Assembly of Clathrin Baskets

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Summary: The creation and internalization of small, protein-coated vesicles is a central factor in the uptake of materials from the surfaces of eucaryotic cells through a process known as receptor mediated endocytosis. Under appropriate in vitro conditions, the principal coat component, viz., clathrin, which is found in the cell as a trimer joined at a common hub, assembles into polyhedral 'baskets' ('cages') containing pentagonal and hexagonal facets. These reconstituted cages have the appearance of miniscule soccer-ball like structures. Their sizes vary over a finite range whose limits depend on the presence or absence of ancillary proteins ('assembly proteins') that are known to increase the tendency for the baskets to form. By fitting data on basket size distributions to simple energetic (thermodynamic) models, one is able to estimate mechanical properties of the clathrin constituents of the baskets and infer the role of assembly proteins in strengthening interactions between the clathrin components of the struts that constitute the edges of the baskets.

Keywords: clathrin; elasticity; endocytosis; self-assembly; triskelia

Introduction

Clathrin is the primary component of the protein coats of transport vesicles involved in receptor-mediated endocytosis ("RME") and various other trafficking activities that occur in eucaryotic cells. In particular, animal cells utilize RME processes to regulate cell surface components [1]. An important example where RME plays a critical role is ErbB signaling involved in cell growth and proliferation. There are at least four members of the ErbB receptor family that form homo- or hetero-dimers which then respond differentially to growth factor ligands, depending on the particular paired combination of receptors [2]. For example, the binding of epidermal growth factor (EGF) ligands to ErbB1 dimers leads to internalization of ligand-receptor complex, but the formation of ErbB2–ErbB1 heterodimers frustrates ErbB1 uptake upon ligand binding, resulting in aberrant signaling and heightened cell growth [3,4]. Overexpression of ErbB receptors is involved in many cancers [3]; in particular, high expression of ErbB2 is a signature of certain forms of breast cancer.

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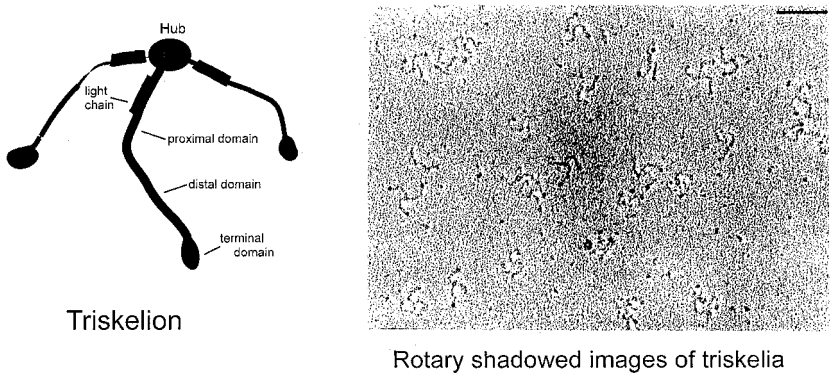

Fig. 1. Cartoon of clathrin triskelion, showing light chains joined to heavy chains near the center hub. (Electron micrograph images adapted from Ref. [10]).

When isolated from cell preparations, clathrin typically is found as a heteropolymer composed of clathrin heavy chains (CHC) of MW ca. 190 kDa and clathrin light chains (CLC) of MW ca. 25-35 kDa [5]. The native, mesoscopic, structure of clathrin is that of a three legged complex known as a "triskelion" that contains three clathrin heterodimers (each composed of a single CHC and single bound CLC), joined at a common hub [6]. The dimensions of the CHC are approximately 2.4–2.8 nm in cross-section and 50 nm when fully extended [7]. The CHC has a tendency to bend midway along its length [6-8] and the lowest energy configuration of the triskelion thus is presumed to be a pinwheel-like structure (see Fig. 1), as evidenced by electron microscopy [9,10]. Under appropriate conditions the triskelia form cage-like supramolecular structures having diameters of the order of 100 nm [9,11] and topologies similar to that of the seams of soccer balls [12]. In this form the cages contain 12 pentagonal faces and an even – but variable – number of hexagonal facets which, for energetic reasons, probably appear in multiples of 4.

