Published in final edited form as:

Methods. 2013 March 1; 59(3): 316–327. doi:10.1016/j.ymeth.2012.12.006.

Unraveling Protein-Protein Interactions in Clathrin Assemblies via Atomic Force Spectroscopy

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Abstract

Atomic force microscopy (AFM), single molecule force spectroscopy (SMFS), and single particle force spectroscopy (SPFS) are used to characterize intermolecular interactions and domain structures of clathrin triskelia and clathrin-coated vesicles (CCVs). The latter are involved in receptor-mediated endocytosis (RME) and other trafficking pathways. Here, we subject individual triskelia, bovine-brain CCVs, and reconstituted clathrin-AP180 coats to AFM-SMFS and AFM-SPFS pulling experiments and apply novel analytics to extract force-extension relations from very large data sets. The spectroscopic fingerprints of these samples differ markedly, providing important new information about the mechanism of CCV uncoating. For individual triskelia, SMFS reveals a series of events associated with heavy chain alpha-helix hairpin unfolding, as well as cooperative unraveling of several hairpin domains. SPFS of clathrin assemblies exposes weaker clathrin-clathrin interactions that are indicative of inter-leg association essential for RME and intracellular trafficking. Clathrin-AP180 coats are energetically easier to unravel than the coats of CCVs, with a non-trivial dependence on force-loading rate.

Keywords

Clathrin Triskelion and Clathrin-coated Vesicles; Single Molecular Force Spectroscopy (SMFS); Single Particle Force Spectroscopy (SPFS); Atomic force microscopy (AFM); Macromolecular Assembly; Protein Interaction and Protein Folding

1. Introduction

Clathrin mediated endocytosis (CME) and clathrin mediated intracellular trafficking are key processes by which proteins move between different compartments in eukaryotic cells (1-6). This is important for many biological activities including receptor mediated endocytosis

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(RME), the down-regulation of cell surface proteins, synaptic vesicle recycling, antigen presentation, and virus and pathogen entry (7-10). CME also has been exploited as a means for drug-delivery (11, 12). Because CME is an essential process in eukaryotes, defects in underlying cellular constituents are associated with a number of human diseases (1, 13).

Central to CME, as well as to intracellular trafficking events, are clathrin triskelia, which are protein structures containing three clathrin heavy chains, each of which is associated with a clathrin light chain. The heavy chains are joined together at their C-termini, giving the molecular complex a distinctive three-legged shape (Fig. 1). In CME and intracellular trafficking, clathrin triskelia and various adaptor proteins, along with numerous regulatory proteins, assemble onto cargo-containing membrane patches to form *ca.* 80-120 nm diameter clathrin-coated vesicles (CCVs)(2). The CCVs are distinguished by the presence of polyhedral cage-like structures in their coats, which typically contain 60-140 triskelia (Fig. 1B-C).

Pure clathrin cages, and cages containing adaptors (which hereafter are referred to as coats) self-assemble in appropriate buffers to form well-defined, closed polyhedra which resemble the clathrin coats found on CCVs (Fig. 1C) (14-16). Many protein-protein and protein-lipid interactions (17-19), some of which are cell and tissue specific, are involved in the assembly and disassembly of a CCV (7, 20). Under physiological conditions, the clathrin coats on CCVs, as well as clathrin coats assembled *in vitro*, form without nucleotide hydrolysis and are stabilized by weak physical interactions between the heavy chains of the clathrin legs (21, 22). However, the disassembly process (often referred to as uncoating) is more complicated, involving an ATP-dependent reaction which is mediated by the Hsc70 chaperone protein and its J co-chaperone auxilin/GAK (20, 23-25). The uncoated vesicles fuse with endosomes and other intracellular compartments, where the cargo is released (1, 2, 6). This complex energy-dependent process, which is not fully understood, drives the internalization of extracellular nutrients and membrane-bound receptors. To dissect this complex process, investigators have reconstituted functional clathrin-containing structures *in vitro* (26) and in simulations and modeling (27-32).

We recently used atomic force microscopy (AFM) to establish the mechanical rigidity of typical CCVs (33), from which the energy associated with the distortion of the vesicles can be determined and compared with the rigidity of triskelion legs and clathrin nets (34). AFM also has been used to examine associations that occur, in solution, between clathrin triskelia prior to cage formation (35). Until now, though, the intra- and intermolecular forces that arise as the clathrin cages are disrupted have received little attention. We now employ AFM single molecule force spectroscopy (SMFS) to assess the forces involved in intramolecular clathrin hairpin folding and hairpin inter-domain interactions within the heavy chains of isolated triskelia. We then use a similar technique, which we refer to as “single particle force spectroscopy” (SPFS), to study the unraveling of macromolecular complexes in CCVs and reconstituted AP180-clathrin coats, and note how the measured force spectra differ from those for the triskelia. AFM requires no fixation or staining but only suitable sample attachment between substrate support and AFM tip, which allows the clathrin and CCVs to be examined under various conditions approximating those of physiological relevance. We show that our implementation of AFM and SMFS, together with appropriate modeling and data analysis, can provide new information about both intra- and intermolecular interactions of clathrin assemblies. We focus on the interactions and energy needed to physically unravel clathrin coats, and point out their potential relationship to the mechanism of physiological uncoating.

