Kinetics of Complexin Binding to the SNARE Complex: Correcting Single Molecule FRET Measurements for Hidden Events

Yulong Li,* George J. Augustine,*† and Keith Weninger‡
*Department of Neurobiology, Duke University Medical Center, Durham, North Carolina; †Marine Biological Laboratory, Woods Hole, Massachusetts; and ‡Physics Department, North Carolina State University, Raleigh, North Carolina

ABSTRACT Virtually all measurements of biochemical kinetics have been derived from macroscopic measurements. Single-molecule methods can reveal the kinetic behavior of individual molecular complexes and thus have the potential to determine heterogeneous behaviors. Here we have used single-molecule fluorescence resonance energy transfer to determine the kinetics of binding of SNARE (soluble *N*-ethyl maleimide-sensitive fusion protein attachment protein receptor) complexes to complexin and to a peptide derived from the central SNARE binding region of complexin. A Markov model was developed to account for the presence of unlabeled competitor in such measurements. We find that complexin associates rapidly with SNARE complexes anchored in lipid bilayers with a rate constant of $7.0 \times 10^6\ M^{-1}\ s^{-1}$ and dissociates slowly with a rate constant of $0.3\ s^{-1}$. The complexin peptide associates with SNARE complexes at a rate slower than that of full-length complexin ($1.2 \times 10^6\ M^{-1}\ s^{-1}$), and dissociates much more rapidly (rate constant $>67\ s^{-1}$). Comparison of single-molecule fluorescence resonance energy transfer measurements made using several dye attachment sites illustrates that dye labeling of complexin can modify its rate of unbinding from SNAREs. These rate constants provide a quantitative framework for modeling of the cascade of reactions underlying exocytosis. In addition, our theoretical correction establishes a general approach for improving single-molecule measurements of intermolecular binding kinetics.

INTRODUCTION

Complexin (also known as synaphin) is a small cytoplasmic protein (~15–21 kD mass) that binds to the SNARE (soluble *N*-ethyl maleimide-sensitive fusion attachment protein receptor) complex with high affinity (1,2) via a central, α-helical domain (3,4). High resolution structures derived from x-ray diffraction measurements show that this central domain of complexin selectively recognizes the interface between the coiled-coil domains of syntaxin and synaptobrevin within the SNARE complex (5,6). Recent in vitro experiments suggest that complexin clamps the *trans*-SNARE complex in a hemifusion state (7,8), which might be important to prime the SNARE complex for synaptotagmin binding (9).

The interaction between complexin and the SNARE complex is an essential step in Ca^{2+}-dependent exocytosis. For example, neurotransmitter release is drastically reduced when complexin genes are knocked out (10) or point-mutated (11,12) or when binding-site peptides are used to inhibit the interaction of complexin with SNARE complexes (13). Complexin is also linked to some neurological disorders (14,15).

Although it is generally accepted that complexin is essential for neurotransmitter release, the timing and mechanism of complexin's role remain controversial (7–10,12,13,16). A key step toward understanding the physiological action of complexin within an in vivo signaling network is the characterization of the interaction between complexin and SNARE complexes in a simplified system.

Here we have determined the kinetics of complexin binding to membrane-anchored SNARE complexes by using single-molecule fluorescence resonance energy transfer (smFRET) measurements to directly monitor the time-dependent FRET signal generated by the interactions between individual SNARE complexes and complexin. We also have developed a theoretical approach based upon Markov modeling to extract kinetic information from such measurements despite the unavoidable presence of the unlabeled competitive molecules. With these approaches we also could determine the binding kinetics of a peptide, derived from the central, SNARE-binding domain of complexin (SBD; residues 46–74), which has been used to inhibit binding of full-length complexin to SNAREs (13,17). Our measurements of the kinetics of the SNARE complex binding to complexin and to the complexin peptide provide strong constraints for models of complexin-dependent exocytosis and provide biophysical insights into the dynamic regulation of membrane fusion. The combination of the smFRET approach and our Markov analysis can be generalized easily to study intermolecular interactions that occur in other biological settings.

