Solution structure of the ESCRT-I complex by small-angle X-ray scattering, EPR, and FRET spectroscopy

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ESCR-T-I is required for the sorting of integral membrane proteins to the lysosome, or vacuole in yeast, for cytokinesis in animal cells, and for the budding of HIV-1 from human macrophages and T lymphocytes. ESCRT-I is a heterotetramer of Vps23, Vps28, Vps37, and Mvb12. The crystal structures of the core complex and the ubiquitin E2 variant and Vps28 C-terminal domains have been determined, but internal flexibility has prevented crystallization of intact ESCRT-I. Here we have characterized the structure of ESCRT-I in solution by simultaneous structural refinement against small-angle X-ray scattering and double electron-electron resonance spectroscopy of spin-labeled complexes. An ensemble of at least six structures, comprising an equally populated mixture of related closed conformations, was necessary to fit all of the data. This structural ensemble was cross-validated against single-cysteine mutants, the yeast ESCRT-0 (13, 16), and human UEV domains bound to ubiquitin (14, 15), and the human UEV domain bound to peptides from HIV-1 and ESCRT-0 (13, 16). Yeast ESCRT-I binds to ESCRT-II via the C-terminal domain (CTD) of Vps28 (17), whose structure has been determined (18, 19) (Fig. 1C). Vps23, Vps28, and Vps37 assemble via a trimeric subcomplex known as the “headpiece” (17, 20) (Fig. 1D). Vps23, Vps37, and Mvb12 form an elongated stalk (21) (Fig. 1D). The stalk and headpiece collectively comprise the core. Four other regions are absent from the solved structures. A 60-residue Pro-rich linker connects the UEV and stalk portions of Vps23 and includes the midbody localization sequence (22). A 30-residue flexible linker connects the headpiece and CTD of Vps28. The N-terminal predicted helix (NTH; Fig. 1E) of Vps37 is basic, predicted to be mostly helical, and contributes to membrane binding (21). Flexible hydrophobic residues at the C terminus of Mvb12 may be a ubiquitin binding domain (23). Fundamental to understanding the mechanism of ESCRT-I-mediated membrane budding is a better understanding of the arrangement of the functional domains in three dimensions.

ESCR-T-I is representative of a class of signaling, trafficking, and regulatory proteins in this size range that contain intrinsically disordered regions. This class presents a fundamental unsolved challenge to structural biologists. The presence of flexible regions has precluded crystallization of intact ESCRT-I, whereas the low molecular weight of the complex (108 kDa) has precluded single particle EM analysis. We report here an effort to construct a definitive solution structure of intact ESCRT-I and develop an approach to the structural analysis of midsize protein complexes with regions

T he endosomal sorting complex required for transport (ESCRT) complexes are required for ubiquitin-dependent receptor down-regulation, multivesicular body (MVB) biogenesis, the budding of HIV-1 and most other enveloped viruses, and cytokinesis (1, 2). From yeast to humans, ESCRTs function as discrete protein sorting

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Structural domains and labeling sites. (A) Domain schematic of the four subunits of ESCRT-I, colored blue (Vps23), red (Vps28), gray (Vps37), and orange (Mvb12) showing a speculative model for their orientation relative to the neck of a membrane bud, adapted from ref. 3. (B) UEV domain of Vps23. (C) CTD of Vps28. (D) Core assembly. (E) NTH of Vps37. Engineered Cys residues are shown in a filled sphere model.}
\end{figure}
of intrinsic disorder. Small-angle X-ray scattering (SAXS) and solution NMR are a powerful combination (24) because the global shape information from SAXS complements short-range NMR constraints. A natural counterpart of this approach for larger complexes is to combine SAXS with spectroscopic techniques that yield longer-range constraints by utilizing site-specific or selective labeling. These techniques include double electron–electron resonance (DEER) (25) and FRET spectroscopy (26). SAXS, DEER, and FRET data were collected for ESCRT-I in solution. A molecular simulation framework was devised for the joint refinement of protein complex structures against SAXS and spectroscopic data, leading to an improved model of the complete ESCRT-I structure. We show that ESCRT-I in solution is in an equilibrium between roughly equal populations of open and closed conformations. We describe these populations in terms of a set of six structures that serve as snapshots of a larger continuum of states.

Results

**SAXS of ESCRT-I.** The solution structure of full-length Cys-free yeast ESCRT-I was analyzed by SAXS. The $I(q)$ vs. $q$ curve showed features to $q = 0.5 \text{ Å}^{-1}$ (Fig. 2A). The maximal protein dimension $D_{\text{max}}$ was determined to be 225 Å (Fig. 2C). SAXS data were initially analyzed by ab initio envelope generation (27). The envelope thus obtained (Fig. 2D) was elongated and had a large lobe at one end and a smaller lobe at the other. The envelope allowed us to make a visually satisfactory fit of the ESCRT-I core by placing the stalk into the elongated center of the envelope and the headpiece into one lobe (Fig. 2E). However, the envelope lacked sufficient detail to position the UEV, CTD, and NTH regions accurately. Indeed, the Kratky plot (Fig. 2B) is characteristic of a partially unfolded or otherwise noncompact protein (28). Given the evidence for intrinsic flexibility in the connecting regions of ESCRT-I, the appropriateness of modeling these regions into the ab initio envelope in a unique conformation is not clear a priori.