2. Materials and methods

2.1 Atomic force microscopy and single molecular force spectroscopy

While other SMFS measurement systems exist (36-41), AFM-based force spectroscopy is arguably the most widely adopted. AFM uses a flexible cantilever with a sharp probe (2-20 nm tip radius) that is scanned over a sample surface, in its imaging mode, sensing inter-atomic forces between the probe tip and the sample. The resulting deflection of the cantilever is converted to a depiction of the surface topography of the sample (42) (Fig 2A). Associated optoelectronics produce extremely high lateral and vertical resolutions of several Ångströms (see Eq. 1A). In addition to high resolution imaging, AFM has also been employed to study intermolecular forces and protein unfolding at pN force resolution, using data obtained as a result of the deflection of the cantilever when it is pressed upon or lifted off the sample (Fig. 2B-E). Oftentimes, however, the force-displacement curve can have regions that are not directly accessible to the measurements because of a “rupture” phenomenon (dashed lines in Fig. 2E) or a “jump-to-contact” instability; moreover, as the AFM cantilever and sample surface move relative to each other in fluid measurements, there can be some viscous drag forces that are more significant at higher cantilever ramping speed and with lower cantilever spring constant. The spring constant of the cantilever can be selected from a wide range of ca 0.005 N/m to 100 N/m, and can be precisely calibrated. Hard cantilevers are used for nano-indentations and tapping mode imaging to achieve greater position sensitivity, and softer ones for contact and force measurements to achieve enhanced force resolution.

Several AFM systems currently are commercially available. For our measurements of clathrin, we used mainly two instrument platforms: (i) MultiMode®-NanoScope V AFM (Bruker, Santa Barbara, CA), and (ii) ForceRobot® AFM (JPK, Dresden, Germany), the latter being a highly automated single molecule force spectroscopy instrument. We followed standard data analysis practices for SMFS (43, 44), but also developed custom optimized force curve categorization and novel statistical analysis to extract information about the unfolding of internal-domains of the triskelia. New programs were developed in MathCad® (Mathsoft, Cambridge, MA) to extend the functionality of the instrument software. Briefly, we adopted the worm-like-chain (WLC) model for free chain extension between unfolding events, viz. (42),

$$f(z) = \left(\frac{k_B T}{P} \right) \left[\frac{1}{4 \left(1 - \frac{z}{L} \right)^2} - \frac{1}{4} + \frac{z}{L} \right], \quad (1)$$

where z is the separation between the sample surface, where the applied force is extrapolated to zero, and the tip of the AFM cantilever, $f(z)$ is the stretching force at that extension, P is the persistence length describing the unfolded peptide chain, $k_B T$ is the thermal energy, and L is the total length of the unfolded peptide chain being stretched. Generalized nonlinear least-squares fitting of individual force curves against this model produced descriptive parameters for clathrin leg elasticity, internal domain barriers, and clathrin-clathrin interactions in CCVs and clathrin-AP180 coats. Because the cantilever arm was raised and lowered when the sample stage was moved by the ForceRobot®, and often was positioned over bare mica substrate, the AFM tip was self-cleaning and thousands of trials could take place before the tip become grossly contaminated and the tip-sample setup was replaced.

2.2. Macromolecular interactions and energy surfaces

To understand domain unfolding and protein bond unraveling in SMFS and SPFS (Fig. 2), we here follow recent analyses by Evans, Ritchie and coworkers (45-47) and others (48-53)

who have extended a general model of reversible macromolecular reactions (54) to dynamic force applications in AFM and related systems. The force-induced rupturing of protein/ligand interactions and protein unfolding may be conceptualized using transition-state theory that describes chemical or physical reactions taking place on a mesoscopic, multi-dimensional, potential energy surface. For many biological processes, the energy surface has countless possible reaction paths from reactants to products and the energy landscapes can be very complex due to the large number of interactions at molecular interfaces. Conceptually one has to limit descriptions to just a few most relevant reaction pathways, and the system energy is visualized by potential surfaces that can have multiple barriers of different widths and heights located at different positions along their reaction coordinates.

To illustrate this conceptual simplification (Fig. 3), it is useful to consider a general three-state unbinding reaction occurring along a reaction coordinate X (46). The bound or folded state corresponds to a deep well, and the unbound or unfolded state is where the energy tends to a reference value of zero (Fig. 3A). When a macromolecular bond is covalently linked, e.g., to disordered polypeptide segments which behave as worm-like chains (WLCs) and is stretched via increasing tip-sample separation (TSS), the AFM force is applied onto this bond as well as onto WLCs and some folded domains (Fig. 2). The associated mechanical stress throughout the folded domain or the macromolecular bond alters the magnitude and stability of non-covalent interactions and, at high enough magnitude, can even weaken the covalent linkages. As a consequence, disruption of intramolecular structure or disassembly of intermolecular associations occurs (Fig. 2E and Fig. 3). When a mechanical force is applied across the bond or the folded domain, the reaction potential energy landscape is distorted multidimensionally and generally tilts toward unfolding by $-f(t)X$, where $f(t)$, which may be a function of X , is the force component at time t along the relevant direction (Fig. 3A). The effect of tilting the potential energy is that molecular rearrangement, linked to an enhanced probability of crossing lowered energy barriers or a complete energy downhill movement, facilitates bond rupture over a range of unraveling force (see Fig. 3B).

