The Juxtamembrane Linker of Full-length Synaptotagmin 1 Controls Oligomerization and Calcium-dependent Membrane Binding*

Bin Lu†§, Volker Kiessling†§, Lukas K. Tamm†§, and David S. Cafiso†§¶

From the Departments of † Chemistry and § Molecular Physiology and Biological Physics and the ¶ Center for Membrane Biology, University of Virginia, Charlottesville, Virginia 22904

Background: Synaptotagmin 1 is the Ca\(^{2+}\) sensor for neuronal exocytosis.

Results: The juxtamembrane linker of synaptotagmin 1 is oligomerized through a glycine zipper motif, and this interaction controls the activity of synaptotagmin 1.

Conclusion: Structural modifications in the linker alter the activity of synaptotagmin 1.

Significance: The oligomerization of synaptotagmin 1 may control the organization at the focal site of fusion.

Synaptotagmin 1 (Syt1) is the calcium sensor for synchronous neurotransmitter release. The two C2 domains of Syt1, which may mediate fusion by bridging the vesicle and plasma membranes, are connected to the vesicle membrane by a 60-residue linker. Here, we use site-directed spin labeling and a novel total internal reflection fluorescence vesicle binding assay to characterize the juxtamembrane linker and to test the ability of reconstituted full-length Syt1 to interact with opposing membrane surfaces. EPR spectroscopy demonstrates that the majority of the linker interacts with the membrane interface, thereby limiting the extension of the C2A and C2B domains into the cytoplasm. Pulse dipolar EPR spectroscopy provides evidence that purified full-length Syt1 is oligomerized in the membrane, and mutagenesis indicates that a glycine zipper/GXXG motif within the linker helps mediate oligomerization. The total internal reflection fluorescence-based vesicle binding assay demonstrates that full-length Syt1 that is reconstituted into supported lipid bilayers will capture vesicles containing negatively charged lipid in a Ca\(^{2+}\)-dependent manner. Moreover, the rate of vesicle capture increases with Syt1 density, and mutations in the GXXG motif that inhibit oligomerization of Syt1 reduce the rate of vesicle capture. This work demonstrates that modifications within the 60-residue linker modulate both the oligomerization of Syt1 and its ability to interact with opposing bilayers. In addition to controlling its activity, the oligomerization of Syt1 may play a role in organizing proteins within the active zone of membrane fusion.

Neurotransmitter release results from the fusion of synaptic vesicles with the presynaptic plasma membrane following an increase in intracellular calcium. This exquisitely regulated process is driven by a number of critical protein components. The SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) form the core of the fusion machinery, and they assemble into a four helical bundle that drive a close approach between the vesicle and plasma membranes (1–4).

There is strong evidence that synaptotagmin 1 (Syt1)\(^2\) is the Ca\(^{2+}\) sensor for neuronal exocytosis. When intracellular Ca\(^{2+}\) levels rise, Syt1 triggers fast synchronous neurotransmitter release, but Syt1 has also been associated with a slower, asynchronous release process (5). Synaptotagmin 1 is a vesicle membrane protein possessing two C2 domains, designated C2A and C2B, which are anchored to the vesicle membrane by a single transmembrane helical segment (see Fig. 1a). These C2 domains bind and insert into negatively charged membranes in a Ca\(^{2+}\)-dependent manner (6, 7) and are also known to interact with SNARE proteins (8). High-resolution structures for the isolated C2A and C2B domains as well as a fragment possessing the two domains in tandem (C2AB) have been obtained (9–13). These structures indicate that C2A and C2B adopt a similar fold formed from eight \(\beta\)-strands and that they bind three and two Ca\(^{2+}\) ions, respectively.

The C2A and C2B domains of Syt1 are connected to the vesicle membrane by a long linker of \(~60\) residues (Fig. 1b). This linker is of variable length, but it is present in all isoforms of the synaptotagmin family except Syt17 (14). Regions of the linker are highly conserved, suggesting that it may play an important role in Ca\(^{2+}\)-evoked exocytosis. It has been noted that the linker contains a segment capable of forming a laterally amphipathic helix, which might mediate dimerization of the protein (15). Inspection of the linker segment also reveals the presence of two motifs, one of which is positively charged near the transmembrane segment, and a second of excess negative charge at the C-terminal end near C2A. This raises the possibility that electrostatic interactions might promote an interac-

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1 To whom correspondence should be addressed: Dept. of Chemistry, University of Virginia, McCormick Rd., Charlottesville, VA 22904-4319. Tel.: 434-924-3067; Fax: 434-924-3567; E-mail: cafiso@virginia.edu.

