Supporting Methods

Molecules

Protein Expression and Purification. All SNARE proteins were expressed from pET28a in the BL21 (DE3) strain of *Escherichia coli* according to standard techniques (1, 2). Bacterial lysis was facilitated by the addition of 6 M urea. The recombinant proteins contained N-terminal His_6 tags that served to affinity purify them on nickel-nitrilotriacetic acid-agarose (Qiagen). After affinity purification, 5 mM DTT and 2 mM EDTA were added. The tags were cleaved off by thrombin. Cleavage occurred overnight during concomitant dialysis (50 mM NaCl/20 mM Tris•HCl, pH 7.4/1 mM DTT). Subsequently, proteins were purified on a MonoQ (syntaxin and SNAP-25) or MonoS column (synaptobrevin) using the Äkta Explorer (Amersham Pharmacia Biotech). The fractions were analyzed by SDS/PAGE and Coomassie staining. Proteins were determined to be at least 95% pure. Expression and purification of munc-18 was the same as for the SNARE proteins with the exception that bacteria were induced for 4 h at 25°C and 0.5% Triton X-100 was used instead of urea in the extraction step. The final purification step occurred on a MonoQ column. Before labeling, DTT was removed from the proteins by gel filtration using PD-10 columns and PBS as elution buffer (10.4 mM Na_2HPO_4/3.2 mM KH_2PO_4/123 mM NaCl).

Calculation of the Species-Weighted Average Fluorescence Lifetime, $\bar{\tau}_{D(A)}$. Detailed analysis of the fluorescence decay of the subensemble of FRET-active molecules indicated a weak reversible fluorescence quenching of the donor dye, resulting in a double exponential fluorescence decay with the fluorescence lifetimes, τ_{DN}, and species amplitudes, x_{DN} (τ_{D1} = 2.82 ns, x_{D1} = 0.61 and τ_{D2} = 5.64 ns, x_{D2} = 0.39). This fast photochemical equilibrium does not perturb the FRET process; however, it has to be taken in account for the calculation of the appropriate FRET parameters. Knowing that the maximum-likelihood estimator used for the calculation of single-molecule fluorescence decay data gives results that are close to a fluorescence-weighted average fluorescence lifetime, we developed a correction procedure, which assumes that only the unquenched donor is deactivated by FRET. The goal was to compute the fluorescence lifetime of the donor not perturbed by reversible quenching, because this is the appropriate variable to be used in the calculations of the FRET efficiency (3). Therefore, fluorescence decays with a exciplex mechanism (4) and FRET were simulated to obtain a linear correction factor of 0.89. This value was used to rescale all experimentally determined $\tau_{D(A)}$ values to their photophysical meaningful ones represented by species weighted average fluorescence lifetimes $\bar{\tau}_{D(A)}$.

Correlation Analysis. The normalized autocorrelation (AC) function, $G(t_c)$, with the correlation time, t_c, allows for the analysis of fluctuations in the fluorescence signal, $\delta S(t)$, about an average value $\langle S(t) \rangle$:

$$G(t_c) = \frac{\langle S(t)\, S(t+t_c)\rangle}{\langle S(t)\rangle^2} = 1 + \frac{\langle \delta S(t)\, \delta S(t+t_c)\rangle}{\langle S(t)\rangle^2} \qquad \textbf{[3]},$$

where $S(t) = \langle S(t)\rangle + \delta S(t)$. The calculation of the correlation function on selected single-molecule bursts is performed as described by Eggeling *et al.* (5). Thereby, the AC curves of the donor AC_{DD} (G_{GG}) and the acceptor AC_{AA} (G_{RR}) are calculated from the signal in green (S_G) or red (S_R) detection channels, respectively.