At low loading rates (i.e. the rate at which the force increases), the dissociation kinetics are dominated by the probability of crossing a barrier farthest from the potential well. As the loading rate is increased there is a crossover where the kinetics is dominated by the probability of crossing an inner barrier (46, 55). Thus, the barrier width X_U of the reaction coordinate is smaller at a higher force than at a lower force (Fig. 3A). In particular, the weak non-covalent interactions found in protein-ligand interactions, as well as those involved in protein folding, are sensitive to the loading rate of the applied force. As the load increases with time, the off-rate increases and the likelihood of bond survival decreases, and there is a force peak in the statistical distribution of rupture events (Fig. 3B) (46). This peak in the measured distribution of unraveling force is often regarded as the apparent bond strength, which increases with higher loading rates in a non-trivial way that depends upon both the bond lifetime and the geometry of the reaction coordinate (Fig. 3B) and is often visualized via a rupture force vs. log (force loading rate) curve (46, 53, 56). At very high loading rates, there is too little time for the system to cross any dynamic barrier and bond rupture occurs when the force exceeds a critical value that abolishes all energy barriers along the separation direction (53). This illustration (Fig. 3) also shows several mechanisms leading to a distribution of unraveling force, ΔF , as measured in SMFS or SPFS. Stochastic dissociation happens when the relevant energy barrier is lowered to the order of thermal energy, so one expects $\Delta F X_U \approx 1 \cdot k_B T \approx 5 \text{ pN} \cdot \text{nm}$; therefore, if the barrier width X_U is subnanometer, ΔF should have a distribution width of the order of 10 pN or greater. In addition, ΔF is increased by global molecular fluctuations or viscoelastic hysteresis that changes in the energy surface. Lastly, in addition to intrinsic heterogeneity in the structure of macromolecular complexes and domains, instrumental factors such as the geometry of

macromolecular attachment and variation across support surfaces can affect the values of measured ΔF at each force-loading rate.

However, qualitative differences in sensitivity to force applied upon proteins and other macromolecules, deriving from differing folding and intermolecular interactions reflecting underlying energy manifolds, can indeed be observed via atomic force spectroscopy. While a strict physical interpretation of the reaction surface is not reasonable for complicated macromolecules and the full process of a domain unfolding or protein bond unraveling may not be visualized without molecular modeling or atomic level simulations (57-60), merely uncovering the presence of hidden barriers can provide insights into binding mechanisms that may be biologically relevant. In principle, the nature of the transition state, the protein-ligand interface, and protein domain structures all can be usefully explored (45, 48-50), although the above-illustrated effects of measurement details highlight the need for care when providing quantitative interpretation of SMFS and SPFS results.

2.3 Clathrin triskelia and clathrin assemblies

In the past we used atomic force microscopy (AFM) to examine the properties of single triskelia and higher order structures, including native CCVs, in both dry and aqueous environments (33, 35). The spatial resolution of AFM in aqueous buffers is high enough to visualize clathrin lattice polygons on the surfaces of native CCVs, and the force measurement capability of AFM allows the quantification of CCV coat rigidity (33). We also have shown that high-resolution AFM can provide new information about the flexibility of clathrin legs, *in vitro* oligomerization of triskelia, and self-assembly of clathrin cages (35).

For the data reported here, bovine brain CCVs and clathrin triskelia were purified and biochemically characterized as previously described (61, 62). They were then suspended in either “CCV buffer” (0.1 M MES-NaOH, pH 6.5) or “triskelion storage buffer” (0.5 M Tris, 3 mM DTT, pH 7.0), in both cases at a concentration of 2.0 mg/ml. AP180 was prepared as described (63, 64). Some measurements of isolated clathrin triskelia were done on fresh samples, but others were performed on material that had been flash frozen by liquid nitrogen into aliquots, stored at -80 °C, and thawed immediately before use. Clathrin cages were assembled by overnight dialysis into CCV buffer of a solution containing 1.2 M clathrin triskelia and 3.6 M AP180. Any aggregates were removed by centrifugation at 10,000 × g for 10 minutes.

For SMFS experiments, 5 to 10 μ l of clathrin triskelia solution, or a suspension of CCVs or clathrin-AP180 coats at concentration 20 - 50 μ g/ml, was incubated on a clean mica support for 3 to 10 min. Following incubation, the sample surface was rinsed with the original buffer and then covered with 200 μ l of the buffer. The sample holder then was added to a fluid cell assembly fitted with a silicon nitride MLCT/MSCT (Bruker, CA), or DNP-S (Bruker, CA), or Biolever (Olympus, Japan) cantilever. After the assembly was inserted into the ForceRobot® instrument (JPK, Germany), a set of force curves was obtained, typically overnight, with various automation controls at room temperature. The spring constant of the cantilever (nominal value 0.006 to 0.06 N/m) was measured precisely for each setup, using the built-in thermal tuning procedure of the AFM instrument. Suitably low coverage of the protein or the assembly on the mica support was verified by imaging the same sample in parallel on the MultiMode® nanoscope AFM instrument (Bruker, CA) (Fig. 1).

2.4 Implementation of SMFS and Analysis

JPK ForceRobot® Workspace software (ver.2.2.9) was used to observe and analyze the SMFS data collected through the ForceRobot® apparatus. This software provides an

interface where the experimental plan can be designed (Fig. 4A, inset). The alignment of the laser on the AFM cantilever, the cantilever spring constant calibration, the location to which the tip moves, and how long it pushes on the sample, etc., can be controlled through this interface. The data can be presented in many ways such as in graphs that show the vertical deflection of the cantilever (in terms of volts) over time. For illustration, we show data collected from a sample of isolated triskelia. As seen in Fig. 4A, the large initial spike in voltage was a result of the sample being pushed on to the tip and then retracted, and the sawtooth-like peaks that followed indicate that the tip had attached to, and unfolded, a single clathrin triskelion. The axes on these graphs can be converted from time to TSS (tip-sample separation), and from vertical deflection to AFM force exerted on the molecular chain, with proper calibrations and viscous drag correction, etc. accounted for within the program.