2 The abbreviations used are: Syt1, synaptotagmin 1; PC, phosphatidylcholine; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; bPS, porcine brain PS; TIRF, total internal reflection.
Structure of the Juxtamembrane Linker of Synaptotagmin 1

A number of studies have attempted to reconstitute Ca\(^{2+}\)-dependent membrane fusion using a minimal number of protein components. In these functional assays, both the full-length protein and a soluble fragment, possessing just the two C2 domains (C2AB), have been employed as a Ca\(^{2+}\) sensor, often producing dramatically different results. For example, it has been observed that the tandem C2AB construct is able to stimulate fusion in a Ca\(^{2+}\)-dependent manner, whereas the full-length protein does not; however, the full-length protein is able to stimulate fusion in a Ca\(^{2+}\)-independent manner (16, 17). Remarkably, this Ca\(^{2+}\)-independent activity of Syt1 is eliminated when steps are taken to prevent the target membrane SNAREs, syntaxin and SNAP-25, from assembling into a non-native-like 2:1 complex (18). In addition, some of the differences that are observed between full-length Syt1 and the C2AB construct have been attributed to a role for Syt1 in other steps that are required for fusion, such as docking (19).

Despite the many function studies that have utilized both full-length and C2AB forms of Syt1, most structural work has utilized the soluble construct of Syt1 containing the C2A and C2B domains. At present, relatively little work has been carried out on the structure of the full-length protein and the organization of the linker. There are reports of oligomerization of Syt1 mediated both by the C2 domains (20) and by the linker region of the protein (21–23). However, some reports of oligomerization driven by the C2 domains (24, 25) have been questioned due to the presence of acidic bacterial contaminants that remain attached to the protein after affinity purification (11). Recently, it has been suggested that association and dissociation of oppositely charged ends of the linker may regulate the ability of Syt1 to extend from the membrane interface (26). The linker region also contains a conserved GXXXG motif, sometimes referred to as a glycine zipper. These glycine zippers are well known to mediate helix interactions in membrane proteins (27, 28), but it has recently been shown that these motifs have a role in promoting helix association at the membrane interface (29).

In the present study, we examine the state of the Syt1 linker using both continuous wave and pulse EPR spectroscopy when the protein is reconstituted into membranes composed of phosphatidylcholine (PC) and phosphatidylserine (PS). The data demonstrate that a large section of the linker is closely associated with or buried within the bilayer interface. In addition, pulse EPR spectroscopy shows that full-length Syt1 is oligomerized within the bilayer and that this oligomerization is mediated by a conserved GXXXG motif in the linker. Using a total internal reflection (TIRF) vesicle capture assay, we demonstrate that this reconstituted full-length Syt1 has the capacity to capture vesicles in a Ca\(^{2+}\)-dependent manner. The vesicle capture rate is dependent upon the density of reconstituted Syt1, and it appears to be significantly reduced by mutations in the GXXXG motif at equivalent protein densities. The results indicate that modifications within the linker connecting the C2 domains to the vesicle membrane play a role in the activity of Syt1.

EXPERIMENTAL PROCEDURES

Mutagenesis, Expression, and Purification of Syt1—The wild-type rat Syt1 (amino acids 1–421) was inserted into a pET-28a plasmid with a His\(_{6}\) tag on its N terminus. The single cysteine mutations (positions 81, 86, 90, 95, 104, 114, 123, and 133), double cysteine mutation (positions 86 and 136), and GA mutant (G92A, G96A, and G97A) were introduced into Cys-free Syt1 (C73A, C74A, C76A, C78A, C82S, and C277S) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). A form of Syt1 lacking the C2 domains, Syt1(1–140), was generated by inducing two tandem stop codons at residues 141 and 142 of Cys-free Syt1. All mutations sequences were subsequently verified by DNA sequencing (Genewiz, South Plainfield, NJ).

