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Alternate Interfaces May Mediate Homomeric and Heteromeric Assembly in the Transmembrane Domains of SNARE Proteins

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The fusion of a vesicle to a target membrane is mediated by temporally and spatially regulated interactions within a set of evolutionarily conserved proteins. Integral to proper fusion is the interaction between proteins originating on both vesicle and target membranes to form a protein bridge between the two membranes, known as the SNARE complex. This protein complex includes the single-pass transmembrane helix proteins: syntaxin and synaptobrevin. Experimental data and amino acid sequence analysis suggest that an interface of interaction is conserved between the transmembrane regions of the two proteins. However, conflicting reports have been presented on the role of the synaptobrevin transmembrane domain in mediating important protein–protein interactions. To address this question, a thermodynamic study was carried out to determine quantitatively the self-association propensities of the transmembrane domains of synaptobrevin and syntaxin. Our results show that the transmembrane domain of synaptobrevin has only a modest ability to self-associate, whereas the transmembrane domain of syntaxin is able to form stable homodimers. Nevertheless, by a single amino acid substitution, synaptobrevin can be driven to dimerize with the same affinity as syntaxin. Furthermore, crosslinking studies show that dimerization of synaptobrevin is promoted by oxidizing agents. Despite the presence of a conserved cysteine residue in the same location as in synaptobrevin, syntaxin dimerization is not promoted by oxidization. This analysis suggests that subtle yet distinct differences are present between the two transmembrane dimer interfaces. A syntaxin/synaptobrevin heterodimer is able to form under oxidizing conditions, and we propose that the interface of interaction for the heterodimer may resemble the homodimer interface formed by the synaptobrevin transmembrane domain. Computational analysis of the transmembrane sequences of syntaxin and synaptobrevin reveal structural models that correlate with the experimental data. These data may provide insight into the role of transmembrane segments in the mechanism of vesicle fusion.

Keywords: SNARE; membrane protein; vesicle fusion; thermodynamics; disulfide crosslinking

Introduction

The fusion of a vesicle to a target membrane is essential to many biological functions in eukaryotes. The mechanism of fusion has been dissected into distinct and essential stages. One essential stage is the bridging of the target and vesicle membranes to form a single membrane, which requires a set of proteins termed the soluble NSF attachment protein receptors (SNARE) complex.[1] The SNARE complex includes SNAP25 and two single-pass transmembrane proteins: syntaxin, which is located on the target membrane (t-SNARE), and synaptobrevin, which is located on the vesicle membrane (v-SNARE).[2] The crystal structure and electron microscopy data demonstrate

Abbreviations used: GpA, glycophorin A; AUC, analytical ultracentrifugation; SNARE, soluble NSF attachment protein receptors; TM, transmembrane domain; CuOP, copper phenanthroline; Syx, syntaxin; Syb, synaptobrevin; SN, staphylococcal nuclease.

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that the SNARE complex is a parallel helical bundle with the transmembrane segments emerging as tails inserted into the two merging membranes.[3,4] Evidence suggests that the SNARE complex creates a protein bridge that draws the two membranes together and overcomes repulsive forces to allow membrane mixing.[5] A *cis* to *trans* transition occurs in the complex when the membranes merge, and the transmembrane segments emerge inserted into the same membrane.[6] The soluble domain complex has enormous stability and the formation of this complex could overcome the energetic barrier to fusion.[7] However, the stability of the interactions of the transmembrane domains has not been quantified, and these segments may provide additional energy and mediate interactions required for the mechanism of intracellular vesicle fusion.

These transmembrane segments may act as more than just membrane anchors. They could be integral to membrane fusion through a direct role in bilayer mixing and by promotion of protein complex formation and oligomerization.[1] Synthetic peptides corresponding to the transmembrane domains of syntaxin and synaptobrevin have been shown to drive fusion *in vitro*, in the absence of their respective cytoplasmic domains.[8] This fusiogenic activity may involve sequence-specific interactions between the SNARE protein transmembrane domains. Biochemical studies suggest that the TM segments of syntaxin and synaptobrevin promote the formation of the SNARE complex,[9] and that sequence-specific interactions occur between these transmembrane domains.[10,11] In addition, mutations in the transmembrane domains of syntaxin and synaptobrevin have been shown to lead to impaired neurotransmission in *Caenorhabditis elegans*.[12,13] Further study into the nature of interactions between transmembrane segments will be necessary to better understand the mechanism of vesicle fusion.

Previous biochemical studies have provided insight into the specificity of interactions for both homo and hetero-oligomerization of the syntaxin and synaptobrevin TMs. SDS–PAGE analysis of full-length synaptobrevin mutants led to a possible motif of interaction for homodimerization.[14] Interestingly, this motif is highly conserved in the syntaxin TM, except for a single amino acid difference at the N terminus.[11] This sequence similarity suggested an analogous region of interaction for the syntaxin/synaptobrevin heterodimer and the syntaxin homodimer. This hypothesis is supported by evidence of heterodimerization of syntaxin and synaptobrevin using SDS–PAGE.[11] Homodimerization of syntaxin and synaptobrevin have also been measured using the ToxR assay *in vivo*.[11] The addition of the synaptobrevin and the proposed syntaxin interaction motif to a polyalanine helix supports dimerization in the ToxR assay, which leads to the conclusion that this interaction sequence promotes homodimerization in both proteins.