The formation of plasma-membrane-derived endocytic vesicles that are involved in receptor mediated endocytosis requires several steps, the exact sequence of which has not yet been established in detail. The canonical scheme involves the binding of ligands to transmembrane receptors, the binding of activated receptors to "adaptor" proteins that move from the cytoplasm to the membrane and then recruit [13] (or perhaps are recruited by [14]) clathrin triskelia to form a coated pit, the growth and invagination of a pit to form a vesicle [15], the closure of the vesicle and its fission from the cell surface, and the

subsequent movement of the vesicle to the interior of the cell where it releases cargo. The universal signature of these endocytic vesicles is a polyhedral clathrin lattice that has the appearance and approximate dimensions of reconstituted clathrin cages. Other components of the coats may be cell or organelle specific.

It is not known how the initial patch of coated membrane invaginates to form the nascent vesicle, but proteins that simultaneously bind to clathrin and certain membrane lipids (mainly phosphoinositides) – thereby possibly bending the membranes – are known to exist [16,17]. Specific proteins – notably dynamin – seem to be involved in pinching of the membrane at the neck where the vesicle detaches from the plasma membrane [18]. Also, proteins that facilitate rearrangement of the clathrin lattice near the neck may be involved [12,19,20]. (Many clathrin-associated proteins that either are integral constituents of vesicle coats, or interact with vesicles transiently during or after their formation, are discussed in a recent review [21].) In addition to being the principal coat component of receptor-linked, plasma-membrane-derived vesicles, clathrin also is found in the coats of certain tubulovesiclar bodies that emanate from the *trans*-golgi network in the interior of the cells. Similarly complex schemes, involving different coat proteins (e.g., COPI or COPII proteins instead of clathrin), give rise to other tubulo-vesicular structures involved in intracellular trafficking [22].

Given the intricacy of this system, one might at first think it impossible to ask questions of a physical nature about the processes occurring during formation of coated vesicles. Although present as isolated triskelia in vitro at pH 7.0, clathrin, alone, spontaneously assembles at low pH ($pH \leq 6.5$) to form closed cages ("baskets"). Such cages also can be assembled in the presence of "adaptors" [23], in which case they are smaller and of more uniform size [24] (see Fig. 2). These assembly factors induce basket formation even at physiological pH ($pH \approx 7.0$). The data shown here were obtained when using an imprecisely characterized fraction of "assembly proteins" (APs), which probably mostly consisted of the AP-2 adaptor complex. However, the behavior shown in Fig. 2 is typical for assembly with adaptors of various kinds [25,26]. It is of interest to note, also, that when native coated vesicles are harvested, one seemingly finds variation in mean vesicle size that depends on the source of the specimens [27].

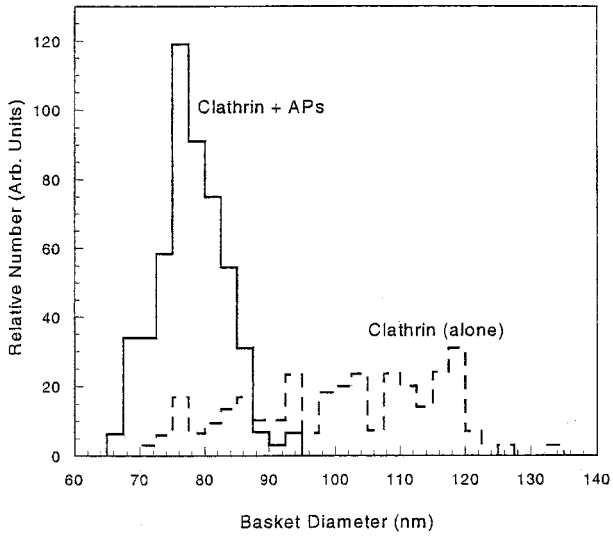

Fig. 2. Size distributions of baskets assembled in the presence (solid line) and absence (dotted line) of APs (adapted from [24]).

