computational design of peptides that target transmembrane helices. *Science* 2007;315:1817–22. [PubMed: 17395823]

6. MacKenzie KR. Folding and Stability of alpha-Helical Integral Membrane Proteins. *Chem Rev* 2006;106:1931–77. [PubMed: 16683762]
7. Senes A, Engel DE, DeGrado WF. Folding of helical membrane proteins: the role of polar, GxxxG-like and proline motifs. *Curr Opin Struct Biol* 2004;14:465–79. [PubMed: 15313242]
8. MacKenzie KR, Fleming KG. Association energetics of membrane spanning alpha-helices. *Curr Opin Struct Biol*. 2008
9. Senes A, Gerstein M, Engelman DM. Statistical analysis of amino acid patterns in transmembrane helices: the GxxxG motif occurs frequently and in association with beta-branched residues at neighboring positions. *J Mol Biol* 2000;296:921–36. [PubMed: 10677292]
10. MacKenzie KR, Prestegard JH, Engelman DM. A transmembrane helix dimer: structure and implications. *Science* 1997;276:131–3. [PubMed: 9082985]
11. Dews IC, MacKenzie KR. Transmembrane domains of the syndecan family of growth factor coreceptors display a hierarchy of homotypic and heterotypic interactions. *Proc Natl Acad Sci U S A* 2007;104:20782–7. [PubMed: 18093920]
12. Doura AK, Kobus FJ, Dubrovsky L, Hibbard E, Fleming KG. Sequence context modulates the stability of a GxxxG-mediated transmembrane helix-helix dimer. *J Mol Biol* 2004;341:991–8. [PubMed: 15289100]
13. MacKenzie KR, Engelman DM. Structure-based prediction of the stability of transmembrane helix-helix interactions: the sequence dependence of glycophorin A dimerization. *Proc Natl Acad Sci U S A* 1998;95:3583–90. [PubMed: 9520409]
14. Doura AK, Fleming KG. Complex interactions at the helix-helix interface stabilize the glycophorin A transmembrane dimer. *J Mol Biol* 2004;343:1487–97. [PubMed: 15491626]
15. Gratkowski H, Lear JD, DeGrado WF. Polar side chains drive the association of model transmembrane peptides. *Proc Natl Acad Sci U S A* 2001;98:880–5. [PubMed: 11158564]
16. Choma C, Gratkowski H, Lear JD, DeGrado WF. Asparagine-mediated self-association of a model transmembrane helix. *Nat Struct Biol* 2000;7:161–6. [PubMed: 10655620]
17. Zhou FX, Merianos HJ, Brunger AT, Engelman DM. Polar residues drive association of polyleucine transmembrane helices. *Proc Natl Acad Sci U S A* 2001;98:2250–5. [PubMed: 11226225]
18. Zhou FX, Cocco MJ, Russ WP, Brunger AT, Engelman DM. Interhelical hydrogen bonding drives strong interactions in membrane proteins. *Nat Struct Biol* 2000;7:154–60. [PubMed: 10655619]
19. Lear JD, Gratkowski H, Adamian L, Liang J, DeGrado WF. Position-dependence of stabilizing polar interactions of asparagine in transmembrane helical bundles. *Biochemistry* 2003;42:6400–7. [PubMed: 12767221]
20. Joh NH, Min A, Faham S, Whitelegge JP, Yang D, Woods VL, Bowie JU. Modest stabilization by most hydrogen-bonded side-chain interactions in membrane proteins. *Nature* 2008;453:1266–70. [PubMed: 18500332]
21. Sulistijo ES, Jaszewski TM, MacKenzie KR. Sequence-specific dimerization of the transmembrane domain of the “BH3-only” protein BNIP3 in membranes and detergent. *J Biol Chem* 2003;278:51950–6. [PubMed: 14532263]
22. Sulistijo ES, MacKenzie KR. Sequence Dependence of BNIP3 Transmembrane Domain Dimerization Implicates Side-chain Hydrogen Bonding and a Tandem GxxxG Motif in Specific Helix-Helix Interactions. *J Mol Biol* 2006;364:974–90. [PubMed: 17049556]
23. Bocharov EV, Pustovalova YE, Pavlov KV, Volynsky PE, Goncharuk MV, Ermolyuk YS, Karpunin DV, Schulga AA, Kirpichnikov MP, Efremov RG, Maslennikov IV, Arseniev AS. Unique dimeric structure of BNIP3 transmembrane domain suggests membrane permeabilization as a cell death trigger. *J Biol Chem* 2007;282:16256–66. [PubMed: 17412696]
24. Sulistijo ES, MacKenzie KR. Structural basis for dimerization of the BNIP3 transmembrane domain. *Biochemistry* 2009;48:5106–20. [PubMed: 19415897]
25. Russ WP, Engelman DM. TOXCAT: a measure of transmembrane helix association in a biological membrane. *Proc Natl Acad Sci U S A* 1999;96:863–8. [PubMed: 9927659]
26. Duong MT, Jaszewski TM, Fleming KG, MacKenzie KR. Changes in apparent free energy of helix-helix dimerization in a biological membrane due to point mutations. *J Mol Biol* 2007;371:422–34. [PubMed: 17570394]