**Ensemble Refinement of ESCRT-I SAXS.** To sample physical configurations of ESCRT-I, we used a coarse-grained model for protein binding (29). The core, Vps23-UEV, Vps28-CTD, and Vps37-NTH were treated as rigid domains. The interactions between the domains were treated at the residue level with amino acid dependent pair potentials and Debye–Hückel-type electrostatic interactions. Flexible linker peptides connecting the four rigid domains are represented as amino acid beads on a polymer with appropriate stretching, bending, and torsion-angle potentials. Monte Carlo simulations of ESCRT-I were carried out using a starting model based on the crystal structures of yeast Vps23 UEV [Protein Data Bank (PDB) ID code 1UZX], yeast Vps28 CTD (PDB ID code 239U), the yeast ESCRT-I core (PDB ID code 2P22), a model of the NTH in a helical conformation, and linkers generated in sterically allowed but otherwise arbitrary conformations. The resulting 10,000 conformations were clustered and fit to the experimental $I(q)$ curve by using the ensemble refinement of SAXS (EROS) program (30). An acceptable fit could be obtained with a minimum of two conformations, weighted equally (Fig. 2F). However, no single conformation was adequate to fit the $I(q)$ data. One of the two conformations is more closed (black, Fig. 2E) and the other more open (yellow, Fig. 2E). In the more closed structure, both the UEV and CTD domains approach close to the stalk in “cis,” such that they are both on the same side of the stalk. The UEV and CTD do not touch one another even in the more closed conformation.

In the more open conformation, the CTD is farther from the stalk, but still on the *cis* face, whereas the UEV has swung away from the stalk to project past the tip of the stalk. In the more open conformation, there are no direct contacts between either the UEV or CTD and the core. Without interactions to hold these domains firmly in position, the open conformation is likely representative of an ensemble of structures that fill a similar scattering volume in solution. This analysis led us to conclude that at least half the population of ESCRT-I complexes in solution adopts a dynamic structure in which the UEV and CTD are mobile with respect to the core.

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**Fig. 2.** SAXS of ESCRT-I. (A) Fit of the simulated scattering curves [$I(q)$] to the observed scattering of ESCRT-I. (B) Kratky plot of the same experimental data and simulated scattering shown in A. Experimental $I(q)$ data points in A and B represent the mean of 10 consecutive measurements of the same sample, and the error bars represent the standard error of the mean. (C) Pair distribution function [$P(r)$] for ESCRT-I. (D) An ab initio molecular envelope for ESCRT-I. (E) Superposition of the two EROS-refined structures of ESCRT-I. (F) Fit of experimental SAXS data of A to values computed from the structures fit to both SAXS and DEER data.
DEER EPR Analysis of ESCRT-I. Four sets of unique Cys pairs were engineered into the Cys-free yeast ESCRT-I construct and labeled with the spin probe (1-oxyl-2,2,5,5-tetramethyl-∆3-pyrorline-3-methyl) methanethiosulfonate (MTSL). Cys pairs were selected using the EROS-refined structures to constrain the most ambiguous interdomain distances. Solvent-exposed residues in crystallized regions were selected to avoid interference with complex assembly. The pair Vps28 Cys27—Vps37 Cys173 was chosen within the crystallized headpiece region to serve as a control. The remaining three pairs were designed to measure the position of one of the major functional domains relative to the core. Vps23 Cys108—Vps23 Cys256 allowed measurement of the UEV-core separation. Vps28 Cys65—Vps28 Cys151 and Vps23 Cys223—Vps37 Cys12 were used to measure the CTD-core and NTH-core separations, respectively.

Each labeled ESCRT-I sample yielded interpretable DEER spectra (Fig. 3). In a Gaussian fit, the control pair Vps28 Cys27—Vps37 Cys173 produced a sharply peaked $P(r)$ distribution centered at 52 Å (Table S1). The positions of the MTSL labels were modeled onto the structures using the appropriate distribution of MTSL conformers (31). For the control pair, the $P(r)$ distribution calculated from the modeled MTSL coordinates is in excellent agreement with experiment (Fig. 3, Top). Thus this MTSL label faithfully reported a known intracomplex distance in ESCRT-I. The Vps23 Cys108—Vps23 Cys256 and Vps23 Cys223—Vps37 Cys12 samples yielded $P(r)$ distributions in Gaussian fits that were about twice as broad as the intracore $P(r)$ described above (Table S1). From the broad $P(r)$ distributions for the UEV-, CTD-, and NTH-core pairs, we conclude that all of these domains are mobile with respect to the core.