Changing the axes of the graphs allows them to be viewed as force curves, which show the changes in force exerted on the cantilever after correcting for the viscous drag force. When the tip pulls on a single clathrin triskelion after being pushed into and lifted out of the sample, the force curve shows several peaks (Fig. 4B). These peaks are followed by snap relaxations of the force between the cantilever and the sample surface. As the tip is pulling on a single protein, the force reaches a point where it is large enough to unfold a new part of the macromolecule, so the force drops because there is less resistance from a longer WLC after unfolding, and a new peak subsequently forms at a larger tip surface extension. Each peak in a force curve corresponds to a domain of the protein or protein complex being disrupted. Therefore, by analyzing the peaks in the SMFS force curves, the structure of clathrin and its assemblies can be examined.

During the sampling process, the cantilever explores a different location on the sample surface every few seconds and the force curve from each such “trial” can differ (Fig. 4B, inset). In many instances, a trial force curve may completely lack peaks, since the protein is randomly dispersed throughout the sample and the instrument cannot pinpoint exactly where it is located. For this reason, SMFS requires numerous attempts and a long data collection period in order to gather useful data; ForceRobot® deletes most of the meaningless data so that only the curves that demonstrate seemingly significant events are transferred to the computer. Since the probability of detecting a single molecule in the sample is already low by design, it can be concluded that the probability of concurrently detecting multiple molecules is negligible, and for this reason SMFS is able to isolate single molecules. Likewise, in each SPFS, only single assembled particle is detected for further analysis by maintaining a low event to trial rate from a low coverage of the particles.

Even after ForceRobot® deletes most of the blank recordings, etc. the remaining data still contains some force curves which demonstrate aberrant characteristics. ForceRobot® Workspace has filtering and categorizing functions that segregate curves having multiple, well-defined peaks. In order to properly analyze these remaining curves, the peak-find function in ForceRobot® Workspace was used to locate the peaks having a size greater than a certain threshold. However, before peak-finding, the data sets were reduced to ca. 4096 data points per curve after using a moving average procedure to minimize extra “noise-induced” features. In correspondence to Fig. 4, regions of increasing force were identified and shaded in red in Fig. 5A; the white areas correspond to incidents in which the force decreases as intra- or intermolecular bonds are disrupted (Fig. 2E).

We extracted information out of a single force curve by fitting each band with a WLC (worm-like chain) regression model (Eq. 1) and recording the peaks, using ForceRobot® Workspace software. We thus determined the force (which we refer to as the “peak force”; see Figs. 6-8) at which structural change takes place in the sample, and noted the tip-to-sample separation (“TSS”) at which that event occurred. (The TSS is the difference between

the cantilever height and the height when the tip initially is attached to the sample at zero force.) The peaks that were detected by the software then were fitted using the WLC function (Eq. 1). Once the peaks are fitted in this way (see Fig. 5B), they can be used to calculate the extension force rate and chain length of each segment of protein. Using a series of programs, mostly written in MathCad® (Mathsoft, MA), we determined the extension force rate for a protein domain by taking the slope of the WLC curve at the unfolding peak. The WLC curves also were used to identify the chain lengths of unfolded domains, being taken as the differences in WLC length between two consecutive peaks. Examples of data fitted with the MathCad® programs are shown in Fig. 5C.

3. Results and Discussion

3.1 SMFS of clathrin triskelia

Here we present an overview of the major unfolding events obtained through SMFS, using a technique which we refer to as “fingerprinting.” After the raw data are processed so that only force curves showing significant peaks remain, results can be presented in several ways (Fig. 6). In Fig. 6A we show data acquired from isolated triskelia, plotted on a 3D scatter plot. The axes are the TSS at which each peak occurs, the force value at each peak, and the peak order of each peak—i.e., whether it was first, second, etc. The peak order scale is color-coded from blue to red in increasing peak order, the purpose being so the “depth” of the points can be seen when viewing the 3D scatter plot. Note that in Fig. 6A the peaks tend to concentrate in the bottom left corner, at lower peak forces, lower TSS values, and lower peak orders.

We also show a scatter plot of peak order vs. peak force, Fig. 6B, which is a 2D projection of the same data as appear in Fig. 6A. This allows convenient observation of the relationship between the order of the protein domain and the force needed to unravel the domain. We similarly provide a 2D scatter plot of TSS vs. peak order (Fig. 6C). Looking at Fig. 6B, we observe that the points are concentrated in an area encompassing peaks of order below 10, and peak forces approximately 400 pN or less. So, for most of the samples, fewer than ten domains are stretched out, at low to relatively high forces, before the triskelia break or are pulled off the mica substrate. However, the data points at peak order higher than 10 are plentiful and, in many force curves, more than 20 peaks are well observed. Figure 6C shows a positive correlation between TSS and peak order, which is logical because the TSS is large by the time multiple domains are stretched. Interestingly, there is a *ca.* 100 nm TSS range into which most data points fall.

While the scatter plots show data over wide distributions, many of the data points overlap. Thus, we provide histograms that show exactly how many data points correspond to each force value (Fig. 6D) and each TSS value (Fig. 6E). As Fig. 6D indicates, generally there is an increase in the number of detected peaks as the force decreases and, overall, more peaks are noted at lower forces than at higher forces. The most common force that led to the unfolding of a triskelial domain is about 150 pN. Similarly, Fig. 6E shows a strong trend indicating that the frequency of observing a peak decreases as the TSS distance increases. We have superimposed a linear fit to this descending histogram over small TSS values, and found its intersection with the abscissa to be about 110 nm. Since each triskelion leg is approximately 50 nm long, even if one leg were attached to the substrate at its terminal domain and another to the probe tip at its terminal domain, as the sample moved away from the probe the TSS distance could not exceed *ca.* 100 nm unless at least one of the legs experienced internal rearrangement. Hence, TSS values greater than 100 nm unequivocally indicate heavy chain unfolding of single clathrin triskelia. Moreover, due to the random attachment of triskelion heavy chain legs between the surface and tip, a linear probability distribution for the captured sizes up to the maximum unfolded length is reasonable.