The ToxR and SDS–PAGE data provided strong evidence for interactions within the transmembrane regions of syntaxin and synaptobrevin. However, these studies have been subjects of controversy in the recent literature. Bowen and co-workers observed association of synaptobrevin using an assay similar to ToxR, the TOXCAT assay. These data showed that the dimerization signal given by the synaptobrevin TM is not significantly greater than their negative control, the glycophorin A mutant GpA-G83I.[15] In further contradiction to the previous studies, the authors also found that dimerization of synaptobrevin observed using SDS–PAGE was insignificant and dependent on the purification protocol. Furthermore, strong dimerization was found only with the substitution of a transmembrane residue by asparagine. The presence of a charged residue is unfavorable in the apolar membrane environment and has been shown to drive association of transmembrane helices.[16,17] In response, the authors of the SDS–PAGE and ToxR studies demonstrated again that the ToxR signal for synaptobrevin was substantially greater than their negative control, GpA-G83A, and that dimerization can be observed by SDS–PAGE in the presence of the crosslinking agents.[18] These authors demonstrated also that the construct used by Bowen and co-workers elicited lower response in ToxR than the construct that was used in the initial study. Although the significance of synaptobrevin dimerization is still unclear, it appears that dimerization propensity depends on the environment, experimental conditions, and exact sequence of the experimental constructs.

Here, we address the role of the transmembrane regions of syntaxin and synaptobrevin and their propensities for homo and hetero-dimerization using quantitative techniques to analyze oligomerization *in vitro*. Sedimentation equilibrium analytical ultracentrifugation (AUC) can be used to determine the free energy of association for a transmembrane protein in a detergent environment.[19] Using this technique, the fundamental measurement is the protein mass. The following properties can be determined definitively: the stoichiometries of any protein complexes; the association propensity; and the detergent concentration dependence on association. If the proteins fail to associate, sedimentation equilibrium will demonstrate the monomer molecular mass, as opposed to a qualitative decrease in dimerization signal as in the TOXCAT and ToxR assays. Sedimentation equilibrium has been used extensively to measure the free energy of association in a detergent environment for glycophorin A and mutants.[19–22] Since this method has been useful in understanding the sequence context of interactions in glycophorin A, it may bring clarity to the controversy surrounding the association propensities of the syntaxin and synaptobrevin transmembrane sequences.

In addition, the presence of conserved cysteine residues in the transmembrane domains permit the use of oxidizing conditions to observe weak oligomerization by SDS–PAGE. These crosslinking

studies provide insight into the permissible interface of interaction for syntaxin and synaptobrevin homodimers and the syntaxin/synaptobrevin heterodimer. Including these experimental constraints in computational modeling, it is possible to better understand the nature of the interaction in homodimers and heterodimers of syntaxin and synaptobrevin transmembrane domains. Our findings suggest strongly that sequence-specific interactions do occur in the transmembrane segments of syntaxin and synaptobrevin, albeit weakly when compared to the stability of the GpA TM. Although the wild-type synaptobrevin transmembrane sequence may have little ability to drive homodimerization, the sequence does encode a sterically permissible interface for association.

Results

Synaptobrevin and syntaxin transmembrane domains have surprisingly different propensities for association

Previous studies have shown that the transmembrane regions of syntaxin and synaptobrevin may be involved in homodimerization.[11] To address the ability of these regions to drive dimerization, appropriate fusion constructs containing the transmembrane domain of syntaxin and synaptobrevin were cloned. The sequences are shown in Table 1, in which the previously determined conserved motif of dimerization is highlighted. There is a single residue difference in the dimerization face in the two proteins: Leu99 in synaptobrevin, which is aligned to the Met267 in syntaxin. Substitutions were made at these positions to create the syntaxin interface in the synaptobrevin background, called SN–SybLM, and to create the synaptobrevin interface in the syntaxin background, called SN–SyxML. These constructs were analyzed by sedimentation equilibrium AUC, using well-developed protocols for SN–GpA.[19,23]

Table 1. Free energy of association for syntaxin and synaptobrevin constructs

Construct	Sequence	ΔG_x° (kcal mol^{-1})
SN–Syb	EPE– ^{98}ILGVICAIILIIIIVYFSTZ	Monomeric
SN–SybLM	EPE– ^{98}IMGVICAIILIIIIVYFSTZ	-3.3 ± 0.2
SN–Syx	EPE–^{266}IMIIICCVILGIIIASLLIZ	-3.5 ± 0.3
SN–SyxML	EPE–^{266}ILIIICCVILGIIIASLLIZ	-3.2 ± 0.1

The fusion proteins were cloned with staphylococcal nuclease N-terminal to the indicated transmembrane sequence. The previously determined dimerization interface for synaptobrevin is highlighted.[14] This sequence is highly conserved in the syntaxin transmembrane region. The experimental free energies were measured by sedimentation equilibrium analytical ultracentrifugation. There was no detectable dimer for the SN–Syb construct, which is listed as monomeric. The error is the standard deviation of at least three independent experiments.