In a reversible two-state system, the crosscorrelation curves (CC) of D and A can be computed as the arithmetic mean of the correlations of the signal in the two spectral detection ranges.

$$CC(t_c) = \frac{\left(G_{GR}(t_c) + G_{RG}(t_c)\right)}{2} = \frac{1}{2}\left[\frac{\langle S_G(t)\, S_R(t+t_c)\rangle}{\langle S_G(t) S_R(t)\rangle} + \frac{\langle S_R(t)\, S_G(t+t_c)\rangle}{\langle S_R(t) S_G(t)\rangle}\right] \qquad \textbf{[4]}$$

Typically, fluorescence fluctuations are caused by translational diffusion and photochemical processes, resulting in decay (bunching) terms in the AC curves. If a spatial three-dimensional Gaussian distribution of the detected fluorescence is assumed, and if translational diffusion through the detection volume (V) is the only noticeable process of the fluorescent molecules generating fluctuations [$\delta S(t)$], $G(t_c)$ is given by

$$G(t_c) = 1 + \frac{1}{N}\left(\frac{1}{1+(t_c/t_{TD})}\right)\left(\frac{1}{1+(\omega_0/z_0)^2 \times (t_c/t_{TD})}\right)^{1/2} = 1 + \frac{1}{N} G_{TD}(t_c) \qquad \textbf{[5]}$$

The diffusion part $G_{TD}(t_c)$ is described by the characteristic time for translational diffusion, $t_{TD} = \omega_0^2/4D_t$ of the fluorescent molecules through V where ω_0 is the radial $1/e^2$ radius of V, and D_t is the translational diffusion coefficient of the fluorescent molecules. The amplitude of the time-dependent term $G(t_c = 0)$-1 is equivalent to the normalized variance of the fluorescence (second-order central moment of light intensity). Ideally, it is given by the inverse average number of molecules in the detection volume ($1/N$). However, in single-molecule experiments, it is crucial to correct for the decrease of the amplitude $G(t_c = 0)$-1 due to the background signal.

Consider a FRET-active molecule, with a donor (green) and an acceptor (red) fluorophore attached to it, which switches between two conformational states (closed/open), and which only emits green when open and red when closed, and where effects of differences in the fluorescent brightness between the green and red dye are neglected. The time-dependent part of the normalized auto- and CC functions AC and CC of the fluorescence fluctuations in the green and red detector channels can then be written as (compare Eq. **2** in the main text):

$$AC_{DD}(t_c)=DC+\frac{1}{N_{AC}}\frac{G_{TD,AC}(t_c)}{(1-A_{DD})(1-B_{cl})}\times\left[1-A_{DD}+A_{DD}\exp\left(-t_c/t_{A_{DD}}\right)\right]\times\left[1-B_{cl}+B_{cl}\exp\left(-t_c/t_r\right)\right]$$

$$AC_{AA}(t_c)=DC+\frac{1}{N_{AC}}\frac{G_{TD,AC}(t_c)}{(1-A_{AA})(1-B_{op})}\times\left[1-A_{AA}+A_{AA}\exp\left(-t_c/t_{A_{AA}}\right)\right]\times\left[1-B_{op}+B_{op}\exp\left(-t_c/t_r\right)\right]$$

$$CC(t_c)=DC+\frac{1}{N_{CC}}G_{TD,CC}(t_c)\times\left[1-B_{CC}\exp\left(-t_c/t_r\right)\right]$$

[6]

In our analysis, $G_{TD}(t_c)$ is determined by the characteristic observation time due to the cutting of the bursts, rather than by the characteristic time for translational diffusion (t_{TD}).

Data Analysis. First, the specific diffusion parameters $G_{TD}(t_c)$ of AC_{DD}, AC_{AA}, and CC were determined from the mutant Sx59/105 (Fig. 2*C* in the main text), where the fluorescence is not influenced by the conformational dynamics, as evidenced by a sharp R_{DA} peak. In the next analysis, steps these parameters were kept constant. Considering the mutant Sx91/225 with a broad R_{DA} peak (Fig. 2*D* in the main text), where the labels are at positions between which distance changes take place, the resulting AC and CC curves were fitted simultaneously, with t_r as a global parameter and the other variables as local partially fixed parameters. Analysis of the CC curves yielded an amplitude B_{CC} = 0.3 ± 50% with a relaxation time t_r = 0.8 ± 0.3 ms for the *CC* rise term (black line) and a characteristic observation time of t_{TD} = 1.8 ± 0.3 ms. The relaxation time was well in agreement with that found from global analysis of the *AC* and *CC* curves of other mutants.