Combined SAXS and DEER Refinement. In order to avoid introducing regularization-dependent artifacts into the structural refinement, the structures were refined directly against the measured $V(t)$ DEER data. The EROS procedure (30) was modified to allow simultaneous fitting of SAXS and DEER data with a minimal ensemble of structures. In the ensemble refinement, the structure weights $w_k$ were varied to improve the agreement between

![Fig. 3. DEER of ESCRT-I. (Left) Experimentally observed (solid) modulation of $V(t)$ together with points calculated from six structural clusters. (Right) Histograms of MTSL distances computed from the models. The green curves in the top panels show the results from Gaussian regularization.](image-url)
the refined ensemble \((I(q), V_{ij}(t))\) and the measured \(I_{\text{obs}}(q), V_{ij}^{\text{obs}}(t)\) signals. To find a minimum set of structures that jointly account for both the SAXS and DEER data, the function \(G = \sum_{ij}X_{\text{DEER}(ij)} + X_{\text{SAXS}} + \mu m\) was minimized numerically using a Monte Carlo search algorithm in which the structure weights were varied between \(m\) under the constraint \(\sum_kw_k = 1\). In the formula above, the parameter \(\mu\) controls the number \(n\) of structures with nonzero weights \(w_k > 0\), allowing us to identify a minimal set of representative structures. For small parameters \(\mu \ll 1\), we obtained very good fits to the experimental data \((\chi^2 < 1)\) with many structures in the refined ensemble \((n \gg 1)\). For large parameters \(\mu \gg 1\), the ensemble contained very few structures with nonzero weights, but the agreement with experimental data was not satisfactory \((\chi^2\text{ was significantly larger than }1)\). The balance was struck at \(\mu \approx 0.2\), when we obtained good fits to all experimental datasets \((\chi^2 \approx 1)\) with only \(n = 6\) structures (Figs. 2F and 3).

Qualitatively, the ensemble of six structures (Fig. 4) resembles the open and closed conformations used to fit the SAXS data and fills the same overall region of space. The most closed of the six SAXS-EPR conformations resembles the closed SAXS conformation (Fig. 2E). Another structure has the UEV, CTD, and NTH displaced from the core in a highly extended state. The remaining four have one or two of the domains in more closed states. The UEV folds back against the cis side of the stalk. In each of the three structures with a closed UEV conformation, the UEV fills a similar region of space, but different surfaces of the UEV interact with slightly different portions of the core. In one of the structures, the ubiquitin binding site \((14)\) is occluded.

The NTH folds back along the stalk such that similar regions of space are filled. Again, in each case the precise angle between the NTH and the stalk varies, as do the details of molecular contacts. Two of the three closed states of the CTD involve packing against the distal tip of the headpiece, which is formed by the N-terminal half of the Vps28 subunit. These closed conformers fill the same region of space and contact the same part of the headpiece, but the CTD four helix bundle is rotated 120° apart in the two states. The UEV folds back against the cis side of the stalk.

In the third state, the CTD also contacts the N-terminal half of Vps28, but in this case a slightly different region of space is filled, and the CTD points back toward the cis side of the core. The precise closed state conformations of each of the three domains vary enough that reliable conclusions about the detailed nature of the closed state, or even whether there is a single closed conformation, cannot be drawn. Notably, even in the most open conformations, the space on the “trans” side of the stalk is avoided. Thus even in the context of a highly flexible and dynamic structure, there are distinct preferences for domains to occupy some regions of space over others.

**Bulk FRET Measurements.** We sought to cross-validate the refined ESCRT-I structure with independent data excluded from the refinement. Bulk FRET lifetime data were obtained on a sample labeled with Atto488 and Atto594 on Cys137 and Cys223 of Vps23, which are on the UEV domain and stalk, respectively. This pair provides an independent check on the UEV-core separation, distinct from the MTSL-labeled Cys108-Cys256 pair used in refinement. The donor lifetime was found to be \(\tau_D = 3.7\) ns, which was reduced in the presence of the acceptor to \(\tau_{D_A} = 3.4\) ns (Fig. S1). The FRET efficiency calculated from the structures (see SI Methods) was \(E = 0.11\), which is in good agreement with the mean efficiency \(E = 0.09\) measured in the bulk FRET experiment.

**Single-Molecule FRET.** To provide an independent check on the Vps28 Cys65–Cys151 pair, the pair was labeled with Alexa488 and Alexa594. Fig. 5 A and B compares the measured FRET efficiency histograms (light brown wide bars) with the histogram predicted from the model structures (black narrow bars). To verify the assembly of ESCRT-I at the 40-pM concentration used for these experiments, FRET was also measured between the intersubunit pair Vps28 Cys27—Vps37 Cys173 (Fig. S2). The experimental FRET efficiencies, \((E_{\text{exp}}) = n_A/(n_A + n_D)\), calculated from 2-ms bins containing more than 50 photons (Fig. S4) or more than 110 photons (Fig. S5B), were converted to the true FRET efficiencies, \((E)\), after correcting for background and donor leakage into the acceptor channel, by using a \(\gamma\)-factor to correct for differences in the detection efficiencies of the donor and acceptor channels and differences in the quantum yields of the donor and acceptor dyes as \((E) = n_A/(n_A + n_D)\), with \(\gamma\) = 2 determined from donor lifetime measurements (32).