Figure 1 presents representative data for AUC experiments on the four constructs used in this study. The wild-type syntaxin construct, SN–Syx (Figure 1(a)), is best fit to a monomer–dimer equilibrium, with a standard state mole fraction free energy of association equal to 3.5($\pm$0.3) kcal mol^{-1}. In contrast, no dimer is observed for the wild-type synaptobrevin construct, SN–Syb, at the highest experimentally accessible protein/detergent mole fraction (Figure 1(c)). Alteration of the single residue difference in the conserved interface, the Met267Leu substitution in syntaxin (SN–SyxML), creates no disruption in the free energy of association (Figure 1(b); Table 1). However, when the corresponding mutation is made in the synaptobrevin dimerization motif, the SN–SybLM construct, dimer is observed at a comparable concentration to both SN–Syx and SN–SyxML (Figure 1(d); Table 1). We therefore find that the substitution to methionine in the synaptobrevin transmembrane domain provides at least an additional 1.3 kcal mol^{-1} in the free energy of association.

It is important to consider the concentration dependence of association, since the conditions in the cell are likely to be vastly different from the experimental conditions. Therefore, the populations of oligomers over a range of concentrations were calculated using the experimentally determined thermodynamic values. Figure 2 demonstrates the dimer population as a function of the mole fraction of protein for all constructs. For reference, these are compared to the distribution for SN–GpA analyzed in the same micellar environment. SN–Syx, SN–SyxML, and the SN–SybLM are significantly less stable than glycophorin A. The free energies of association for these SNARE constructs can be considered identical, since they are within error of each other (Table 1). Since there was no dimer observed experimentally, the preferential interaction in the synaptobrevin transmembrane domain is likely to be too weak to measure under equilibrium conditions using the method described. Measuring the association in detergent micelles is limited, because at high protein to detergent ratios random association becomes the driving force for oligomerization.[24] The distribution for SN–Syb, shown in Figure 2, was calculated on the basis of the maximum possible experimental free energy value, which approximates the random distribution and is shown as a dotted line in Figure 2.[24] The apparent concentration of these transmembrane proteins during the process of fusion could be much greater than what is considered experimentally, since the interactions between the soluble domains will drive them into close proximity. Therefore, presumably weak interactions could still be influential in the mechanism of fusion *in vivo*.

Crosslinking reveals differences in the reactivity of cysteine residues in synaptobrevin and syntaxin homodimers

It has been shown that synaptobrevin will form a dimer in the presence of oxidizing agents.[14,18]