Expression and purification of Syt1 followed a procedure described previously (30). Briefly, His\(_{6}\)-tagged Syt1 was expressed in *Escherichia coli* BL21 (DE3) CodonPlus-RIL (Stratagene). The cells were grown at 37 °C in LB with glucose (2 g/liter), kanamycin (34 μg/ml), and chloramphenicol (50 μg/ml) until \(A_{600}\) reached 0.8–1.0. Protein expression was induced by 0.5 mM isopropyl 1-thio-β-D-galactopyranoside, and the cells were grown for an additional 6 h at 16 °C. The cells were harvested and lysed, and the membrane fraction was solubilized using phosphate-buffered saline (PBS), pH 7.4, containing 0.1% v/v Triton X-100. The protein was purified from the solubilized membrane preparation using nickel-nitrotriacetic acid beads (Qiagen). To remove possible nucleic acid contaminants from Syt1, the column was washed with two column volumes of 0.1% Triton X-100 in 25 mM HEPES buffer containing 20 mM Ca\(^{2+}\), pH 7.4 (31). All purified proteins were checked for purity by SDS-PAGE.

Spin Labeling and Reconstitution of Syt1—The cysteine mutants of Syt1 were reacted with (1-oxyl-2,2,5,5-tetramethylpyrrolinyl-3-methyl)methanethiosulfonate spin label at 4 °C overnight while the protein was bound to the nickel-nitrotriacetic acid beads (Qiagen). To remove free spin label, the beads were extensively washed with PBS containing 0.1% (v/v) Triton X-100, 20 mM imidazole, pH 7.4. The protein was subsequently eluted with 500 mM imidazole in PBS containing 1% (w/v) β-octyl glucoside. For all samples, the labeling efficiency was more than 70%.

Purified Syt1 was reconstituted into membrane bilayers at a protein/lipid molar ratio of 1:500. This was accomplished by adding the purified protein/micelle mixture into preformed 100-nm diameter large unilamellar vesicles composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) and 20 mol % 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylserine (POPS). All lipids used in this study were obtained from Avanti (Alabaster, AL). These large unilamellar vesicles were prepared in detergent-free PBS by lipid extrusion (Avanti). The mixed micelle/large unilamellar vesicle mixture was incubated for 10 min at room temperature, and the sample was then diluted 2-fold to lower the β-octyl glucoside below its critical micelle concentration. The remaining detergent was removed by dialysis overnight against a buffer containing 25 mM HEPES, 150 mM KCl, pH 7.4. To prepare samples for EPR spectroscopy, the dialyzed proteolipid solution was concentrated using a 30
kDa cut-off centrifugal filter (EMD Millipore). Reconstitution efficiencies for Syt1 were determined using a Western blot co- 
floation assay (32) and were found to be greater than 95%. Unless otherwise noted, Syt1 was reconstituted in a calcium-
free state.

Circular Dirochroism Spectroscopy—CD spectra of 0.1 mg/ml full-length and truncated Syt1 reconstituted proteoliposomes were collected on an Aviv model 410 spectropolarimeter in a 0.5-mm quartz cell at 22 °C in PBS buffer (pH 7.4). Data were collected at 1-nm intervals from 260 to 196 nm. Spectra were converted to mean residue molar ellipticity units, and the percentage of secondary structure was estimated using the DICHROWEB analysis software (33).

Electron Paramagnetic Resonance—EPR spectroscopy was performed on a continuous wave X-band EMX spectrometer (Bruker Biospin, Billerica, MA) equipped with an ER 4123D dielectric resonator. All EPR spectra were recorded with 100-G magnetic field sweep, 1-G modulation, and 2.0-milliwatt incident microwave power at a temperature of 298 K. The measurements were performed on 10-μl samples in glass capillary tubes (0.60 mm inner diameter × 0.84 mm outer diameter round capillary; VitroCom, Mountain Lakes, NJ). The phasing, normalization, and subtraction of EPR spectra were performed using LabVIEW software provided by Dr. Christian Altenbach (UCLA, Los Angeles, CA).

Continuous wave power saturation experiments were performed using a TPX-2 capillary, and results were used to calculate a depth parameter, Φ, based upon the saturation behavior of the samples containing oxygen and 10 mM Ni(II)EDDA (34). The value of ΔP<sub>3</sub> for Ni(II)EDDA was scaled to an effective concentration of 20 mM, and distances were calculated using an equation obtained previously describing the correlation of distance (x) and Φ values (6).