**Model Validation Against FRET Measurements.** FRET efficiencies were calculated for the Vps28 Cys65/Cys151 double mutant and are in good overall agreement with experiment (Fig. 5 A and B). The low efficiency tail of the measured distribution is not observed in the predicted histogram. More importantly, the predicted histogram contains two distinct peaks at \(E \approx 0.60\) and \(E \approx 0.85\) that are enveloped by the single broad peak of the experimental histogram. This difference is more pronounced in the distribution obtained from the bins with a higher photon threshold \((n_T)\) of 110 photons within the shot noise width is narrower (Fig. 5B). Possible origins of this difference in the measured and predicted histograms are \((i)\) interconversion among the clusters of conformations during the 2-ms bin time that result in bins with FRET efficiencies intermediate between the closed and open conformations, \((ii)\) orientational motion of the dyes is comparable to or slower than the bin time due to dyes sticking to the protein to produce a range of values for the orientation factor \(\kappa^2\), and \((iii)\) the predicted structures do not include conformations with intermediate FRET efficiencies.

The first possibility was evaluated using a simple kinetic model of reversible conformational changes between two states, with each of the three clusters within either the open or closed set of conformations considered as one state. This model was compared to FRET efficiency histograms constructed with varying bin times as described in SI Analysis, from which we conclude that dynamics are most probably not responsible for broadening the experimental histogram. The second possibility was addressed by measuring the polarization anisotropy values for the donor and acceptor dyes. The anisotropy value of the donor \((r_D)\) is higher than expected from the donor lifetime \((r_D)\) and the reorientational correlation time \((\tau_r)\) of approximately 0.74 ns for freely reorienting dyes determined from time-resolved anisotropy measurements (33) (see Table S2). The reorientational correlation...
time of the dyes ($\tau$) calculated from $\tau = \frac{3 \cos^2 \theta - 1}{5 \cos \theta + \frac{1}{3}}$, and $\theta = 0$ (the angle between the absorption and emission dipoles) is 1–2 ns, indicating that the dye dynamics do not add width to the FRET efficiency histogram in excess of the shot noise contribution (SI Analysis), but could, because $\chi^2 \neq 2/3$, produce a small shift in the FRET efficiency peaks compared to those corresponding to freely reorienting dyes. The conclusion from the above analysis is that the single broad peak of the measured histogram probably reflects the presence of additional conformations not yet accounted for in the modeling.

Discussion

Here we have applied a combination of techniques—SAXS, DEER, and FRET—that probe overall shape along with distances between specific residue pairs within ESCRT-I. The observations here support the expectation that each complementary technique adds to the overall information content and defines the solution structure more sharply. A set of three $R_{H}$ values for ESCRT-I constructs could previously be interpreted in terms of a single open structure (21). The SAXS data could be fit by a minimum of two structures, one open and one closed. The SAXS and DEER data together could be fit by a minimum of six, spanning a spectrum of more open and closed conformations of the UEV, CTD, and NTH relative to the core. In the earlier hydrodynamic model, ESCRT-I was entirely in an open state. Indeed, the information content of the previous hydrodynamic study, with only three data points, was insufficient to define more than a single state for the three domains. The major insight to emerge from the present analysis is the unanticipated existence of a roughly 50% population of closed conformations.

One of the most unexpected aspects of the analysis was the constraint imposed by the distribution information in the DEER spectra. For the three-domain-core pairs, these spectra each led to a broad $P(r)$ distribution in Gaussian fits. Although these distributions were not used in the refinement, the broad collection of six structures emerging from the refinement reflects the same underlying conformational heterogeneity. Single-molecule FRET histograms provide a direct measurement of the conformational space sampled. The core-CTD label pair showed a single very broad peak in the histogram that had excellent overlap with the calculated histogram, giving us confidence in the model derived from SAXS and DEER. The broad and relatively featureless character of the FRET efficiencies highlights the likely presence of intermediate conformations in between the six structures used to fit the SAXS and DEER data. It is important to note that there is no evidence that there are precisely six (or some other particular number) discrete conformations. We view these six structures as snapshots that span and represent the larger conformational space sampled by ESCRT-I (Fig. 5C), a concept supported by the FRET histograms and the broad $P(r)$ distributions from the DEER.

In the current model of ESCRT-driven membrane budding, multiple copies of ESCRT-I and -II assemble to form a ring at the bud neck (3). ESCRT-I localization to bud necks in vitro has been directly observed (5), but the nature and structure of the putative membrane-bound ESCRT-I-II assembly is unknown. In yeast, the intraluminal vesicles (ILVs) in MVBs are 22–26 nm in diameter (34). Assuming the bud neck is of similar dimensions to the bud itself implies a bud neck circumference of approximately 75 nm. With a maximum dimension of 22 nm seen here, at least four ESCRT-I complexes aligned end to end would be needed to span the bud neck. The internal flexibility observed for approximately 50% of ESCRT-I complexes in solution (Fig. 5C, Top) would allow the complex to adjust its structure along the pathway of bud biogenesis. Once incorporated with ESCRT-II into a membrane-bound assembly, a single conformation is predicted, possibly one corresponding to the closed state seen in the other approximately 50% of the solution population (Fig. 5C, Bottom). The solution structure will be useful as a reference point for the structural pathway of ESCRT-I-II assembly responsible for bud neck induction. Although SAXS is not feasible in the membrane setting due to scattering from liposomes, the DEER and FRET techniques can be carried over. Thus a comparison of the spectra in solution and in membrane-bound settings could provide a first step to understanding conformational changes involved in forming the bud neck assembly. The role of conformational changes in ESCRT-I-mediated HIV-1 budding is an even more urgent question. These spectroscopic techniques should also be portable to the HIV-I setting, once the HIV budding reaction can be reconstituted in vitro.