Inferences from Theory

Why is there dispersion in the sizes of reconstituted baskets? Clathrin cages are closed biological polymers, other examples being virus capsids [28], peptide-conjugated tubulin rings [29] and microtubules [30] (whose lengths vary widely, but whose cross-sections are closed tubes). That the baskets are closed and of finite size might be explained by the shapes of the building blocks – i.e., the triskelia. To assemble into baskets, the triskelia might have a natural "pucker." That is, they would be capable of assuming a posture where, if the ends of the legs were touching a plane, the central hub (Fig. 1) would be elevated about the surface. Indeed, such form has been inferred from carefully prepared electron microscopy images [31]. However, it is difficult to quantify the pucker from such images and, in any event, *solution* structures cannot be determined in this way. Confirmation of the existence of puckered shape in solution results from the application of

static and dynamic light scattering [32]. The notion of "intrinsic pucker" of *flexible* triskelia can be used to rationalize the dispersion in basket sizes.

A simple way to characterize the energy of such a puckered entity at the mesoscopic level is

$$g^{dist} = \kappa f(\xi - \xi_0) \qquad (1)$$

where ξ is the "curvature" of the structure (integrated over the entire triskelion) and ξ_0 is the curvature of the lowest energy shape of the triskelion. κ plays the role of a rigidity modulus (or Hooke's Law constant) and, in general, $f(x \rightarrow 0) = 0$; $f(x \rightarrow \text{large}) \rightarrow$ *inf.* Since we are interested in using an expression of this nature to describe the energy of clathrin cages of "size" N, it is useful to index ξ_0 to N_0, the cage of lowest distortion energy. That is, in order to assemble a basket containing N triskelia, some triskelia need to be expanded (opened) or contracted (squeezed), or stretched or twisted (or all of these). Not enough is known about the detailed tertiary structure of a triskelion (MW ~ 650 kDa) to enable us to calculate this complex dependence which, additionally, probably depends on the position of a triskelion in an assembled cage.

To first approximation we lump these factors together, take κ to be a phenomenological constant, and consider ξ to vary inversely with N (so that f, in effect, is a function of the difference between the average solid angle subtended by a triskelion in a cage of size N and the solid angle subtended in the cage of lowest distortion energy). In this case the first term in an expansion of f (x) is $g^{dist} = \kappa (1/N - 1/N_0)^2$, which diverges when $N << N_0$ and tends to zero when N is close to N_0. This expression clearly fails to represent the "large N" distortion energy of a typical triskelion because $g^{dist} \rightarrow$ *constant* as N gets very large. But, the total distortion energy of a basket varies as $G_N^{dist} \sim Ng^{dist}$, so even in this approximation the total distortion energy involved in assembling a basket diverges, as expected, when the basket gets very large. Of course, baskets would not form unless there were some net favorable interaction energy between triskelia which, to first order, can be taken as $G_N^{stab} = -bN$, where b is a positive constant containing both enthalpic and entropic components. When these terms are combined, we obtain

$$G_N = aN (1/N - 1/N_0)^2 - bN \qquad (2)$$

where we have replaced the general term κ with the symbol a.

The expression given by Eq.(2) contains only three parameters: rigidity coefficient, a; the basal shape parameter N_0; and a term signifying the inter-leg interaction energy, b. Upon assuming that the baskets assemble by a thermodynamically reversible process, it follows that the equilibrium distribution of basket sizes is given by $P_N \sim \exp(-G_N/k_BT)$, which can be used to obtain expressions that relate the energy parameters {a, N_0, b} to the width of the distribution ($W_{1/2}$), the value of N where the distribution is maximal (N*), and to the values of N where P_N effectively becomes zero [33]. These can be used to fit the basket assembly data shown in Fig. 2, thereby providing estimates of various energies associated with cage assembly. Good fits are obtained [33], and one finds, for example, that under conditions used for in vitro assembly, the net energies of adding or removing triskelions to "growing" baskets are of the order of k_BT. This result agrees with observations that significant nucleotide hydrolysis apparently does not take place during coated vesicle (CV) formation, other than GTP hydrolysis associated with freeing the CVs from the plasma membrane (although ATP hydrolysis is needed to uncoat the vesicles). One also finds that the free energy change occurring when baskets form in the presence of assembly proteins ("APs") is greater than that occurring without APs [33]. It can be shown that the critical clathrin concentration for assembly, C_c, depends on the free energy change in an inverse manner. That is, the deeper is the "energy well" associated with assembly, the lower is C_c and more stable the cages that are formed ([34]), which is why the presence of APs favors basket formation.