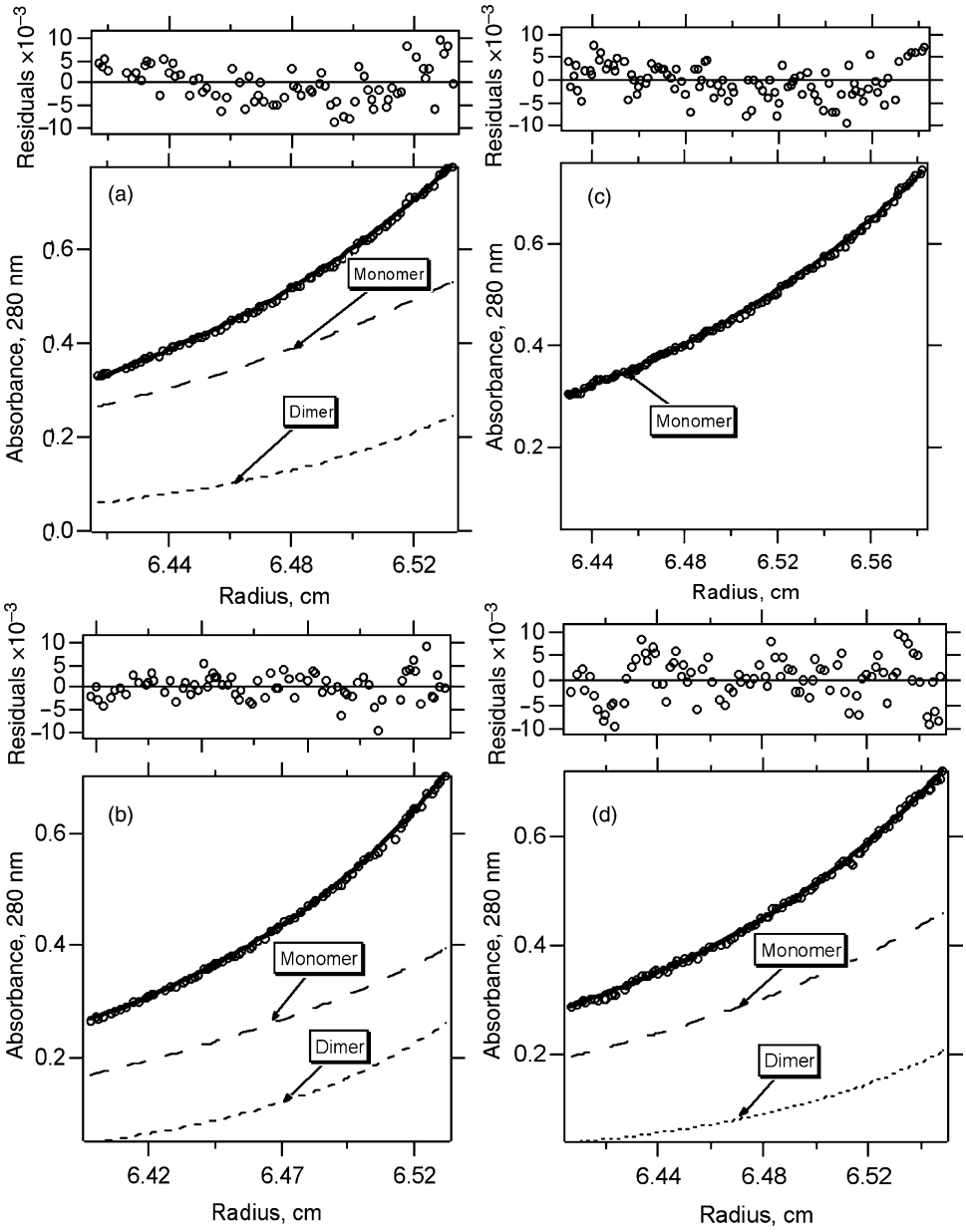

Figure 1. Sedimentation equilibrium data for synaptobrevin and syntaxin constructs. The raw data for a representative data set is shown as open circles. The continuous line is the fit for which the above residuals appear small and random. The concentration of monomer (broken line) and dimer (double broken line) are calculated on the basis of the globally determined equilibrium constant. (a) SN–Syx, (b) SN–SyxML and (d) SN–SybLM best fit to a monomer–dimer equilibrium. (c) SN–Syb is best fit to a single monomeric species.

Under these conditions, a single cysteine residue in the transmembrane region reacts to form a disulfide-bonded dimer, which can be visualized using SDS–PAGE. Syntaxin has two cysteine residues in the transmembrane region that may be important for homo and hetero-dimerization, and that may indicate the interface of interaction (Table 1). Furthermore, because cysteine 271 is a conserved residue in the synaptobrevin interface, it may be involved in heterodimerization of syntaxin and synaptobrevin. To test the position of these cysteine residues in the interface of transmembrane dimers and to determine if that role is altered in the SN–SyxML and SN–SybLM mutants, crosslinking experiments were carried out. If the same interface were involved in homodimerization for both syntaxin and synaptobrevin, all constructs would be expected to dimerize in the presence of oxidizing agents. Furthermore, since the syntaxin constructs and the SN–SybLM construct demonstrate a similar free energy of association, a similar motif for interaction may exist in these proteins, distinct from the wild-type synaptobrevin construct.

Using copper phenanthroline (CuOP) (Figure 3) and iodine (data not shown), mutant and wild-type fusion proteins were subjected to oxidizing

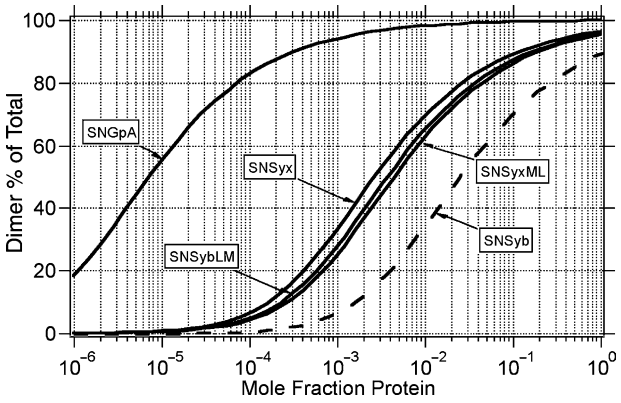

Figure 2. Relative dimer population of syntaxin and synaptobrevin constructs. The fraction dimer is plotted *versus* the mole fraction of protein. The population distribution is calculated on the basis of the free energy of association measured by analytical ultracentrifugation. For reference, the calculated distribution for the strong dimer glycophorin A is shown. The broken line shows the maximum possible dimer distribution for the SN–Syb construct, which approximates the random distribution for a dimer in a micellar environment.[24]

conditions that promote the formation of favorable disulfide bonds. Both oxidizing agents promoted dimer formation for SN–Syb and SN–SybLM proteins. Notably, SN–Syb does not populate dimer at time zero, but dimer formation appears after exposure to CuOP. SN–SybLM populates dimer at time zero and becomes predominantly dimeric after exposure to CuOP. Additionally, the rate of crosslinking for SN–SybLM appears more rapid than the rate for SN–Syb. This may be explained, in part, as being due to the increased propensity for dimerization in the SN–SybLM protein, but may also be due to a better accommodation for disulfide bond formation in the structure of the SN–SybLM dimer. These data are consistent with previous studies, and demonstrate that Cys103 is at the dimer interface and forms a disulfide bond across the interface in synaptobrevin.

Here, we test the ability of cysteine residues in the syntaxin transmembrane domain to promote dimerization. Under conditions that promote dimerization for synaptobrevin, neither SN–Syx nor SN–SyxML formed crosslinked dimer (Figure 3). Since syntaxin has a greater affinity than wild-type synaptobrevin, there is a visible dimer band at time zero for wild-type and mutant SN–Syx proteins. This dimer band is due to the inherent affinity of the transmembrane sequences, and does not represent a crosslinked dimer. After exposure to an oxidizing agent, there is no increase in dimer concentration for SN–Syx or SN–SyxML, which is consistent with an absence of crosslinking in these dimers. The oxidizing agent, CuOP, is a promiscuous catalyst of disulfide bond formation, even for cysteine residues that are only proximal due to molecular motion.[25,26] The lack of reactivity of the cysteine residues in the syntaxin dimer indicates that these cysteine residues are in a stable conformation that does not permit disulfide bond formation across the interface. Furthermore, at high concentrations of protein, SDS–PAGE reveals higher-order oligomers for both SN–Syx and SN–SyxML, which are also not affected by oxidizing agents (data not shown). These higher-order oligomers are not unique to our experimental conditions, but have appeared on immunoblots of full-length syntaxin protein in rat brain membranes.[27] In contrast, observations show that the SN–SybLM mutant, which has the putative syntaxin dimer interface, does not populate higher-order oligomers at similar concentrations. These data indicate that, although SN–SybLM demonstrates a free energy of association equivalent to the syntaxin constructs, the interface for interaction in this construct is distinct. Therefore, inherent differences exist in the homodimerization interfaces for the syntaxin and synaptobrevin constructs.