MATERIALS AND METHODS

Sample preparation

Proteins: plasmids, mutations, expression, purification, and labeling

A cDNA construct for full-length complexin fused to a hexahistidine tag was provided by T. Abe (Niigata, Japan). Plasmids for glutathione S-transferase

Submitted November 15, 2006, and accepted for publication May 10, 2007.

Address reprint requests to Keith Weninger, E-mail:keith_weninger@ncsu.edu.

Yulong Li's present address is Dept. of Molecular and Cellular Physiology, Stanford University, Stanford, CA 94305.

Editor: David W. Piston.

0006-3495/07/09/2178/10 $2.00

doi: 10.1529/biophysj.106.101220

fusions of the cytoplasmic domain of synaptobrevin (1–94) and full-length SNAP-25 have been described previously (18). These fusion proteins were expressed and purified by glutathione-Sepharose with standard methods. Hexahistidine-tagged, full-length rat syntaxin-1A in pet28a and His-tagged, full length complexin were expressed, purified, and labeled as described earlier (19). Thrombin treatment followed by ion-exchange chromatography was used to remove glutathione S-transferase and hexahistidine tags.

In these plasmids, all cysteine residues in wild-type syntaxin-1A and complexin were mutated to serine to allow for site-specific dye labeling via engineered cysteine residues. The mutations $E^{39}C$ (complexin) and $E^{41}C$ (synaptobrevin) were created using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA). Mutations were selected with guidance from the crystal structure of the truncated neuronal SNARE complex (20) and the structure of the complexin/SNARE complexes (5,6).

Proteins were labeled as described earlier (19) and labeling efficiency was determined by absorbance spectroscopy. The $E^{41}C$ synaptobrevin mutant was labeled with Alexa 647 maleimide (Invitrogen, Carlsbad, CA) at 84% efficiency. Full-length complexin with the $E^{39}C$ mutation was labeled with Alexa 555 maleimide at 30% ± 4% efficiency based upon the published extinction coefficients for the dyes (Invitrogen). We estimate the uncertainty of labeling from the observed spread of several independent absorption measurements of a single sample. Alexa-555-labeled complexin peptide was synthesized by a commercial source (Global Peptide, Fort Collins, CO) and dye-labeling efficiency of this peptide was >90%. The sequence of the peptide was: Alexa555-CERRKEKHRKMEEEREEMRQTIRDKYGLKK.

Formation of SNARE complexes

SNARE complexes were formed in solution as described previously (19). Briefly, syntaxin was mixed with full-length SNAP-25 at a 1:2 molar ratio followed by addition of a 1:5 molar ratio of the synaptobrevin cytosolic domain. After overnight incubation, SNARE complexes were purified away from free synaptobrevin by anion exchange on monoQ resin in Tris-buffered saline (TBS) (20 mM Tris at pH 8.2, 200 mM NaCl, 1 mM dithiothreitol) containing 100 mM β-d-octyl glucoside (Anatrace, Maumee, OH). Formation of the SNARE complex was confirmed by SDS-PAGE without boiling. Further boiling dissembled the SNARE complex (data not shown).

Reconstitution into liposomes

A chloroform solution of egg phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) was dried under flowing argon inside a glass culture tube and then placed in a vacuum for several hours. TBS was added to yield a final lipid concentration of 30 mg/ml and the solution was passed 21 times through 50-nm pore-size filters with the Mini-Extruder (Avanti Polar Lipids).

Preformed SNARE complexes were reconstituted into liposome solutions as described earlier (19). Briefly, protein solutions (80 nM) in TBS containing 100 mM β-d-octyl glucoside were mixed at a 1:4 ratio with 30 mg/ml liposome samples and incubated at 4°C for 30 min. These mixtures were then diluted 1:1 with detergent-free TBS and separated from detergent and unincorporated protein using size-exclusion chromatography on a Sepharose CL4B column (GE Healthcare Bio-Sciences, Piscataway, NJ) in detergent-free TBS.