27. Lemmon MA, Flanagan JM, Treutlein HR, Zhang J, Engelman DM. Sequence specificity in the dimerization of transmembrane alpha-helices. *Biochemistry* 1992;31:12719–25. [PubMed: 1463743]
28. Lemmon MA, Treutlein HR, Adams PD, Brunger AT, Engelman DM. A dimerization motif for transmembrane alpha-helices. *Nat Struct Biol* 1994;1:157–63. [PubMed: 7656033]
29. Brosig B, Langosch D. The dimerization motif of the glycoporphin A transmembrane segment in membranes: importance of glycine residues. *Protein Sci* 1998;7:1052–6. [PubMed: 9568912]
30. Langosch D, Brosig B, Kolmar H, Fritz HJ. Dimerisation of the glycoporphin A transmembrane segment in membranes probed with the ToxR transcription activator. *J Mol Biol* 1996;263:525–30. [PubMed: 8918935]
31. Lemmon MA, Flanagan JM, Hunt JF, Adair BD, Bormann BJ, Dempsey CE, Engelman DM. Glycophorin A dimerization is driven by specific interactions between transmembrane alpha-helices. *J Biol Chem* 1992;267:7683–9. [PubMed: 1560003]
32. Hessa T, Kim H, Bihlmaier K, Lundin C, Boekel J, Andersson H, Nilsson I, White SH, von Heijne G. Recognition of transmembrane helices by the endoplasmic reticulum translocon. *Nature* 2005;433:377–81. [PubMed: 15674282]
33. Caputo GA, London E. Cumulative effects of amino acid substitutions and hydrophobic mismatch upon the transmembrane stability and conformation of hydrophobic alpha-helices. *Biochemistry* 2003;42:3275–85. [PubMed: 12641459]
34. Krishnakumar SS, London E. The control of transmembrane helix transverse position in membranes by hydrophilic residues. *J Mol Biol* 2007;374:1251–69. [PubMed: 17997412]
35. Wimley WC, Creamer TP, White SH. Solvation energies of amino acid side chains and backbone in a family of host-guest pentapeptides. *Biochemistry* 1996;35:5109–24. [PubMed: 8611495]
36. Orzaez M, Salgado J, Gimenez-Giner A, Perez-Paya E, Mingarro I. Influence of proline residues in transmembrane helix packing. *J Mol Biol* 2004;335:631–40. [PubMed: 14672669]

Figure 1. Schematic of the TOXCAT assay for helix-helix interactions in membranes

The TOXCAT fusion protein is expressed in the inner membrane of *E. coli*, with its DNA-binding ToxR domain (diamond) in the cytoplasm and the maltose binding protein domain (circle) in the periplasm. Association of the TMDs (cylinders) generates a ToxR domain dimer that can bind to the cholera toxin promoter (*ctx*) and drive transcription of the reporter gene chloramphenicol acetyltransferase (CAT).

Figure 2. Phenylalanine scanning mutagenesis of TOXCAT constructs bearing the BNIP3 TMD and deletion variants

A. Sequences of the transmembrane regions of the ToxR-TMD-MBP fusion construct and deletion variants. Residues from the vector cloning sites are offset and in smaller font. **B.** Raw activity data from single TOXCAT assays are shown for the positive and negative controls (GpA and GpA G83I, dark gray), the full-length BNIP3 TMD (white), and phenylalanine point mutations at residues 172 through 184 (light gray). The dotted horizontal line indicates the TOXCAT value for the wild-type construct. Panels **C** through **E** show the controls and full-length BNIP3 TMD for comparison, as well as the indicated deletion variants of the BNIP3 TMD and the corresponding scanning phenylalanine point mutation sets. Each panel contains

a single experiment completed on the same day (triplicate CAT activity measurements from a single culture preparation $\pm$ standard deviation) to show the precision of the method. When replicated for three independent cultures, the averaged results do not differ significantly from the single trials shown here.

Figure 3. Effects of 101 single point mutations on BNIP3 TMD TOXCAT signals

Averages of three independent TOXCAT assays are presented for 101 single point mutations, expressed as a percentage of the TOXCAT score for the wild-type construct (NΔ6-BNIP3). Data within a given panel correspond to constructs in which successive positions have been mutated to the same residue (F, L, I, V, A, G, S, or P) as given in the top right-hand corner of the panel. Error bars indicate the standard deviation for three independent cultures of each mutant, grown, harvested, and assayed in parallel with a wild-type culture to which it has been scaled. Dotted horizontal lines at 90%, 75%, 50%, 25%, and 10% of wild-type indicate the boundaries used to bin the mutants into phenotype classes (see text). The bottom right-hand panel shows the average score for each position (excluding scores for mutations to P), and the standard deviation of that score across the available substitutions at that position.