By fitting data to this model, one also obtains an estimate of an effective "rigidity modulus" associated with distorting the triskelia. Is such assessment consistent with estimates obtained by other means? Due to its small size, it is difficult, if not impossible, to directly measure the response of a triskelion to an applied force. The only previously published estimate of triskelion rigidity relates the shape fluctuations of triskelion legs, as inferred from electron microscope images, to the flexural rigidity, EI, of the legs [8]. In that analysis the images of triskelions placed on grids by glycerol nebularization were assumed to be reasonably accurate representations of triskelion shapes in solution. The Boltzmann equation and a Hooke's Law description relating the bending energy of a thin wire to differences between stressed and relaxed shapes were used, yielding expressions for variances of Fourier mode amplitudes of triskelial shape in terms of the mode number, the length of the triskelion leg, and EI. Decomposition of the fluctuating shapes into normal modes thus yielded a value of $EI_{clath} \approx 35\ k_BT$-nm for an individual leg that makes intuitive sense, in that EI_{clath} in this case is intermediate between that of cytoskeletal

elements like actin filaments and microtubules, and that of soft network components such as elastin. Additionally, after making plausible assumptions about the cross sections of the composite strut linking vertices in a basket, a value of E ≈560 k_BT-nm was obtained for the flexural rigidity of the strut. Then, the energy necessary to bend a patch of clathrin lattice was compared with that necessary to bend plasma membrane of equivalent area. The magnitudes of these quantities were found to be similar, suggesting that the clathrin lattice can assist in the in vivo budding process but, by itself, is unlikely to be able to cause a patch of membrane to form the highly curved structures characteristic of coated vesicles.

Given the number of assumptions that enter into the analysis described in the foregoing paragraph, it is desirable to obtain another estimate for the flexural rigidity of a basket strut, EI_{strut}. By assuming that ξ in Eq.(1) represents the average *curvature* of the strut (some "lumped" characteristic of triskelion shape), an expression analogous to that of Eq.(2) can be obtained [34], viz.,

$$G_N = hN\,(1/N^{1/2} - 1/n^{1/2})^2 - bN\,, \qquad (3)$$

where h is directly proportional to EI_{strut}. Upon using this expression to reduce the data shown in Fig. 2 we find, remarkably, that the value of $EI_{strut} \approx 600\ k_BT$-nm for baskets assembled in the presence of APs is in close agreement with that obtained from the earlier analysis [8] when it was assumed that the triskelion legs do not move with respect to each other within a composite strut. Moreover, the value of EI_{strut} for baskets formed in the *absence* of APs is an order of magnitude lower than in the presence of APs, but several times greater than that of an individual triskelion leg. Taken together, these results suggest that a mechanical role for APs is to prevent slippage of legs with respect to one another when a basket is stressed [manuscript in preparation].

In summary, these simple thermodynamic/energetic models rationalize the finite size-range of reconstituted cages. The use of such models to analyze basket assembly data confirms that basket growth can involve lattice rearrangements driven only by thermal mechanisms. It also provides numerical estimates of clathrin bending rigidity that agree with values inferred by analyzing electron micrographs of triskelial shapes. The models enable us to infer ways that molecular variables affect basket stability and, in particular, confirm (and provide numerical estimates of) the influence of adaptor complexes (i.e.,

assembly proteins) on inter-leg stabilizations. Similar models can be adapted to investigate the conditions affecting the critical concentration for basket assembly, and to extend the analysis to considerations of compound vesicles that contain membrane and/or cargo.

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