Influence of the Habc Domain on SNARE-Complex Formation Measured by Steady-State Fluorescence Anisotropy. All measurements were carried out in a Fluoromax 1971 fluorometer (Jobin-Yvon Longjumeau, France) with automated polarizers. Excitation and emission wavelengths were 488 and 522 nm, respectively. The slit widths were set at 7 nm and the integration time at 1 sec. The *G*-factor was determined according to $G = I_{HV}/I_{HH}$, where I_{HV} and I_{HH} are the fluorescence intensities of the vertically and horizontally polarized emissions when the sample is excited with horizontally polarized light. After measuring the intensities of the vertically and horizontally polarized emissions upon vertically polarized excitation (I_{VV} and I_{VH}, respectively) the anisotropy values were computed automatically by the software according to the following relationship: $r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$ (3). The temperature was kept at 25°C throughout the experiment. All assembly reactions took place in polystyrol cuvettes from Sarstedt in a final volume of 3 ml.

SNAP-25, labeled at position 84 (SN84) with Alexa 488-maleimide was added to preheated (25°C) buffer (PBS/0.1 mg κ-casein/ml/0.1 %Triton X-100). After 10-20 measuring points were taken (≈5-10 min), synaptobrevin, and either syntaxin with [Sx(1-262)] or without Habc domain [Sx(180-262)] were added. The final concentration of SNAP-25 was always 5 nM. Synaptobrevin and syntaxin were used at equal concentrations that increased from 250 nM up to 4 μM, depending on the reaction studied.

The anisotropy values taken prior to complex formation were averaged and set to zero. Each curve was fitted by a monoexponential rise function, according to pseudo first-order reaction kinetics. The individual pseudo first-order rate constants of each set of experiments were plotted against the concentration of syntaxin/synaptobrevin. The rise of the curves yielded the second-order rate constants.

Free Energy Calculation. Assuming that the dissociation rates of the ternary complex of the different constructs are equal and slow compared with their formation (k_2 <1 $M^{-1}s^{-1}$), the second-order rate constants served to compute free energy differences for the various assembly reactions. According to $\Delta G = -RTlnK$, binding of syntaxin's N terminus to the H3 domain involves the release of ≈1 kcal/mol. This value again was used to estimate the ratio between open and closed states. Computing the equilibrium constant for the backfolding of syntaxin (according to $e^{-(\Delta G/RT)} = K$) revealed that only ≈16% of the protein are closed.

Results. While it is well established that the closed conformation of syntaxin is inactive and cannot assemble with other SNAREs, it remains to be clarified whether the H3 region in the open configuration is unimpeded in its ability to form SNARE complexes. We have therefore determined the effective pseudo first-order rate coefficients, k_1, of SNARE complex formation by steady-state fluorescence anisotropy measurements using labeled SNAP-25, varying concentrations of unlabeled synaptobrevin and either complete syntaxin, Sx(1-262), or isolated H3 domain, Sx(180-262) (Fig. 4). The obtained second-order rate coefficient, k_2, for complete syntaxin (890 ± 30 l $mol^{-1}s^{-1}$) was 7.1-fold smaller than that for the isolated H3 domain (6,300 ± 400 l $mol^{-1}s^{-1}$). Assuming equal dissociation rates of the complex, a free-energy difference of ≈1 kcal/mol of the two assembly reactions can be calculated. If the H3 domain in the open conformation is unimpeded, this energy corresponds to the energy difference between the open and the closed state of syntaxin. Determination of the equilibrium constant (according to $K = e^{-\Delta G/RT}$) revealed that at least 12% of syntaxin is in the closed conformation, which is in good agreement with the values obtained by our single-molecule measurements. This result indicates that the adoption of an open conformation of free syntaxin is a rate-limiting step in SNARE-complex formation.