Altogether, the scatter plots and the histograms generate a characteristic “fingerprint” based on SMFS peaks that is unique for a given sample.

3.2 SPFS of native CCVs

SPFS fingerprinting from clathrin coated vesicles (CCVs) is next presented, in a parallel fashion with that of single triskelia (compare Fig. 7 with Fig. 6). As shown in Fig. 7, a much narrower distribution of peaks is discerned in the CCV fingerprint than in the single triskelion fingerprint. Moreover, based on the 3D scatter plot (Fig. 7A), it would appear that the peaks are also concentrated around lower peak orders, lower TSS values, and lower peak force values. In particular, many of the peaks seem to have a TSS close to 100 nm, a maximal force less than 200 pN, and peak orders between 1 and 4. Because the single triskelia also show a grouping of TSS values below 100 nm, this may indicate the lifting of heavy chains off the CCVs. However, the force also may reflect distortions of the overall shape of a vesicle. There also are a few data points spread out over a wide TSS range but associated with low peak forces. Low force likely is indicative of clathrin lattice unraveling, especially at the higher TSS when interconnected triskelia are stretched. The 2D scatter plots of force peak vs. peak order (Fig. 7B) and TSS vs. peak order (Fig. 7C) allow the distribution of the peaks to be more clearly examined. Most of the data points in both graphs have peak orders between 1 and 3. As shown in Fig. 7B, the data points are also clustered below peak force 200 pN, a lower peak force than the single triskelia clustering. On the other hand, Fig. 7C indicates that the TSS values are concentrated below 200 nm, similar to the single triskelia data. In contrast with the single triskelion data, there appears to be no correlation of TSS and peak order for the CCVs in Fig. 7C.

The histogram of peak force (Fig. 7D) demonstrates a notably high count around 100 pN. The peak count at 100 pN is three to six times as large as any of the other peak counts. In Fig. 7E, the TSS with the highest count also is obvious: the most frequent TSS at which CCVs seem to unravel is 100 nm, occurring with much higher frequency and being more narrowly distributed than at other separations. Overall, it would appear that CCV domains unravel at a lower peak force than do single triskelia, although they have similarly low TSS values. This indicates that clathrin-clathrin interactions in CCVs are weaker than intramolecular bonds in single triskelia.

3.3 SPFS of clathrin-AP180 coats

SMFS force curves were also used to investigate reconstituted clathrin-AP180 coats (Fig. 8). Results presented in Fig. 8 show a broad range of both CCV characteristics (cf. Fig. 7) and those of single triskelia (cf. Fig. 6). Additionally, unique signature events at larger TSS and lower peak forces are detected, which can be attributed to the unraveling of the clathrin-AP180 lattice. Again, only a single coat complex was examined during each trial. In this case, the 3D scatter plot (Fig. 8A) exhibits heavy clustering of data points in the front lower left quadrant. The size of the area in which the points are clustered suggests domain unbinding within a broad range of TSS and peak force values, the points being clustered below *ca.* 400 pN, like for single triskelia. In contrast to the case for single triskelia, though, these results may in part reflect the breaking of bonds between AP180 molecules and associated clathrin cages.

The observed clustering pattern of native CCVs also is noticed in Fig. 8A. However, the clathrin-AP180 coat data as well show unique signature patterns, differing from both single triskelia and CCVs. In addition to data clustering at low TSS and relatively high peak force, there is a prominent band of data points that extends along the bottom of Fig. 8A, eventually reaching high TSS values but at an extremely low peak force. These points can be attributed to the unraveling of clathrin lattices unique to clathrin-AP180 coats, since the lattices are

made of several triskelia which can form a long chain when pulled out, but the required force is low because the non-covalent intermolecular interactions between triskelia are relatively weak. The color of the majority of the data points in Fig. 8A ranges from blue to blue-green, but a few points are seen to range from green to red, implying that, primarily, the peak order is low. Both the peak force scatter plot (Fig. 8B) and the TSS scatter plot (Fig. 8C) support this observation.

Moreover, whereas the points are closely clustered in the peak force scatter plot (Fig. 8B), Fig. 8C shows data points evenly distributed through the entirety of the TSS range. The TSS range that corresponds with lattice unraveling, 200-250 nm, contains points from all ten peak orders. This suggests that the lattice unraveling occurring in the clathrin-AP180 coats is present in all stages of stretching rather than unraveling only at a certain peak order, in agreement with inferences from a recent analysis of Hsc70-driven disassembly of clathrin coats (20). It is also interesting to point out that Fig. 8C suggests a relationship between TSS and peak order, because peaks of different order first begin to appear on the plot at increasingly large TSS values. Also, the histogram of force peaks (Fig. 8D) indicates a marked increase in frequency as the force decreases, with the highest count at about 50 pN indicating the prevalence of low-force lattice unraveling in clathrin-AP180 coats.

Thus, there are clear quantitative differences in characteristics between clathrin-AP180 coats and CCVs or single triskelia. For example, the peak force data cluster below 300 pN (Figs. 8B, 8D) whereas there are many peak force data points beyond 400 pN for single triskelia (Figs. 6B, 6D). Moreover, the TSS histogram, rather than exhibiting a monotonically decreasing trend, almost looks like a skewed bell curve, increasing to a maximal value when TSS is close to 100 nm. This wide range of TSS values also is indicative of clathrin lattice unraveling.