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Φ = 3.4 \tanh(0.11(x - 8.56)) + 1.1 \quad (\text{Eq. 1})
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Pulse EPR experiments were performed on a Bruker Elexys E580 EPR spectrometer running at Q-band. Measurements at Q-band used an EN5107D2 dielectric resonator with 10–15 μl of sample that was loaded into quartz capillaries having a 2-mm inner diameter × 2.4-mm outer diameter (Fiber Optic Center, Inc., New Bedford, MA). Single- or double- spin-labeled samples of Syt1 had protein concentrations that varied from 50 to 100 μM and also contained 10% glycerol.

DEER samples were flash-frozen in liquid nitrogen, and the data were recorded at 80 K. Data in pulse mode were acquired using a four-pulse DEER sequence with a 16-ns τ/2 and two 32-ns τ observe pulses separated by a 32-ns τ pump pulse. The dipolar evolution times were typically 1.2–2.0 μs. The pump frequency was set to the center maximum of the nitroxide spectrum, and the observer frequency was set to the low field maximum, typically 15–25 MHz higher.

The phase-corrected dipolar evolution data were processed, and distance distributions were determined using either Gaussian fitting or Tikhonov regularization incorporated into the DeerAnalysis2013 software package (35). This program also contains an error analysis routine that was used to assess the effect of the background subtraction upon the distance distribution. In several cases, the spin-labeled protein was diluted with unlabeled wild-type protein in a 1:2 or 1:5 ratio (protein/wild type) to determine the contributions of inter- versus intramolecular dipolar coupling.

TIRF Microscopy Binding Assay—Detailed information about the TIRF microscopy was described previously (36). In brief, the fluorescence of rhodamine B-labeled liposomes close to the surface was detected by a Zeiss Axiovert 35 fluorescence microscope (Carl Zeiss), equipped with a ×40 water immersion objective (Carl Zeiss; numeric aperture = 0.7) and prism-based TIRF illumination. The light source was an argon ion laser (Innova 300C; Coherent, Palo Alto, CA) tuned to 514 nm. Fluor- escence was observed through a 610-nm band pass filter (D610/60, Chroma, Brattleboro, VT) by electron-multiplying CCDs (DU-860E or DV887ESC-BV, Andor Technologies). The prism-quartz interface was lubricated with glycerol to allow easy translocation of the sample cell on the microscope stage. The beam was totally internally reflected at an angle of 72° from the surface normal, resulting in an evanescent wave that decays exponentially with a characteristic penetration depth of ~100 nm. An elliptical area of 250 × 65 μm was illuminated. The laser intensity, shutter, and camera were controlled by a home-made program written in LabVIEW (National Instruments, Austin, TX).

Supported bilayers containing wild-type Syt1 or the GA mutant were formed at varied protein/lipid ratios as described previously (36). These bilayers were perfused with 2 ml of liposomes that were fluorescently labeled with 1 mol% rhodamine B-labeled lipid, and the fluorescence intensity on the supported bilayer was then followed using TIRF microscopy. In these experiments, the supported membrane contained bPC/choles- terol, 4:1 (or bPC/porcine brain phosphatidyethanolamine/ bPS/cholesterol, 4:3:1:2), whereas the target membrane vesicles contained 32 mol% bPC, 30% phosphatidyethanolamine, 15% bPS, 20% cholesterol, 3% phosphatidylinositol 4,5-bisphos- phate, and 1% 1,2-dioleoyl-sn-glycero-3-phosphoethanol- 
amine-N’-(lissamine rhodamine B sulfonyl). Experiments were carried out in one of two ways. In one binding experiment, the Ca<sup>2+</sup> concentration was varied in the presence of the labeled vesicles to produce data that yielded the Ca<sup>2+</sup> dependence of the vesicle binding. In a second experiment, vesicles at increasing concentrations were titrated into the sample chamber at a fixed Ca<sup>2+</sup> concentration. Between titration steps, the reaction was monitored for ~20 min to allow the system to approach equilibrium. The average fluorescence intensity of all camera pixels was recorded every 30 s. The fluorescence changes between titration steps were fit to first order kinetics to extract saturation intensities and initial rates.