The successes and limitations of this analysis suggest lessons for the integration of global and site-specific structural data on partially flexible complexes. EROS analysis of the SAXS data revealed an ensemble of structures capable of fitting the data, but lacked information about the distinct behavior of individual domains. Site-specific labeling provided powerful complementary information. Site-specific spin labeling and DEER spectroscopy provided direct evidence that each individual domain was conformationally heterogeneous relative to the core, a conclusion that we could not have drawn from the SAXS analysis alone. We used another site-specific structural probe, FRET spectroscopy, to validate conclusions drawn from the two other techniques.
and to reveal the presence of conformational intermediates that were not detected by the other techniques. Multisubunit complexes represent a major challenge in structural biology, and the integration of restraints from multiple methods is likely to be critical in developing a molecular understanding of function.

**Materials and Methods**

In the ensemble refinement, the number \( N \) and weight \( w_k \) of structures \( k \) in the ensemble were obtained by simultaneous fits to SAXS and DEER data. The scattering intensity \( I(q) \) of structure \( k \) was calculated as in ref. 30 and averaged over the ensemble,

\[
I(q) = \sum_{k=1}^{N} w_k I_k(q).
\]

Deviations from the measured SAXS intensity \( \bar{I}_{\text{obs}}(q) \) were quantified by

\[
X^2_{\text{SAXS}} = N_q^{-1} \sum_{m=1}^{N_q} \left[ I(q_m) - \bar{I}_{\text{obs}}(q_m) \right]^2 / \sigma^2_{I_q}(q_m),
\]

summed over the \( N_q \) points in the intensity curve, with \( c \) a constant determined by the condition \( \chi^2_{\text{SAXS}}/c = 0 \). To refine the DEER data, possible conformations of the six MTSL labels on ESCRT-I domains were generated by the multiscale modeling of macromolecular system Matlab module (31). By appropriate translation and rotation of the MTSL coordinates, the rotamers were positioned on the rigid domains of all the ESCRT-I structures in the ensemble. For each MTSL label pair \((i,j)\) and configuration \( k \) of the ESCRT-I complex in the ensemble, the dipolar evolution function

\[
V_{\langle i,j \rangle}(t) = \left( \int_0^\infty dx \cos[2\pi(1-3x^2)D_\text{dip}/r_{ij}^3] \right)
\]

was calculated with \( D_{\text{dip}} = 52.04 \text{ MHz nm}^2 \). The average is over all MTSL label conformations, with \( r_{ij} \) the distance between the spin labels \( i \) and \( j \) attached to residues \( i \) and \( j \), respectively. To validate the DEER model, MTSL labels were attached to residues 27 and 173 on the same rigid domain. The computed dipolar evolution function \( V_{\langle 27,173 \rangle}(t) \) is thus the same for each structure \( k \) and is found to be in good agreement with the measured DEER signal (see Fig. 3, Top). For the other three label pairs, \((i,j)=(108,256), (12,223), \) and \((65,151)\), the dipolar evolution function was averaged over the ensemble,

\[
V_{\langle i,j \rangle}(t) = \sum_{k=1}^{N} w_k V_{k,\langle i,j \rangle}(t).
\]

Deviations from the measured DEER signals \( V_{\text{DEER}}^{\text{obs}}(i)(t) \) were quantified by

\[
X^2_{\text{DEER}}(i,j) = N_i^{-1} \sum_{m=1}^{N_i} \left[ 1 - \lambda V_{\langle i,j \rangle}(t_m) - \left( \frac{V_{\text{DEER}}^{\text{obs}}(i)(t_m) - \lambda}{\sigma(\langle i,j \rangle)} \right)^2 / \sigma^2_{\langle i,j \rangle}(t_m) \right],
\]

which is summed over the \( N_i \) points in the \( V_{\text{DEER}}^{\text{obs}}(i)(t) \) curve, with the modulation depth \( \lambda \) obtained from \( \chi^2_{\text{DEER}}(i,j)/\lambda = 0 \). The statistical error \( \sigma(\langle i,j \rangle) \) was estimated from the noise level in the experimental data.

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Supporting Information

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SI Text

**SI Analysis. Dynamics of single-molecule FRET.** To evaluate the contribution of dynamics to the width of the measured FRET efficiency histogram, we used the maximum likelihood method of Gopich and Szabo (1). In this method, the optimal values of two mean FRET efficiencies and two rate coefficients for transition between the two states were determined that maximized the likelihood of observing the photon trajectories, consisting of a color for each photon and the interval between it and the preceding photon (2, 3). The FRET efficiencies determined by this method are 0.53 and 0.85 for the open and closed forms, respectively, which are in excellent agreement with the mean FRET efficiencies calculated for the model structures of 0.63 and 0.85, corresponding to differences in mean distances of approximately 0.3 nm and approximately 0.2 nm, respectively. The fraction of the closed form is 0.46, which is also close to the modeling result of 0.50. The sum of the two rate coefficients $k_2$ is 1.04 ms$^{-1}$, which is similar to the 2-ms bin time as expected from the broadening.