3.4 Molecular interactions in AP180-Clathrin coats

Both CCVs and clathrin-AP180 coats contain many interlinked triskelia. As a result, as compared with isolated triskelia, spectroscopy curves of greater complexity can be expected during SPFS, since multiple clathrin triskelia are being stretched at the same time. In addition, the spectra may contain features linked to the unraveling of long lattice chains. As indicated in Figs. 7 and 8, points corresponding to larger TSS are more prominent in the clathrin-AP180 coat data than in the CCV data. We now more fully analyze the events noted for clathrin-AP180 coats (Fig. 9).

The event size associated with lattice unraveling (see Fig. 5C) is plotted against TSS in Fig. 9A. This scatter plot indicates a significantly larger number, and wider distribution, of shorter events than larger events. Interestingly, the majority of the shorter events have TSS values below 200 nm. On the other hand, the very largest, albeit less frequent, event sizes -- i.e., the ones that most strongly indicate lattice unraveling -- do not start appearing until a TSS value above 200 nm. Therefore, clathrin lattices seem to unravel mostly when the coats are quite elongated. These detected long-size events, which correspond to large TSS values, are most noticeable when clathrin-AP180 force curves are compared to those found with single triskelia and native CCVs.

In order to get a better idea of the relative amounts of each fragment length present in the clathrin-AP180 coat samples, a histogram (Fig. 9B) was constructed for event lengths. By "event length" we mean the "size" between events as indicated in Fig. 5C (not to be confused with the TSS, which is the focus of Figs. 6-8). The histogram shows detected sizes primarily below 200 nm, with most of the distribution ranging from 20 nm to 100 nm. The event sizes that appear most frequently lie between 10 and 30 nm and are well matched by "single" and "cooperative" hairpin unfolding events detected in single triskelion force

spectroscopy (data not shown). However, this might be a coincidence, because the coats are a complex containing AP180 as well as clathrin and slippage between heavy-chain legs might occur if the AP180-clathrin bonds are broken. The larger events, at size greater about 40 nm, are detected only with clathrin-AP180 coat samples and are evidence of lattice unraveling.

As shown in Fig. 9C, the force curves contain many peaks with lengths almost up to 500 nm TSS. These peaks are initially more closely spaced, indicating shorter event size lengths. As the TSS increases, the peaks become more spread out and the event sizes are longer, indicating long lattice unraveling. Peaks like these, which are spread out at high TSS values, cannot be found in the single triskelia force curves and are hardly seen in the CCV force curves. Interestingly, the heights of the peaks appearing at larger TSS are lower than those at shorter values of TTS, signifying that lower forces are needed to disrupt the sample at large extensions.

3.5 Lattice stability in AP180-clathrin coats

When studying clathrin-AP180 coat unraveling, it is of interest to assess the stability of the clathrin lattice and the strength of its clathrin-clathrin interactions. These properties can be probed by further examining the effects of applied force (Fig. 10). When the peak force for each event is plotted against the size of the event (Fig. 10A), the points cover a broad range, mostly clustering where both the event length and peak force are low. However, a negative correlation is also discernible, since it appears that as the event length increases the range of peak force shifts slightly downwards (Fig. 10A). If this correlation holds true, then shorter events such as heavy-chain unfolding take more force, but longer events such as lattice unraveling require significantly less. This statement is supported by the lower peak forces noted at larger TSS, as already discussed in Section 3.4 (see Fig. 9C). Most likely, linkage forces between heavy chains when AP180 is involved are larger than the clathrin-clathrin interactions in a partially disrupted lattice.

We also plot peak force against the force loading rate, i.e., the rate at which the force is applied on the sample during SPFS. The scatter plot in Fig. 10B indicates that the relationship between peak force and force loading rate follows two linear trends. This behavior is emphasized by the two linear lines which have been superimposed on the data. Unbinding seems to increase at the rate dictated by the first line until the force loading rate reaches the intersection of the two lines, at which point the required force begins to increase at a higher rate. The intersection between the two lines suggests a change in how loading rate impacts the force required to unravel a clathrin-AP180 coat. The plot shows that the dependence of lattice stability on force-loading rate that is well described by recent theoretical models, which are briefly mentioned in Sec. 2.2.

4. Concluding Remarks

The clathrin folding energetics and interactions examined here illustrate the usefulness of AFM SMFS and SPFS for bio-macromolecular characterizations. In principle, characteristics of the transition state, protein-ligand interfaces, and protein domain structures all can be usefully explored with atomic force spectroscopy. Differences in sensitivity to applied forces reflect variations in underlying energies of intra- and intermolecular interactions, potentially providing biologically relevant insights concerning binding mechanisms and macromolecular function. Force measurements in SMFS and SPFS are convertible to energy scales measured in other macromolecular interaction assays upon multiplication by an action length which, although generally unknown in detail, mirrors the position of the energy barrier Xu along the reaction coordinate (Fig. 3A), with a typical value ranging from 0.1 nm to a few nm. Therefore, for the peak force of a few hundred pN

seen in clathrin assemblies, the corresponding energy is of the order of $10k_B T$ (see Sec. 2.2) and within a physiologically relevant range for clathrin and CME (1, 2).