Crosslinking promotes heterodimerization of syntaxin and synaptobrevin

Previous data show that the transmembrane regions of syntaxin and synaptobrevin can form

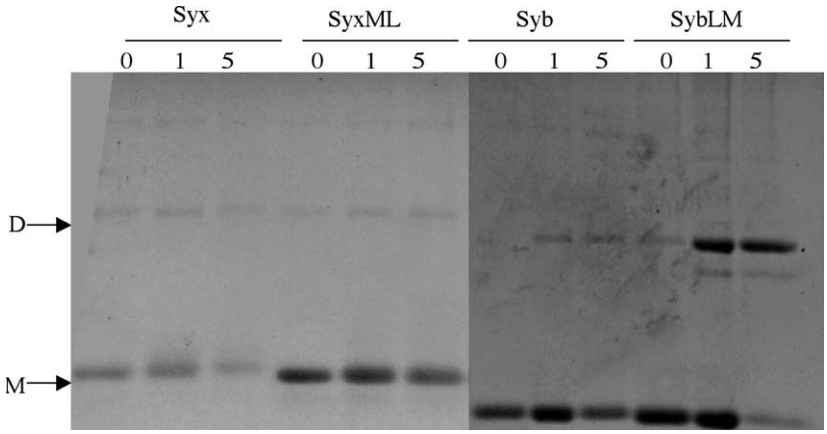

Figure 3. Homodimer crosslinking for syntaxin and synaptobrevin constructs. Disulfide crosslinking was carried out on purified protein using CuOP as an oxidizing agent. Both mutant and wild-type syntaxin and synaptobrevin constructs were exposed to oxidizing agent and samples were taken at 1 min and 5 min, and observed by SDS–PAGE. SN–Syx and SN–SyxML did not crosslink under these conditions. SN–Syb shows no dimer at time zero and an increase in dimer concentration after exposure to CuOP. SN–SybLM shows dimer at time zero and a larger increase in dimer concentration after exposure to oxidizing agent. An increase in dimer concentration is indicative of the formation of disulfide bonds across a dimer interface. D, dimer; M, monomer.

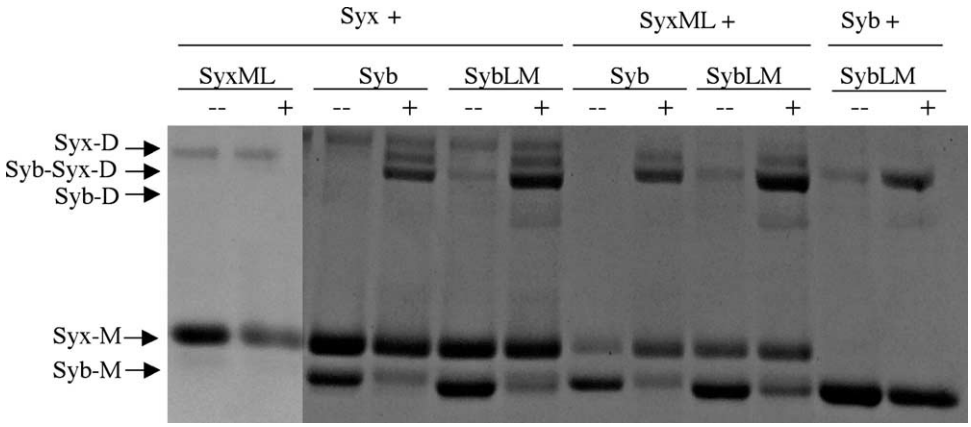

Figure 4. Heterodimer crosslinking for syntaxin and synaptobrevin constructs. The inherently different migration properties of the SN–Syb and SN–Syx fusion proteins on SDS–PAGE allow the visualization of a SN–Syx/SN–Syb heterodimer. Proteins were mixed and exposed to CuOP for 5 min. The effect of mutation on crosslinking is also tested by inclusion of the SN–SyxML and SN–SybLM constructs. Heterodimer formation occurs between SN–Syx and SN–Syb proteins and does not seem to be affected by the mutations at the interface. D, dimer; M, monomer.

heterodimers under oxidizing conditions.[11] Using CuOP as an oxidizing agent, it is possible to crosslink all combinations of syntaxin and synaptobrevin constructs (Figure 4). Inherent differences in the migration of the constructs allow the visualization of heterodimer formation by SDS–PAGE. The three upper arrows indicate the migration of the dimer species in the gel, with the middle arrow showing the heterodimer. The lower arrows indicate the SN–Syx and SN–Syb monomers. SN–SyxML and SN–Syx do not crosslink to each other, but both variants do crosslink with SN–Syb and SN–SybLM to form disulfide-linked heterodimers. It appears that neither mutation in the SN–Syb or SN–Syx transmembrane region affects the rate of heterodimer formation under oxidizing conditions. Although the conserved cysteine residue cannot promote homodimerization in syntaxin, these residues promote heterodimerization of syntaxin and synaptobrevin. In contrast to the syntaxin homodimer, the cysteine residues in the heterodimer are reactive and can form a disulfide bond across the dimer interface. This is similar to the result for the synaptobrevin homodimer, which permits a disulfide-bonded dimer. Therefore, the crosslinking data suggest that the heterodimer interface may be similar to the synaptobrevin crosslinked homodimer interface, but distinct from the syntaxin homodimer interface.