RESULTS

Reconstitution and Vesicle Capture by Synaptotagmin 1—

Full-length cysteine-free Syt1 and a shortened version of Syt1 (amino acids 1–140) lacking the two soluble C2 domains were reconstituted into POPC/POPS (4:1) vesicle bilayers as described under “Experimental Procedures.” Circular dichroism spectra of both versions of the protein were recorded in the absence of added Ca<sup>2+</sup> and are shown in Fig. 1c along with a table indicating the approximate secondary structure compo-
positive and negative charged regions are taggedmin 1, and the sequence of the linker (residues 80–140), where the expected secondary structure of Syt1. As expected, the form with smaller fractions of helix, which is roughly consistent with the position of the two forms obtained by a fitting to the CD spectra. The full-length protein exhibits high levels of sheet and coil with smaller fractions of helix, which is roughly consistent with the expected secondary structure of Syt1. As expected, the form lacking the C2 domains shows a much lower sheet composition and a higher fraction of helix, although in this shortened form (residues 1–140) the content of helix and sheet is higher than would be expected from the single transmembrane helical stretch near the N-terminal end.

To determine whether this full-length form of Syt1 is functional, the reconstituted protein was incorporated into supported bilayers, and TIRF microscopy was used to assay the attachment of fluorescently labeled vesicles in the subphase surrounding the supported surface as depicted in Fig. 2a. The target vesicles contained 15 mol % bPS and 3 mol % phosphatidylinositol 4,5-bisphosphate, and the equilibrium binding of these vesicles was monitored as a function of increasing Ca\(^{2+}\) concentrations as shown in Fig. 2b. When fit to a simple Langmuir isotherm, these data yielded an apparent Ca\(^{2+}\) affinity for vesicle attachment of 33 ± 1 μM. As seen in Fig. 2c, significant vesicle attachment to the supported bilayer does not take place in the absence of Syt1. In Fig. 2c, target lipid vesicles were titrated into the subphase in the presence of 100 μM Ca\(^{2+}\), where Syt1 at protein/lipid molar ratios of 0, 1:16,000, 1:4000, and 1:2000 was present in the supported bilayer. The initial rate of vesicle binding in Fig. 2c depends both on the lipid vesicle concentration in the subphase and on the concentration of Syt1 in the supported bilayer, and, as shown in Fig. 2d, the slopes of these initial rates increase as a function of the Syt1 density in the supported bilayer. The increase in binding rate on vesicle (lipid) concentration seen in Fig. 2c indicates that vesicle binding depends upon the frequency of encounters between the vesicles and the surface. The increase in rate with Syt1 concentration seen in Fig. 2d indicates that vesicle attachment is limited by the availability of Syt1 in the supported surface. When the target vesicles contained only bPC, no vesicle attachment was observed.

**The Linker Is Closely Associated with the Lipid Interface**—
The spin-labeled side chain, R1 (Fig. 3a) was incorporated into eight sites in the linker. Shown in Fig. 3b are the resulting EPR spectra obtained from Syt1 when reconstituted into POPC/POPS (4:1) lipid bilayers. These spectra were acquired in the absence of Ca\(^{2+}\), but neither the addition nor the removal of Ca\(^{2+}\) with EGTA alters these spectra. The EPR spectra from the linker are all composed of two motional components. The faster component (labeled \(i\) in Fig. 3b) resembles that for a nitroxide-labeled protein residing in the aqueous phase with little secondary structure (37). At each site, this component represents the more minor component (less than 50% of the total spins), and it can be simulated by isotropic motion of the side chain having a correlation time (\(\tau_c\)) ranging from 0.5 to 0.7 ns, except near the transmembrane segment, where the values of \(\tau_c\) range from 0.7 to 1.4 ns. A broader component (labeled \(m\) in Fig. 3b) results from a population of R1 undergoing slower motion and must arise from a label in tertiary contact with the backbone or another part of the protein. This could be due to additional secondary or tertiary structure in the linker or the association of the linker with the membrane interface. The correlation times for this slower motional component are relatively consistent across the linker and range from 3.1 to 4.4 ns.

Progressive power saturation of the EPR spectra in the presence and absence of either Ni(II)EDDA or oxygen was used to
assess the membrane depth of these labeled positions. The depth parameters are shown in Fig. 3c, and they indicate that the label is at the level of the lipid phosphates or deeper in the bilayer for every position except 133. This indicates that the linker must be located in or near the membrane interface up through residue 123. The longest correlation time for the mobile components in the EPR spectra from the linker (Fig. 3b) is found for site 81, which is buried deepest in the bilayer. This is consistent with the finding the R1 side chain for hydrocarbon-exposed labels has a strong tendency to interact with the protein surface (38, 39).