Although the loading-rate dependent rupture and unraveling forces, as well as the rupture size distributions, reported in this work for clathrin triskelia, CCVs, and clathrin coats are informative, it is necessary to point out, that SMFS and SPFS for bio-macromolecular characterizations has limitations. For many biologically relevant processes, the energy landscapes are too complex to allow strict physical interpretation of protein stability or protein-protein binding. Usually, large scale data analysis is necessary to unravel interactions of particular interest and SMFS studies need to start with relatively simple macromolecular constructs and build towards native proteins and their complexes. In addition to the clathrin constructs, we are undertaking such SMFS approaches to aid the development of malaria vaccines (65). Moreover, SMFS/SPFS is only one of many methods for characterizing macromolecular interactions, others, for example, being calorimetry, mass spectrometry (66, 67), analytical ultracentrifugation (68), NMR (69), surface binding assays (70-72), and *in vivo* protein binding assays (73, 74). Each method for probing protein and macromolecular interactions has its own strengths and weaknesses, and combining complementary approaches is particularly useful when targeting complicated biomedical phenomena.

While using AFM to study clathrin involves several challenges, important information have been obtained by using AFM to examine clathrin and its structural brethren. We previously demonstrated that triskelial dimers and small oligomers form when solution conditions favor cage assembly, and were able to examine fluctuations in the shapes of triskelia (35). We also were able to directly measure CCV rigidity (33). In both instances we made use of the applicability of AFM to study wet samples. The clathrin cycle, from assembly on cell membranes to vesicle uncoating, is exceedingly dynamic (75-78). It is of interest to consider how the energetics determining molecular interactions between clathrin triskelia in solution affect various clathrin functions in eukaryotic cells (79).

Acknowledgments

We thank Dr. Svetlana Kotova (National Institutes of Health (NIH), currently Laboratory of Modified Polymer Systems, N.N.Semenov Institute of Chemical Physics of the Russian Academy of Sciences, Moscow, Russia) and Dr. Kondury Prasad (University of Texas Health Science Center at San Antonio) for contributions during the early stage of this study and technical assistances. We also are very grateful to Dr. Dan Sackett (NIH) for helpful comments and technical assistances. This work was supported in part by an extramural grant (EML, NIH-NINDS NS029051) and by the intramural research programs of the National Institute of Biomedical Imaging and Bioengineering (NIBIB) and the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD).

Abbreviations

AFM	Atomic force microscopy
SMFS	Single Molecular Force Spectroscopy
SPFS	Single Particle Force Spectroscopy
CME	Clathrin Mediated Endocytosis
CCV	Clathrin-coated Vesicle
TSS	Tip-Sample Separation

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Highlights

Single molecule/particle force spectroscopy (SMFS/SPFS) is introduced.

SMFS/SPFS is used to investigate clathrin triskelia and clathrin assemblies.

Cooperative unfolding of clathrin alpha-helix hairpin domains is revealed.

Force spectra of triskelia and clathrin coats provide characteristic fingerprints.

SPFS with varying force loading rate differentiates protein-protein interactions.

Figure 1.

Structural characterization of clathrin triskelia and clathrin complexes. (A) AFM image of clathrin triskelia deposited on mica from 0.5 M Tris-HCl buffer (pH 7.0) and viewed in air (35). Similar samples were used in this single molecule force spectroscopy (SMFS) study. The insets show two height profiles (denoted by 1 and 2) of a representative triskelion at a 3x enlargement. (B) Representative AFM image in air of clathrin-AP180 coats deposited from assembly buffer (see Methods) on mica and used in this single particle force spectroscopy (SPFS) study. The scale on the right of the image shows the height profiles of the coats, which are smaller than the widths of the coats (note 100 nm scale bar), due to flattening during sample preparation. (In contrast, wet images of CCVs under buffer show little, or no, flattening (33).) (C) Structural knowledge of clathrin and its assemblies: Left - EM reconstruction of a small clathrin-AP2 coat showing facets created by the interlinked triskelions (14); Upper right - a representative structure of the clathrin heavy chain (from 1XI4 of PDB and (15)); Bottom right - the crystal structure of several hair-pin domains from the proximal segment of a triskelial leg (from 1B89 of PDB and (16)).

Figure 2.

AFM-based single molecule force spectroscopy (SMFS). (A) Schematic showing the elements of an instrument used in a SMFS measurement. The sample is pushed onto the cantilever tip and is subsequently pulled away. If the tip adheres to a target attached to its surface, when the surface is pulled away the cantilever bends, its deflection being measured by the focused projected laser beam. The force necessary to maintain the tip-to-sample separation (TSS) thereby can be measured and a force-extension curve can be determined. The tip is moved about the sample surface in order to collect data from a large number of individual targets. (B)-(D) As the sample surface is moved away from the cantilever, increased stretching force is applied on the target (either a single molecule such as a clathrin triskelion, or a molecular complex such as an assembled coat) which will be stretched due to domain unfolding and then pulled apart by the breaking of a final bond. (E) Schematic illustration of the resulting force-extension curve for a single realization of the measurement. As some internal domains are tethered between unstructured peptide chains and are readily extended under increasing force (point 1 to 2, and point 3 to 4), the force vs. separation relationship is characteristic of that of the extension of a worm-like-chain (WLC) model of an entropic spring. Sudden force reduction occurs and the cantilever snaps back toward lower bending when a given internal domain is unfolded and a new segment is added to the WLC (point 2 to 3), or when the final bond ruptures (point 4 to 5).

Figure 3.