Extraction of Structural Information. For the calculation of FRET distance histograms, the C^{α} positions, x_i , and the dye positions, y_i , of the labeled residues were considered (Fig. 3 *Inset*, in the main text). Fluctuations of the protein and the dye molecules were described by a potential function $V = \sum_{ij} V_{ij}^{CC} + \sum_l V_l^{dye}$, including soft distance restraints $V_{ij}^{CC} = \frac{1}{2} k_{ij}^{CC} \left(\left| \boldsymbol{x}_i - \boldsymbol{x}_j \right| - d_{ij} \right)^2$ for all 15 residue pairs, *ij*, as well as terms $V_l^{dye} = \frac{1}{2} k^R \left(\left| \boldsymbol{x}_l - \boldsymbol{y}_l \right| - R \right)^2 - k_l^{dye} \hat{\mathbf{r}}_l \cdot \hat{\mathbf{r}}_l^0$, for the link between each dye *l* and residue *l*, where R = 16 Å is the average dye-C^{α} distance, k^R describes its fluctuation, and $\hat{\mathbf{r}} = (\boldsymbol{x}_l - \boldsymbol{y}_l) / \left| \boldsymbol{x}_l - \boldsymbol{y}_l \right|$ is the dye linker direction. Optimized were the structural parameter vectors μ, containing average C^{α} distances, d_{ij}, the stiffnesses, k_{ij}^{CC}, describing the fluctuations around these average distances, and the average orientations, $\hat{\mathbf{r}}_l^0$, of the dye

molecules with respect to the C^{α} positions. For the average fluctuations of these orientations as seen in a burst, $k_l^{dye} = nk_BT/\theta_{\min}^2$ was chosen with a wobbling cone angle, $\theta_{\min} = 40^0$, and the average photon number/burst, *n*.

Structure sets were generated from Metropolis Monte Carlo runs, yielding distributions, p_{ij}^m, for the dye pair distances *ij* (binned in 1-Å intervals, *m*) and averages $\langle ... \rangle$. A full set of FRET-compatible structural parameter vectors was identified by multiple optimization runs, each starting from randomly perturbed initial values for μ obtained from the previous run until convergence. To account for the widening of the measured distributions due to shot noise, p_{ij}^m was numerically convoluted with a distance-dependent normalized Gaussian function, $g(m-m') \propto \exp\left[-\frac{1}{2}\Delta^2 (m-m')^2/(a+mb)\right]$, where the coefficients a = 2.0244 Å^2 and b = 0.035 Å^2 were obtained from a fit-to-shot noise data. Prior to each steepest descent step, gradients were computed from a complete Monte Carlo cycle (8,000 steps each) using the actual structural parameters, μ. Typically, 20-30 steepest descent steps were required to reach convergence. Optimization was carried out by minimizing the total root-mean-square difference, $\Delta = \sum_{ij} \sum_m \left(\tilde{p}_{ij}^m - p_{ij}^m\right)^2$, between measured $(\tilde{p}_{ij}^m)$ and simulated distance distributions (p_{ij}^m) via steepest descent using $\partial\Delta/\partial\boldsymbol{\mu} \propto \sum_{ij} \sum_m \left(\tilde{p}_{ij}^m - p_{ij}^m\right)\left[\langle \partial V/\partial\boldsymbol{\mu} \rangle \tilde{p}_{ij}^m - \left\langle \partial V/\partial\boldsymbol{\mu}\phi_{ij}^m \right\rangle\right]$, where $\phi_{ij}^m = 1$ if $\left|\boldsymbol{y}_i - \boldsymbol{y}_j\right|$ is in bin m, and zero otherwise. The resulting average distances between C^{α} atoms were used to generate atomic models for the open conformations (Fig. 3*B* in the main text) with the programs MOLMOL (6) and QUANTA (7). The models were energy-minimized with EGO (8).

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