Alternate interfaces for syntaxin homodimer and syntaxin/synaptobrevin heterodimer can be calculated using computational modeling

Using the previous mutagenesis data, a model for the structure of the synaptobrevin homodimer (Figure 5(a)) was calculated using the program CHI,[22] which has been successful for prediction of other transmembrane homodimer structures.[28,29] This model is consistent with our experimental data, predicting that Leu99 is located at the interface in close contact with the opposing helix. Furthermore, the model shows that Cys103, the residue necessary for crosslinking, is also located at the interface and could be predicted to form an

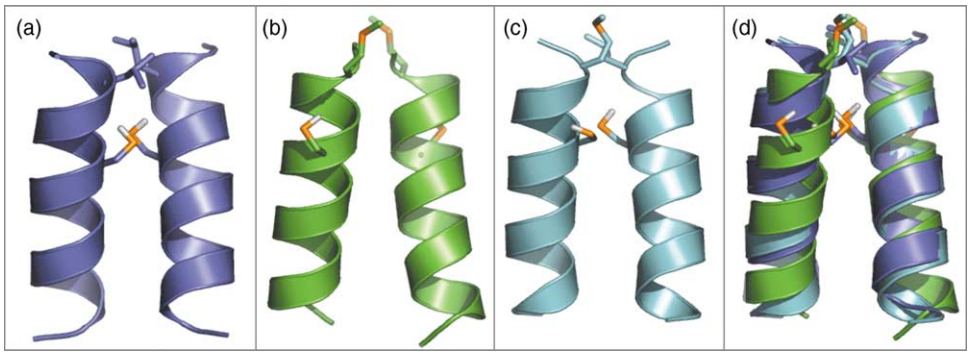

Figure 5. Computational modeling for syntaxin and syntaxin/synaptobrevin heterodimer. Models were generated using the program CHI. Each model is represented as a cartoon helix with the important experimental residues shown as sticks. (a) Computational model for synaptobrevin transmembrane domain homodimer.[22] Shown in sticks are Leu99 and Cys103. (b) Computational model for syntaxin transmembrane homodimer. Shown in sticks are Met267 and Cys271. (c) Computational model for the syntaxin/synaptobrevin heterodimer, syntaxin is the helix on the left and synaptobrevin is the helix on the right. (d) Alignment of syntaxin, synaptobrevin, and heterodimer models.

intermonomer disulfide bond under oxidizing conditions. Using the same modeling protocol, the syntaxin homodimer and syntaxin/synaptobrevin heterodimer structures were calculated and the experimental data were used to distinguish between the models. Although there are conserved residues in syntaxin that can mediate dimerization in the synaptobrevin transmembrane domain, thermodynamic and crosslinking data suggest that these residues are not necessarily important in homodimerization of the syntaxin transmembrane domain. Crosslinking data show that Cys271 and Cys272 do not form a disulfide linkage between two syntaxin monomers. The lack of reactivity of these cysteine residues in the homodimer under conditions that promote disulfide linkages in the heterodimer and synaptobrevin homodimer can be due to multiple structural features. The cysteine residues may be at the interface, but too distant to form a disulfide linkage. The cysteine residues may be at the interface, but in a rotamer orientation inadequate to promote crosslinking. Finally, the cysteine residues could be located on the lipid face and therefore not reactive at the interface. The last explanation seems to be the most likely. CuOP is a promiscuous catalyst and would be able to promote disulfide linkages in the dimer between cysteine residues that are distant and in a rotamer conformation that is transient. Additionally, mutagenesis data for syntaxin suggest that Met267 is not a critical interface residue. Therefore, a likely interface for the syntaxin dimer would not include Met267, Cys271, or Cys272. The interface for interaction for the synaptobrevin/syntaxin heterodimer should include either Cys271 or Cys272 of syntaxin and Cys103 of synaptobrevin, since crosslinking data shows that these residues promote a disulfide-bonded heterodimer, and therefore that the syntaxin/synaptobrevin heterodimer may use an interface similar to that of the synaptobrevin homodimer.