Energy landscape of protein folding and interactions in SMFS/SPFS measurements (modified from (46)). (A) The force applied by an AFM probe increases with time t and tilts the energy surface of a folded domain or a biological bond, which frequently contains metastable states with local minima. The force $f(t)X$, along the reaction coordinate X , changes to favor unfolded or unbounded states at large separation. (B) The domain unfolding or bond rupture event happens probabilistically when the lowered energy barrier between the native bound state and the separated/unbounded state becomes comparable to the unit thermal energy fluctuation, $k_B T$, or when the barrier is totally abolished at higher force. Typically, during a measurement, the applied force is increased at a varying force loading rate. At low loading rate, the force needed to bring about unfolding or unbinding is distributed around a value commensurate with the outer energy barrier noted in the top curve in (A) at a relatively large value of X_u . However, at high loading rates, the force needed to overcome the energy barrier that prevents movement from the bound to unbound states is, on average, higher.

Figure 4.

SMFS measurements of clathrin triskelia. (A) As an illustration of the SMFS method, ForceRobot® workspace software (JPK instrument, Germany, ver. 2.29) is used to control the parameters of the experiment and to obtain high frequency voltage data indicating cantilever deflection vs. time as the AFM cantilever and sample were pushed together and then pulled apart. The inset depicts some typical steps of instrument automation, including laser alignment, cantilever spring constant calibration, sample surface engagement, and force spectroscopy measurement repeats. (B) The force curves are inspected automatically for signature features and only a subset typically is saved. The data are converted in a number of ways to better identify various variables. The inset shows six of a few hundred individual measurements recorded on different triskelia as the cantilever searches over the sample surface.

Figure 5.

Analysis of a SMSF measurement of a clathrin triskelion. (A) The “peak-find” function in ForceRobot® analysis software (ver. 2.29, JPK, Germany) was used to detect the peaks from filtered spectroscopy curves after setting a peak height threshold at, typically, 3-4 times that of the standard deviation of the baseline force at the larger separations. The red bands are segments where increasing force is applied during stretching, whereas white gaps indicate unraveling events when the stretching force drops significantly. (B) The force curve within each red band is fitted with a worm-like chain (WLC) model with a constant persistence length of 0.4 nm, simulating an unfolded peptide chain, and used to calculate the effective peptide chain length pertaining to a disrupted region of the target which straightens under the applied force until the next domain unfolds. (C) Additional analyses of the force curves were carried out using mostly MathCad® (Mathsoft, MA) programs to analyze the unfolding event sizes. The chain lengths of the unfolded segments were determined from the horizontal distances between adjacent WLC fits (illustrated between two peaks, order 5 and 6), and force loading rates (illustrated for the peak, order 8) were calculated from the slopes of the WLC curves at the tops of each peak. (In total, 26 peaks are indicated in A and B: note the dim, continuous curves which represent the primary data).

Figure 6.

SMFS fingerprinting of individual triskelia. (A) Triskelion 3D scatter plot showing the detected force peaks for all force curves. The figure shows data from 95 curves extracted from a total of ~27,000 measurements, which yields ~950 unfolding events. The axes represent the tip-to-sample separation (TSS), the force, and the peak order (color-coded scale) of each peak. (Note: In visualizing this figure and Figs. 7 and 8, the grey plane is the rear or back plane) (B) and (C) show 2D scatter plot projections of the data presented in the 3D scatter plot, showing the relationships between peak order vs. peak force, and peak order vs. TSS. (D) and (E) are histograms of the number of peaks identified by peak force or TSS value. Because the maximal size of the target isolated between the substrate and probe is approximately two legs of a triskelion linked at the hub, events seen in Fig. 6E at with TSS greater than about 110 nm line must correspond to partially unfolded clathrin heavy chains.

Figure 7.

SPFS fingerprinting of CCVs, similar to that shown in Fig. 6. The figure shows unfolding events from 206 force curves extracted from a total of ~ 5,100 measurements. Signature events (A-E) are clustered around a TSS of 100 nm and a peak force of around 100 pN. Only a few events at larger TSS are detected, suggesting that native CCVs are fairly resistant to clathrin lattice unraveling.

Figure 8.

SPFS fingerprinting of Clathrin-AP180 coats (cf. Figs. 6 and 7). Individual clathrin-AP180 coats (cf. Fig. 1B) are unraveled. The figure shows unfolding events from about 1260 saved force curves, obtained from a total of ~ 4200 measurement trials. Signature events (A-E) encompass a broad range of both CCV and single triskelion characteristics. Peak force events at larger TSS and lower peak force are attributed to the unraveling of the clathrin lattice of the coats.

Figure 9.

Unraveling of clathrin-clathrin bonding in clathrin-AP180 coats. (A) Scatter plot of event size vs. TSS from 138 force curves showing unfolding events beyond TSS value of 150 nm, extracted from a total of ~ 4200 trials. (B) Histogram distribution of event size, showing events of small size associated with single hairpin unfolding. Events of larger size are associated with cooperative unfolding of up to 4 hairpins, as is similarly seen in SMFS of individual triskelia (Figs. 4-6). The inset depicts part of the clathrin lattice, where one clathrin heavy chain (magenta color) is highlighted, indicating events of large unraveling size not seen in single triskelion force spectroscopies and attributable only to clathrin-clathrin bonding in the lattice where the inter-vertex distance is ca 20 nm. (C) Representative force curve showing more than a dozen events of small unraveling size at TSS of less about 250 nm, and four events of large unraveling size at larger TSS.

Figure 10.

Clathrin lattice stability and dependence on force loading rate. (A) Scatter plot showing that the unraveling force of the coat lattice is negatively correlated with the size of lattice unraveling, extracted from the data set discussed in Fig. 9. (B) Scatter plot along with grouped mean force values, (blue dots) showing the dependence of lattice stability on force loading rate, with the lines showing a representative fit.