Figure 4. TOXCAT and SDS-PAGE mutagenesis identify the residues at the NMR structure dimer interface

A. Effects of mutations on BNIP3 dimerization in TOXCAT, with each mutant represented by a symbol for the phenotype bin to which it was assigned (see key). **B.** Effects of mutations on BNIP3 dimerization on SDS-PAGE as previously reported²², with each mutant represented by a symbol for the bin to which it was assigned (see key). **C.** Comparison of position-averaged effects of mutations in TOXCAT and SDS-PAGE with residue intermonomer packing. The average TOXCAT bin scores (excluding prolines) at each position along the BNIP3 TMD are shown as diamonds and solid lines, and the average SDS-PAGE bin scores for the same mutations are shown as squares and dashed lines. The number of intermonomer contacts that each residue makes with the opposing monomer in PDB ID 2KA2 (see text and ref 24) are shown as open triangles and dotted lines.

Figure 5. Comparison of intermonomer packing with TOXCAT or SDS-PAGE scores
Plots of the number of intermonomer contacts per residue in the BNIP3 TMD dimer (PDB ID 2ka2) versus (A) the average TOXCAT phenotype score as reported in Figure 4 and (B) the average SDS-PAGE phenotype score as reported in Figure 4. Both position score averages exclude substitutions to proline.

Figure 6. TOXCAT and SDS-PAGE scores for effects of mutations on dimerization are correlated
A. Position-averaged SDS-PAGE scores correlate with position-averaged TOXCAT scores, although not in a linear fashion. Averages are for mutants that appear in both panel A and B of Figure 4, excluding prolines. A linear least-squares fit gives $R^2 = 0.684$, although the data deviate systematically from linearity. The Spearman rank order correlation is $R_S = 0.821$ for a probability $P < 10^{-4}$. **B.** Contingency table for mutants measured using both TOXCAT and SDS. Each of the 99 mutants that appear in both panel A and B of Figure 4 gives rise to a dimerization score data pair (SDS-PAGE, TOXCAT); the number of occurrences of each of the 36 pairs is tabulated.

Figure 7. Unified mutagenesis score maps the BNIP3 TMD dimer interface

Mutant unified phenotypes are portrayed for each of 99 mutants tested in both TOXCAT and SDS-PAGE using circles ranging from white to black, with darker colors representing more disruptive phenotypes. The unified mutagenesis phenotype score for each mutant, which is the sum of the TOXCAT and SDS-PAGE phenotype scores for each mutant from Figure 4, is indicated inside the circle; for mutants that do not disrupt at all, the '0' is not shown. Wild-type residues are indicated by a white square. The number of mutants that are binned into each of the eleven unified phenotype scores are indicated below each symbol in the key.

Figure 8. Equilibrium models for dimer dissociation in detergents

Dissociation of a detergent-solubilized dimer into two helical monomers described by the dissociation constant K_D can be influenced by the partial unfolding or kinking of each monomer with the unfolding constant K_U . In the absence of unfolding, the fraction of dimer (Y_D) is a function of K_D and the total protein concentration C_T :

$$Y_D = \frac{4C_T + K_D \pm \sqrt{K_D^2 + 8C_T K_D}}{4C_T}; Y_D = 0.5 \text{ when } C_T = K_D.$$

Unfolding causes the fraction of dimer to be a function of K_D , C_T , and K_U :

$$Y_D = \frac{4C_T + K_D(1+K_U)^2 \pm \sqrt{K_D^2(1+K_U)^4 + 8C_T K_D(1+K_U)^2}}{4C_T}; Y_D = 0.5 \text{ when } C_T = K_D(1+K_U)^2.$$

Table 1
Phenotype score distributions for 99 BNIP3 mutants tested in both TOXCAT and SDS-PAGE.

assay	0	1	2	3	4	5	total
TOX	59	8	6	14	5	7	99
SDS	26	6	8	7	9	43	99
χ^2	12.8	0.29	0.29	2.33	1.14	25.9	42.8

Chi-squared values for each score pair are calculated as: $\chi^2 = (\text{TOX}_i - \text{SDS}_i)^2 / (\text{TOX}_i + \text{SDS}_i)$, $P \sim 1.3 \cdot 10^{-7}$, the probability of randomly choosing the SDS-PAGE ratio of scores from a parental distribution with the TOXCAT ratio of scores, is calculated from total χ^2 using the incomplete gamma function and 6 degrees of freedom (the number of bins in the phenotype distributions).

Table 2
Statistical correlations among position-averaged mutagenesis scores and residue intermonomer packing

position score	excluded positions	Spearman rank order		correlation
		R_s	P	
TOX pack	None	0.701	$1.8 \cdot 10^{-3}$	0.572
	177, 181	0.937	$1.2 \cdot 10^{-6}$	0.896
SDS pack	None	0.763	$9.3 \cdot 10^{-4}$	0.391
	177, 181	0.879	$3.8 \cdot 10^{-5}$	0.502
TOX SDS	None	0.821	$8.7 \cdot 10^{-5}$	0.684
	177, 181	0.812	$3.8 \cdot 10^{-4}$	0.704

One-tailed P values are calculated from values for Student's t test returned by Spearman analysis and 13 degrees of freedom (11 if residues 177 and 181 are excluded). P values represent the random chance of selecting a set of the same size with the same R_s (or higher) from an uncorrelated distribution.