Models were generated for the syntaxin homodimer and the syntaxin/synaptobrevin heterodimer, and subjected to the structural interpretation of the experimental data. Using CHI, an exhaustive search was performed to find low-energy structures. The structures obtained in this search include both symmetric and non-symmetric dimers. Only symmetric dimers with the interface implicated in the experiments are chosen. In this study, there was a single model that fit these criteria for the syntaxin homodimer and synaptobrevin/syntaxin heterodimer (Figure 5(b) and (c)). The important experimental residues are shown as sticks in Figure 5. For syntaxin, those residues are Met267 and Cys271; and for synaptobrevin, those residues are Leu99 and Cys103. In accordance with the experimental results, the crosslinking residues Cys271 point away from each other and would be unable to form a disulfide bond between the monomers. The symmetric model for the syntaxin dimer that demonstrated an inability for interaction at the cysteine residues also demonstrated that Met267 is not at the dimer interface, and in the model these residues are directed away from each other and are perpendicular to the interface. This is consistent with the experimental result that the Met267Leu substitution has no energetic consequence. As discussed, the heterodimer interface is expected to be distinct from the syntaxin homodimer interface. Therefore, in the heterodimer model, the synaptobrevin dimerization interface is used and a disulfide bond could form between Cys271 on the syntaxin monomer and Cys103 on the synaptobrevin monomer. The conformations of the helix backbone in these models are very similar to each other, which can be seen when all three models are aligned (Figure 5(d)). It is important to note that these are not experimentally determined structures, but are chemically reasonable models that explain the experimental data.

A more detailed comparison of the models can be performed by examination of van der Waals packing interactions using the occluded surface algorithm.[30] Buried surface area and occluded surface have been shown to correlate to energetics in membrane proteins using structure-based parameterization.[20,31] The occluded surfaces for the synaptobrevin and syntaxin models are comparable, each homodimer occluding approximately 330 $Å^2$. The heterodimer model occludes approximately 350 $Å^2$. Our results demonstrate that, at equilibrium, syntaxin populates dimer more readily than synaptobrevin. This result cannot be explained using the theory that buried surface area is the primary determinant for the propensity of helices to dimerize. Nevertheless, the computational structures provide a structural framework compatible with the existing experimental data. Therefore, these models may provide insight into the possible interface of interaction for these transmembrane proteins.

Discussion

Synaptobrevin dimerization is not driven by the transmembrane domain packing interactions

Although the significance of synaptobrevin dimerization has been disagreed upon in the literature, the TOXCAT and ToxR assays have both shown that the synaptobrevin transmembrane domain associates with a greater affinity than the weak dimers GpA83A and GpA83I.[15,18] Using AUC, the free energy of association measured for the synaptobrevin transmembrane domain is weaker than both GpA controls, which have a standard state free energy of association of approximately 3 kcal mol^{-1}.[20] In fact, at limiting protein to detergent ratios, we find that the synaptobrevin transmembrane domain does not drive association. Although ToxR data have been shown to correlate with *in vitro* data for the model transmembrane

domain glycophorin A,[32] recent work has shown that not all transmembrane sequences that produce a positive result in these *in vivo* assays show significant affinity for association *in vitro*.[33] However, consistent with previous studies, we do find that synaptobrevin dimerization can be driven by disulfide crosslinking. In fact, oxidizing conditions were used in the previous study to promote association and to determine the dimerization interface.[14] The formation of a disulfide bond is integral to this dimerization, and sequence context may facilitate this bond by promoting a sterically permissible interface and allowing proper geometry for disulfide linkage. Interestingly, the substitution Leu99Met (SN–SybLM), which creates the putative syntaxin interface, promotes synaptobrevin dimerization in the absence of oxidizing agents. Furthermore, the rate of disulfide bond formation under oxidizing conditions is increased as compared to the wild-type synaptobrevin sequence. This mutant provides insight into the equilibrium population for the heterodimer, since the interface of interaction would have only a single amino acid difference in one monomer. These results may indicate a greater propensity for interaction between the transmembrane domains of synaptobrevin and syntaxin than for two synaptobrevin monomers. The implication for the role of the transmembrane region of synaptobrevin in protein–protein interactions is still unclear. The cysteine residue that promotes crosslinking in synaptobrevin has also been reported to be palmitoylated in adult rat brains.[34] However, these authors show that palmitoylation of synaptobrevin 2 does not occur in embryonic rat brains and that the palmitoylation in adult rat brain is substoichiometric *in vivo*, indicating that a significant population will not be palmitoylated in adult synaptic vesicles. Although palmitoylation may disrupt association of a synaptobrevin dimer, the fact that unpalmitoylated species exist indicates that this association may still be significant *in vivo*. Furthermore, in addition to disulfide linkage, other factors may drive the association *in vivo*, which is optimized by a sterically permissible interface that stabilize the structure or specify the conformation.

Much of our understanding about the association of membrane proteins is derived from the study of stable α-helical complexes. For instance, the well-studied transmembrane dimer glycophorin A is predominantly dimeric *in vivo*. This protein has served as a model for transmembrane helix interactions in part due to the fact that an extremely stable dimer allows extensive mutagenesis. Transmembrane helices for which association occurs as a part of a cellular process, such as vesicle fusion, would not be expected to associate as strongly as GpA. An accessible equilibrium of species is necessary to participate in dynamic events. Therefore, a thermodynamically weak propensity for association may be integral to cell function. Although the native synaptobrevin sequence may have little ability to drive association, it may still play a major role in important protein–protein interactions.