Microscopy and data analysis

Fluorescence microscopy of supported lipid bilayers

Bilayers containing SNARE complexes were formed on the surface of a flow cell between a quartz microscope slide and a coverslip (see Fig. 1 *b*). Ultraviolet curing optical adhesive (Norland Products, Cranbury, NJ) sealed the edges of the chamber, and buffers were exchanged through holes drilled in the quartz slide. Bilayers formed by self-assembly during incubation of liposomes containing reconstituted SNARE proteins (3 mg/ml lipid for 10 min) in the flow channel. Liposomes reconstituted with SNAREs were diluted with protein-free liposomes before bilayer formation to make sure that the spacing between adjacent SNARE complexes in the bilayer was greater than the spatial resolution of the microscope. The incubation with SNARE-containing liposomes was followed by a second incubation with protein-free liposomes (15 mg/ml lipid for 10–30 min), which improved resistance to nonspecific binding of soluble proteins to the surface.

These supported bilayers were illuminated by prism-type total internal reflection of coaxial 532-nm and 635-nm lasers. To allow sequential excitation of both donor and acceptor fluorophores, laser illumination was alternated in the sequence 635 nm for 1 s, 532 nm for 45 s, then 635 nm for 5 s. The illuminated region was observed by a 60× 1.2-NA water immersion objective (Olympus America, Center Valley, PA). A cy3/cy5 emission filter (Chroma Technology, Rockingham, VT) blocked laser excitation light in the emission path. A 645dcxr dichroic mirror (Chroma) split the emitted fluorescence light into two spectral bands that were relayed side by side onto a charged-coupled device detector (Cascade 512B, Roper Scientific, Tucson, AZ). Single fluorophores were identified based on their fluorescence intensity, quantized photobleaching, and spatial characteristics. All observations were conducted at room temperature in TBS buffer augmented with 2% glucose and an enzymatic oxygen scavenger system (100 units/ml glucose oxidase, 1000 units/ml catalase, and 200 μM cyclooctatetraene) to reduce photobleaching. FRET efficiency (E) was calculated from the background-subtracted intensities of the acceptor (I_{acceptor}) and donor (I_{donor}) as $E = I_{\text{acceptor}}/(I_{\text{acceptor}} + I_{\text{donor}})$ from fixed locations in the bilayer identified to contain an acceptor during the initial 635-nm illumination phase.

Measurement of dwell-time distributions

To examine the kinetics of complexin binding, fluorescence was measured at locations where preidentified SNARE complexes were present. The beginning of a binding event was defined by the rapid appearance (within a single image frame) of donor or acceptor emission with intensity signal levels typical of a single molecule, whereas the rapid disappearance of emission denoted unbinding. In a small subset of traces, fluorescence intensity recorded from a fixed location drifted or faded away without such rapid transitions. For example, the intensities for the recordings on the left-hand side of Fig. 2 *b* smoothly drift during the complexin-binding event. These cases presumably arise from the mobility of SNARE complexes reconstituted into the supported bilayers. In our measurements, ~90% of the SNARE complexes were immobile, within our experimental resolution, whereas the remaining 10% exhibited detectable movement. This small mobile fraction is consistent with a previous fluorescence recovery after photobleaching study that observed that 3–7% of syntaxin molecules reconstituted into supported bilayers were mobile, with a diffusion coefficient of 0.07 $\mu m^2/s$ (21). All of the kinetic data reported in our article were derived from traces with stable intensities during binding events. However, including traces with drifting intensities did not produce significant changes in the measured kinetic parameters.

All data were acquired at 100 ms/frame with the exception of some complexin peptide results, which were acquired at 15 ms/frame where indicated. The k_{off} rate was determined at 20–100 nM for complexin or complexin peptide. The duration of the bound state was taken from the dwell time in the high FRET efficiency state and k_{off} was determined from the time constant of exponential fits to histograms of dwell times.

The k_{on} rate constant was determined at 100–200 nM complexin or complexin peptide. Because the high background fluorescence associated with high concentrations of complexin or the complexin peptide obscured donor emission, we measured the time interval between consecutive high FRET efficiency events as the ''dwell time'' in the unbound state under these conditions. To test whether photobleaching affects determinations of binding kinetics, fluorescence dwell-time data obtained during the first 20 s of illumination were compared to data acquired from 20 to 40 s of illumination. No differences were observed (data not shown), which indicates that