The recolored histogram using these parameters reproduces the experimental results much better than the static model as shown in Fig. S3.4. However, this possibility of two-state conformational dynamics is ruled out by the inconsistency in the bin time dependence. Fig. S3 shows the histograms constructed with bin times of 0.5 ms (Fig. S3C), 1.0 ms (Fig. S3B), and 2 ms (Fig. S3A) while keeping the photon threshold at 50. If dynamics were responsible for the broad experimental histogram constructed from 2-ms bins, then the histogram should change with bin size and show signs of two distinct peaks emerging as the bin size decreases. No such difference in the measured histograms is observed, suggesting that dynamics are not responsible for the broad measured histogram. We should mention, however, that conformational dynamics is not completely ruled out, and the model structures might be even more consistent with the measured histograms with a much more complex model in which there are dynamics among all six clusters (4).

**Fluorescence anisotropy.** The second possibility for the difference in measured and predicted smFRET histograms is slow orientational averaging of dyes. Most FRET measurements assume there is an isotropic distribution of donor and acceptor dye transition dipoles that reorient faster than the fluorescence lifetimes. This fast averaging results in the orientational factor $x^2 = 2/3$. If the dye reorientational times are longer than the fluorescence lifetimes, caused, for example, by transient sticking of dyes to the protein, but shorter than the time interval between detected photons (approximately 20 μs), or if the space accessible to the dyes is restricted, $x^2$ will not be $2/3$. In these cases there will be no broadening in excess of the shot noise. However, if the dye reorientational time is comparable to or longer than the interphoton time, not only is $x^2 \neq 2/3$, but there will be broadening of the FRET efficiency peaks in excess of the contribution from shot noise (5).

To test for slow orientational averaging of dyes, we measured the steady-state polarization anisotropy ($r_a$) defined as

$$r_a = \frac{G m_1 - n_x}{G m_1 (1 - 3k_2) + n_x (2 - 3k_1)}.$$

[S1]

Here, $n_x$ and $n_z$ are the number of photons detected in parallel and perpendicular polarization channels for a given bin, $k_1 = 0.11$ and $k_2 = 0.20$ are objective calibration parameters, and $G I = 0.78$ (donor), 0.92 (acceptor) is the correction factor for the detection efficiency of the two polarization channels (6, 7). The anisotropy of a freely rotating donor dye is given by

$$r_a = \frac{3 \cos^2 \theta - 1}{5(1 + \tau_D/\tau_c)}$$

[S2]

where $\theta$ is the angle between absorption and emission dipoles, assumed to be zero, $\tau_D$ is the donor lifetime, and $\tau_c$ is the reorientational correlation time for the transition dipole moments of the dyes. Therefore, the anisotropy values can be different depending on the FRET efficiency with different $\tau_D$ even though $\tau_c$ is the same. The anisotropy values determined from the photons in bins at different FRET efficiency ranges are compared in Table S3. Because the acceptor dye was not excited directly by a laser and the excitation energy was transferred from the donor, $r_a$ is not the exact anisotropy defined in Eq. S1. However, the value is small, suggesting fast orientational averaging of the acceptor.

**SI Methods. Protein expression and purification.** Wild-type yeast ESCRT-I complex and all mutants were expressed and purified as described previously (8). Double cysteine mutants were prepared by QuikChange site-directed mutagenesis kit (Stratagene) in the background of a construct in which all naturally occurring Cys had been mutated to Ala (Mvb1zG18A/C54A/C61A, Vps37C123A, Vps28C101A, Vps23C110A/C344A). Resulting proteins were >95% pure as judged by SDS-PAGE. Pure proteins were concentrated to approximately 5 mg/mL, flash frozen in liquid nitrogen, and stored at ~80°C until use.

**Small-angle X-ray scattering (SAXS).** To inhibit aggregation, the Cys-free ESCRT-I complex was used for all SAXS experiments. Samples were dialyzed overnight at 4°C against 50 mM Tris pH 7.4, 150 mM NaCl, 3 mM PME, 1 mM EDTA, 1% glycerol, and 0.18% ascorbic acid. Data were collected at concentrations of 0.5 to 5.0 mg mL$^{-1}$ at Stanford Synchrotron Radiation Lightsource beamline BL4-2. Data reduction and analysis were performed using the beamline software SAStool. The program AutoGNOM of the ATSAS suite (9) was used to generate $P(r)$ curves and to determine the maximum dimension ($D_{max}$) and radius of gyration $R_g$ from the scattering intensity curve ($I(q)$ versus $q$) in an automatic, unbiased manner, and rounds of manual fitting in GNOM (10) were used to verify these values. Ab initio molecular ensembles were computed by the programs DAMMIN (11). Multiple iterations of DAMMIN were averaged using DAMAVER (12).