The Linker Is in Conformational Exchange between at Least Two States—The two motional components that are seen in the EPR spectra from the linker might have several different origins. The two components could result from two rotameric states of the R1 side chain, or they could reflect two or more conformational states of the Syt1 linker. To test whether the two motional components in the EPR spectra of the Syt1 linker arise from different structures that are in conformational exchange, the EPR spectra were recorded in the presence of a stabilizing osmolyte. Stabilizing osmolytes, such as sucrose and polyethylene glycol (PEG), do not alter label rotamers but may modulate conformational exchange in proteins (40–43). Shown in Fig. 4 are EPR spectra for Syt1 104R1 recorded as a function of added PEG 3350. The progressive addition of PEG 3350 reduces the mobile component in the EPR spectrum of 104R1 and increases the immobile component (Fig. 4a), indicating that the linker is in dynamic equilibrium between at least two different conformations. The fractions of each motional component were determined by spectra subtraction, and the free energy for this equilibrium was plotted as a function of solution osmolality as described previously (44). As expected for a protein converting between two states, a linear dependence of conformational free energy upon PEG concentration was observed (Fig. 4b).

Full-length Synaptotagmin 1 Is Oligomerized through Its Juxtamembrane Linker—Previous work suggested that Syt1 might be oligomerized through its linker (21–23). To test for the possibility that Syt1 is oligomerized in the bilayer, we examined both single and double spin-labeled linker mutants using double electron-electron resonance (DEER). If pairs of nitroxides are in proximity at defined distances, this pulse EPR measure-
ment produces a signal that is modulated at the frequency of the dipolar interaction (45).

Shown in Fig. 5a are DEER signals from single spin labels placed at three positions along the linker. The signals exhibit strong modulation depths, indicating that there are significant dipolar interactions among these labeled mutants and that pairs of spins are being excited in the DEER experiment. The distributions obtained from these data are shown in Fig. 5b, and they are broad and complex. Generally, these distributions display both a shorter and longer distance component, where the longer distance component appears to diminish as the label is moved toward position 95.

The strong DEER signals indicate that the protein is oligomerized within the bilayer. As a control experiment, 86R1 was mixed with unlabeled full-length Syt1 during the reconstitution procedure, and the result is shown in Fig. 5c (blue trace). As expected, the modulation depth in the DEER signal is eliminated, indicating that the signal arises from Syt1 aggregation. Synaptotagmin 1 has been reported to oligomerize through its C2 domains (20), and to determine whether the linker or the C2 domains are mediating this interaction, DEER data were recorded on the membrane-reconstituted protein construct lacking the C2 domains (residues 1–140). The resulting DEER signal and corresponding distribution are shown in Fig. 5, c and d, and they are similar to that obtained for the full-length protein shown in Fig. 5, a and b, indicating that the aggregation observed is mediated by the linker or the TM domain and not by the C2 domains. The distances do not fit a pattern expected for a simple dimer or a symmetric tetramer, and they indicate the presence of an oligomer that is heterogeneous.

As demonstrated above (Fig. 4), the linker is in equilibrium between two or more states, and this may represent an equilibrium between different oligomerized states of the linker. To test the idea that oligomerized states of the linker are in equilibrium, DEER signals were compared in the presence and absence of PEG 3350. PEG 3350 was found to increase the modulation depths (data not shown), indicating that this osmolyte increases the number of spin pairs being excited and enhances the size or number of Syt1 participating in an oligomer. This suggests that the equilibrium seen in the continuous wave EPR spectra (Fig. 4) reflects an equilibrium between different oligomeric states of the Syt1 linker.

The Oligomerization of Syt1 Is Promoted by a Glycine Zipper—An inspection of the sequence of the linker indicates the existence of a region near the transmembrane segment (positions 80–91) that is rich in basic residues and positively charged and another region near C2A (positions 130–140) that is acidic and negatively charged. These charged segments have been noted previously and proposed to play a role in controlling the extension of the C2 domains from the interface perhaps by an intramolecular association (26). A pair of spin labels was placed into the linker, one in each region at positions 86 and
are suppressed in the GA mutant and that this motif helps promote the oligomerization of full-length Syt1.