Implications for multiple interfaces in the syntaxin transmembrane domain

Our data demonstrate that the syntaxin transmembrane domain has a thermodynamic measurable propensity for self-association. Although syntaxin contains a sequence motif similar to that proposed for synaptobrevin, our results suggest that an alternate interface promotes the dimerization observed *in vitro*. The substitution Met267Leu, which creates the putative synaptobrevin interface, has no effect on the ability to dimerize, although the converse mutation promotes dimerization in the synaptobrevin transmembrane domain. Most importantly, the conserved cysteine residue, which promotes dimerization in the synaptobrevin transmembrane domain under oxidative conditions by formation of an intermonomer disulfide linkage, does not form a disulfide linkage in syntaxin under the same experimental conditions. Although the syntaxin sequence corresponding to the previously determined synaptobrevin dimer interface can drive association in other sequence contexts,[11] the entire syntaxin transmembrane sequence may use an alternate interface for homodimerization. Interestingly, the conserved cysteine residues do crosslink in the heterodimer, suggesting an alternate interface for homo and heterodimerization for the syntaxin transmembrane domain. The formation of higher-order oligomers visualized by SDS–PAGE also indicates multiple interfaces for interaction. These higher-order oligomers may be functionally important, since studies suggest that an arrangement of five to eight syntaxin transmembrane segments may compose a proteinaceous fusion pore.[35]

Syntaxin is known to interact with numerous proteins using both transmembrane and soluble domains in the protein. The membrane-proximal domain (H3 domain) is known to promote homo-oligomerization[36] and is the region in the solved structure of the SNARE complex.[3] It has been predicted that large conformational rearrangements occur in the H3 domain to account for the promiscuity of interactions.[37] Full-length syntaxin is also known to interact with many proteins other than SNARE proteins.[38–41] Furthermore, the transmembrane domain is known to promote interactions of the full-length protein.[42] It is conceivable that in addition to the soluble domains, the transmembrane domain has an inherent plasticity that accounts for interactions with multiple proteins. At the physiological level, these alternate interfaces could act as a molecular switch that participates in dynamic interactions that facilitate vesicle fusion. Further biochemical and structural data are necessary to verify the

existence of multiple interfaces in the syntaxin transmembrane domain and the greater role of the transmembrane domains syntaxin and synaptobrevin in vesicle fusion.

Materials and Methods

Cloning, expression, and protein purification

All proteins used in this study were expressed as fusion constructs with staphylococcal nuclease.[43] The syntaxin1A construct, SN–Syx, was cloned by using the pET11A–SNGpA99 construct as a template for successive rounds of site-directed mutagenesis using a Quikchange kit (Stratagene, LaJolla, CA) with the appropriate primers. The synaptobrevin2 or VAMP-2 construct, SN–Syb, was cloned by ligation of extended oligonucleotides containing the synaptobrevin transmembrane sequence into the pET11A–SN vector. Substitutions were made using a Quikchange kit with the wild-type constructs. Proteins were purified using extractions in the detergent Thesit (Fluka, Switzerland) with a single salt extraction in 1M NH_4OAc[44] followed by further purification on an SP column by FPLC.

Sedimentation equilibrium analytical ultracentrifugation

Proteins were exchanged into the detergent C_8E_5 for AUC.[19] Sedimentation equilibrium experiments were carried out at 25 °C in a Beckman XL-A analytical ultracentrifuge. Protein distributions were monitored using the absorbance at 280 nm. For an accurate and precise measurement of the free energy of association, a minimum of 12 data sets were used in a global fitting of the data using MacNonlin.[45] The data used in analysis consisted of three significantly different initial protein concentrations run at four significantly different speeds. The buoyant molecular mass (σ) was calculated from the amino acid composition using SEDNTERP[46] and held constant during global fitting. Each free energy was measured independently a minimum of three times with at least two different concentrations of detergent. The standard state free energy value (ΔG_x°) is calculated from the apparent equilibrium constant determined resulting from the experiment (K_{app}) by assuming an ideal dilute solution using the following equation:[47]

$$\Delta G_x^\circ = -RT\ln\left(K_{app}[\text{micellarDet}]_w\right) \quad (1)$$

Disulfide crosslinking under oxidative conditions

Crosslinking experiments were carried out using CuOP, at a final concentration of 1 mM $CuIISO_4$ and 2 mM 1,10 phenanthroline, or iodine at a final concentration of 0.1 mM (data not shown) with 20 mM Tris–HCl (pH 8.0), 250 mM NaCl, 1% (v/v) Thesit. The oxidizing agents produced comparable results under similar experimental conditions. Reactions were quenched by the addition of 40 mM *N*-ethyl maleimide (NEM), 20 mM EDTA, and SDS–PAGE gel loading buffer. Samples were immediately boiled for 4 min at 95 °C and observed by SDS–PAGE. Although the length of the amino acid sequences of the SN–Syx and SN–Syb clones are identical, the SN–Syb proteins migrates more quickly than the SN–Syx proteins, allowing visualization of the heterodimer by SDS–PAGE.

Computational modeling

Structural models were generated for the syntaxin homodimer and the syntaxin/synaptobrevin heterodimer for the wild-type sequences (Table 1; syntaxin residues 267–292; synaptobrevin residues 98–113) using the program CHI,[28,48] with the CHARMM parameter set.[49] CHI uses simulated annealing and energy minimization to find clusters of low-energy structures. The average structure that represents each cluster is then calculated. A full search for all possible homodimers was performed a minimum of five times to fully explore all possible models.[22] A representative structure for the syntaxin homodimer and the syntaxin/synaptobrevin heterodimer was determined by consideration of symmetric structures that correlate to the experimental data. Structures were analyzed using the occluded surface algorithm version 7.2.2[30] to quantify packing interactions, and WHATIF[50] to calculate helix crossing angles. Figures were constructed using MacPymol (DeLano Scientific LLC).

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