**Labeling by MTSL and fluorescent dyes.** To prepare MTSL-labeled ESCRT-I complex, the engineered double cysteine mutants in the background of the Cys-free construct were incubated overnight at 4°C with 5 mM MTSL. Unreacted dye was removed by size exclusion chromatography on a Superdex 25 column (GE Healthcare). To prepare fluorescently labeled ESCRT-I complexes for single-molecule fluorescence experiments, double cysteine mutants were incubated overnight at 4°C with a 5-fold molar excess of the appropriate dyes. The ratio of donor dye to acceptor dye was 1:3. Unreacted dyes were removed by size exclusion chromatography on a Superdex 25 column (GE Healthcare). The labeling efficiency was approximately one dye per cysteine residue as judged spectroscopically with a donor to acceptor ratio ~1:2. For bulk time-resolved fluorescence experiments, the
ESCRT-I complex was first incubated for 3 h at 4 °C at 1:0.2 molar ratio with donor dye. After the incubation, it was repurified using size exclusion chromatography at Superdex 25 column. At this stage only 5% of cysteine residues were labeled. Assuming a binomial distribution, virtually no doubly donor labeled protein was expected. Singly donor labeled ESCRT-I complex was used to measure the donor lifetime. To prepare doubly labeled ESCRT-I, donor-labeled protein was incubated with a 20-fold molar ratio of the cysteine reactive form of the acceptor dye and was used to measure the lifetime of donor fluorescence in the presence of acceptor. Labeling efficiency was approximately one dye per cysteine residue with 1:15 ratio of donor to acceptor as judged spectroscopically. Labeled proteins were concentrated to approximately 1 μM, flash frozen in liquid nitrogen, and stored at −80 °C until use. Cysteine reactive dyes Alexa488, Atto488, Alexa647, and Atto647 were used in this study.

**DEER EPR spectroscopy.** Pulse-EPR measurements were performed on 25–30 μL of sample loaded into quartz capillaries with 2.00 mm i.d. by 2.40 mm o.d. (Fiber Optic Center, Inc.). The protein concentration of double spin-labeled ESCRT-I mutants was in the 20- to 50-μM range in 20 mM Tris pH 7.4, 100 mM NaCl, and 10% wt/vol glycerol buffer. Prior to inserting into the instrument, the sample-containing capillaries were flash frozen in a dry ice/isopropanol bath. The DEER data were recorded at 80 K on a Bruker Elexsys-E580 spectrometer fitted with an ER4118X-MS3 split ring resonator (Bruker Biospin) at X-band frequency. Data were acquired using a four-pulse DEER sequence (13) with a 16 ns τ/2 and two 32-ns τ-observe pulses separated by a 28-ns τ-pump pulse. The dipolar evolution times were typically 2.0–2.5 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 65–70 MHz higher. The phase-corrected dipolar evolution data were processed assuming a 3D background and Fourier Transformed using the DeerAnalysis2009 package (14).

**Time-resolved ensemble fluorescence measurements.** Förster resonance energy transfer was observed between the atto488 and atto594 dyes covalently attached to Cys in the ESCRT-I protein complex. Fluorescence intensity decays were measured on a laser-based time-correlated single photon counting apparatus with multichannel plate photomultiplier detection described in detail previously (15) and analyzed by the maximum entropy method as discussed in detail in (16). Briefly, fluorescence intensity decays were acquired under the “magic angle” conditions where the measured intensity decay 1(τ) is independent of a rotational diffusion of the chromophore and provides unbiased information about fluorescence lifetimes. The samples were placed in a thermostatic holder, and all experiments were performed at 22 °C in buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM EDTA. ESCRT-I concentration was 1 μM. Atto488 fluorescence was excited and collected at 310 nm and 520 nm, respectively. Fluorescence decays were processed using the singular value decomposition maximum entropy method, and mean lifetimes were calculated as described previously (16). The averaged efficiency of energy transfer 〈E〉 was calculated from the mean donor lifetime in the presence (τDA) and in the absence of acceptor (τD) according to 〈E〉 = 1 − τDA/τD.

The effective distance between the donor–acceptor pair R was calculated from R = R0(1−1/6), where R0 is the critical Förster distance, in this case equal to 5.7 nm.

**Single-molecule experiments.** Single-molecule FRET experiments were performed using a confocal microscope system (MicroTime200, PicoQuant). The donor fluorophore (Alexa Fluor 488) were excited by a linearly polarized dual mode (CW/pulsed) 485-nm diode laser (LDH-D-C-485, PicoQuant) at 30 μW through an oil-immersion objective (PlanApo, NA 1.4, x100, Olympus). Donor and acceptor (Alexa Fluor 594) fluorescence, emitted from molecules freely diffusing through the illuminated volume, was collected by the same objective, divided into two channels, and emitted through optical filters onto single-photon avalanche diodes (SPAD, PerkinElmer Optoelectronics SPCM-AQR-15). ESCRT-I (Vps28 Cys65-Cys151 and Vps28 Cys27—Vps37 Cys173) was diluted to 40 pM, which is sufficient to avoid having two molecules simultaneously in the illuminated volume (17), into a 20 mM Tris buffer (pH 7.4) with 100 mM NaCl and 3 mM β-mercaptoethanol. Bovine serum albumin was added (1 mg/mL) to prevent sticking of protein molecules to the glass coverslip. For steady-state polarization anisotropy measurements, the donor dye was excited with a laser in the pulsed mode at 20 MHz and fluorescence of the parallel and perpendicular polarizations was separated by a polarization cube. Donor and acceptor fluorescence of each polarization was further separated and collected by four SPADs. Other experimental details can be found in ref. 18.