**Glycine Zipper Mutations in the Linker Reduce the Ability of Full-length Syt1 to Capture Lipid Vesicles**—To determine whether oligomerization might be playing a role in the ability of full-length Syt1 to interact with target lipids, the vesicle capture assay (Fig. 2) was used to compare the activity of full-length Syt1 to the Syt1 GA mutant. Shown in Fig. 6a are data for the initial rate of vesicle binding for both Syt1 and the Syt1 GA mutant at equivalent protein densities as a function of the concentration of lipid vesicles. The reconstitution efficiencies and relative levels of Syt1 in the reconstituted system for full-length Syt1 and Syt1 GA were checked using a Western blot co-flotation assay described previously (32) and found to be equivalent. For the Syt1 GA mutant and Syt1, the vesicle capture rates increase as a function of vesicle concentration; however, the binding rates for the Syt1 GA mutant are reduced to ~50% of the value obtained for the wild-type protein (Fig. 6b). In addition, the equilibrium level of bound vesicles reached is also lowered for the Syt1 GA mutant, although the binding rates rather than the extent of vesicle binding appear to be most affected by the GA mutations.

To determine whether lipid composition plays a role in the vesicle capture activity of the Syt1 GA mutant, the experiments were repeated using a lipid mixture containing 10 mol % PS (see “Experimental Procedures”). Shown in Fig. 6c is a comparison of both the vesicle capture rate and the vesicle binding at equilibrium for the wild-type protein and the Syt1 GA mutant. Although the effect of the GA mutations on capture rate is similar between planar bilayers without and with PS, the effect of the mutations on the extent of vesicle binding is more strongly affected in the PS-containing membranes. As indicated above, the GA mutations reduce the modulation depth in the DEER signals from 86R1 by about 90% (Fig. 5b) in membranes composed of PC/PS. When the experiment is repeated in the bPC/cholesterol mixture, the modulation depth is also depressed, but by about 60% (data not shown). These data indicate that the tendency of the Syt1 GA mutant to oligomerize is influenced by lipid composition.

**DISCUSSION**

At present, the precise molecular role of Syt1 in neuronal exocytosis is not understood. Synaptotagmin 1 is known to interact with both SNAREs and membranes (8), and it has been proposed that Syt1 makes simultaneous membrane and SNARE interactions in the presence of Ca^{2+} to mediate synchronous neurotransmitter release (46). However, there is also evidence that the SNARE interactions made by Syt1 are non-specific (47) and that Syt1 may act as a distance regulator to bring the vesicle and target (plasma) membranes into close proximity (48). Distance restraints between the two C2 domains of Syt1 show that it may align in an antiparallel fashion to bring the two membranes into close proximity (49), thereby allowing the SNARE proteins (synaptobrevin, syntaxin, and SNAP-25) to complete their assembly from an intermediate state.

Full-length Syt1 has been used to reconstitute Ca^{2+}-dependent membrane fusion, and it appears to be correctly folded at
FIGURE 5. a and b, background-subtracted DEER signals and distance distributions for single spin-labeled full-length Syt1 86, 90, and 95 in the juxtamembrane linker region, where the red traces represent the best fits to the dipolar evolution that was used to generate the distribution. The shaded regions in the probability distributions represent the range of solutions that can be achieved by variation of background subtraction (due to intermolecular spin interactions). These include all fits having root mean square deviation values within 15% of the best fit. c, DEER signals for single spin-labeled WT Syt1 86 with 5 times unlabeled Syt1 (blue), double spin-labeled WT Syt1 86/136 with 5 times unlabeled Syt1 dilution (magenta), single spin-labeled GA mutant of Syt1 at position 86 (green), and spin-labeled position at 90 of the truncated Syt1 (residues 1–140) (black, data; red, fit). It should be noted that the improved signal/noise ratio in the spin-dilute samples is due to a longer phase memory time for the sample. d, distance distribution for truncated Syt1 (residues 1–140) spin-labeled at position 90.
least when expressed in SF9 cells (50). However, most structural studies have been carried out on the globular portion of Syt1 only containing its two Ca\(^{2+}\)-binding C2 domains (Syt1 C2AB), and little is known regarding the structure of the full-length protein or the 60-residue linker connecting Syt1 C2AB to the synaptic vesicle. In the present study, we demonstrate that the bacterially expressed full-length protein is correctly folded and functional. A combination of continuous wave and pulse EPR spectroscopy shows that the linker, which attaches C2A and C2B to the vesicle membrane, is closely associated with the membrane interface and is structurally heterogeneous. This membrane interaction of the linker will limit the distance that C2A and C2B can extend from the membrane interface at the focal site of fusion. As seen in Fig. 3, positions up to 123 are closely associated with the interface (Fig. 3c), and the distance that C2A extends from the interface will be determined by the configuration of residues on the N-terminal end of the linker and by any conformational equilibrium exhibited by this segment.

The DEER data in Fig. 5 indicate that full-length Syt1 is oligomerized and that a glycine zipper in this region contributes to the interaction. The observation that Syt1 is oligomerized is consistent with earlier findings. It has been reported that a segment of Syt1, which includes residues 1–111, mediates the tetramerization of Syt1 (22). This tetramerization might be mediated in part by free cysteines that are present in the wild-type protein; however, this oligomerization is found to occur under conditions where disulfide cross-links cannot take place (23). It has been reported that the region encompassing residues 1–96 has the ability to induce clustering (21), and, on a more macroscopic scale, stimulated emission depletion microscopy shows that Syt1 is clustered in patches (51), although it is not clear from this study what features of Syt1 or its interactions with other components in the synaptic vesicle might be mediating clustering. The distances observed here by DEER are broad, with both short and long components. This would not be expected for a well defined symmetric helical tetramer, but such a pattern might result from linkers arranged in an antiparallel but structurally heterogeneous manner. The effective $T_2$ times measured in our pulse experiments are not exceptionally short (they are usually on the order of a few ms), indicating that the oligomer being observed is not a large aggregate.

The TIRF vesicle binding assay introduced here provides a method to examine the capacity of Syt1 to capture vesicles and to bind an opposing bilayer in the presence of Ca\(^{2+}\). Under the conditions used for this TIRF assay (Fig. 2b), the apparent Ca\(^{2+}\) dependence for vesicle association (33 $\mu$M) is comparable with the Ca\(^{2+}\)-dependent membrane affinity that has been reported for the binding of soluble Syt1C2AB domains to bilayers (52). The Syt1 GA mutant yields both slower binding rates and lower saturation levels when compared with the wild-type Syt1, indicating that modifications within the 60-residue linker have the ability to modulate Syt1 activity. The reduced activity of this mutant suggests that oligomerization of Syt1 facilitates vesicle capture (Fig. 6). Oligomerization will increase the local density of C2 domains, and this should increase the number of energet-
ically favorable membrane contacts made at the site of contact between the vesicle and the supported bilayer.

In membrane fusion, there are a number of roles that the oligomerization of Syt1 might play. A clustering of Syt1 and an increase in the density of Ca\(^{2+}\) sensors might facilitate vesicle-plasma membrane attachment during fusion. It has been observed that the Syt1 C2AB fragment responds to and alters fusion. In the physical state of the lipid that have been proposed to drive changes in the linker. The tendency of Syt1 to oligomerize may interact with target membranes is modulated by structural genetics of Syt1 oligomerization. There have been reports that phosphorylation occurs at three threonine residues (112, 125, and 128) within the linker of Syt1 (50, 59), and it is conceivable that phosphorylation within the linker alters the energetics of Syt1 oligomerization.

In summary, using EPR spectroscopy and a liposome capture assay, we investigated the structure of the region that links the two C2 domains to the vesicle membrane and the activity of full-length Syt1. A large portion of the juxtamembrane region is closely associated with the membrane interface, which will limit the extent to which the C2A and C2B domains might extend off the vesicle membrane. In addition, full-length Syt1 is oligomerized, a process that appears to be mediated by a glycine zipper region within the linker. Mutations within the glycine zipper diminish the capacity of Syt1 to capture liposomes in a Ca\(^{2+}\)-dependent manner, indicating that the ability of Syt1 to interact with target membranes is modulated by structural changes in the linker. The tendency of Syt1 to oligomerize may play an important role in mediating membrane fusion. The oligomeric state of Syt1 may control its ability to interact with target membranes, it may alter the protein and lipid composition at the focal site of fusion, and it may help facilitate changes in the physical state of the lipid that have been proposed to drive fusion.

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