**Calculation of FRET efficiencies from model coordinates.** Dyes attached to protein sites were previously simulated (19) and the distribution of distances between the Cys Cα atom and the active site in the dye was computed. We used the latter distance distribution to sample possible dye locations relative to the protein complex in the six selected conformations of ESCRT-I. Locations of the dye that overlapped with the proteins or with the other dye were excluded from the analysis. We assumed that all orientations of the donor and acceptor transition dipoles are equally probable. To calculate a FRET efficiency distribution from the model structures, the mean FRET efficiencies (Table S2) were calculated for each of the six selected structures. Using these six mean efficiencies and populations, the FRET efficiency histogram was constructed by the “recoloring” method described in refs. 1 and 2 in order to correctly account for shot noise. In this method, the colors (donor or acceptor) of the photons in each bin of the experimental data were removed. The cluster state was randomly assigned to the bin according to its relative population, and the photons were recolored using the apparent FRET efficiency of the assigned cluster state as the probability to observe an acceptor photon. Because this method uses exactly the same number of photons as that of the experimental data, it is the most rigorous way to account for the shot noise widths of each peak in the predicted histogram.

The mean FRET efficiency for dye pair (i, j) in ESCRT-I structure k = 1…n was calculated as 〈E_k(i,j)〉 = (1 + (r_0/R_0)^6)^{-1/6}, where R_0 is the Förster distance (R_0 = 5.7 nm and R_0 = 5.4 nm for the bulk and single-molecule FRET measurement, respectively) and the average is over all positions α and β of the dyes attached to sites i and j, respectively. For the label pair (ij) = (153,227), we calculated the ensemble average E = Σ_k=1^6 E_k. For the label pair (ij) = (65,151), single-molecule FRET efficiency histograms were calculated by the recoloring method (1, 2) to account for shot noise to compare directly to experiment.

**Monte Carlo (MC) simulations.** To create ESCRT-I structural ensembles, unbiased replica exchange Monte Carlo simulations were performed. After equilibration for 10^5 MC sweeps, 10^5 configurations were saved at regular intervals during a production run covering 10^7 MC sweeps. These configurations were grouped into clusters using the QT-clustering algorithm with DRMS as a metric (20) and a 1-nm cutoff. Structures at the cluster centers were used as representatives of their clusters in the ensemble refinement.


Fig. S1. Bulk FRET of the Vps23 Cys137—Cys223 pair.

Fig. S2. ESCRT-I was assembled at 40 pM concentration. Single molecular FRET efficiency for the labeled Vps28 Cys27—Vps37 Cys173 pair is shown in light brown thick bars for $n_T = 110$ with a bin time ($T_{bin}$) of 2 ms, corrected as described in the legend to Fig. 5. The dashed red line shows the mean interdye distance calculated from the model structures.
Fig. S3. The bin time dependence of the experimental FRET efficiency histogram (light brown thick bar) and the recolored histogram (black narrow bar) using maximum likelihood parameters (two FRET efficiencies and two rate coefficients) of the two-state kinetic model. The FRET efficiency of the recolored bin was $\gamma$-corrected in the same way as for the experimental values.

Table S1. Distance distribution parameters for ESCRT-I double mutants based on single Gaussian regularization of DEER data

<table>
<thead>
<tr>
<th>Mutant pair</th>
<th>Mean distance Å</th>
<th>Standard deviation Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>27/173</td>
<td>52</td>
<td>7</td>
</tr>
<tr>
<td>12/223</td>
<td>28</td>
<td>16</td>
</tr>
<tr>
<td>108/256</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>65/151</td>
<td>23</td>
<td>17</td>
</tr>
</tbody>
</table>

Table S2. Relative populations and FRET efficiencies of 6 representative structural clusters found in the model structures

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Population</th>
<th>$\langle E \rangle$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (closed)</td>
<td>0.19</td>
<td>0.83</td>
</tr>
<tr>
<td>2 (open)</td>
<td>0.19</td>
<td>0.59</td>
</tr>
<tr>
<td>3 (closed)</td>
<td>0.23</td>
<td>0.90</td>
</tr>
<tr>
<td>4 (open)</td>
<td>0.10</td>
<td>0.70</td>
</tr>
<tr>
<td>5 (open)</td>
<td>0.21</td>
<td>0.60</td>
</tr>
<tr>
<td>6 (closed)</td>
<td>0.08</td>
<td>0.83</td>
</tr>
</tbody>
</table>

Table S3. Anisotropy of Alexa 488 and Alexa 594 attached to ESCRT-I

<table>
<thead>
<tr>
<th>FRET efficiency range</th>
<th>$&lt;0.01$</th>
<th>0.4–0.74</th>
<th>0.74–1</th>
</tr>
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<tbody>
<tr>
<td>$r_D$</td>
<td>0.13</td>
<td>0.21</td>
<td>0.249</td>
</tr>
<tr>
<td>$r_D$, ns</td>
<td>3.4</td>
<td>1.7</td>
<td>NA</td>
</tr>
<tr>
<td>$r_c$, ns</td>
<td>1.6</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>$r_A$</td>
<td>NA</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>$r_A$, ns</td>
<td>NA</td>
<td>4.2</td>
<td>3.9</td>
</tr>
</tbody>
</table>

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