Clathrin assembly into coated pits and vesicles is promoted by accessory proteins such as auxilin and AP180, and disassembly is effected by the Hsc70 ATPase. These interactions may be mimicked in vitro by the assembly and disassembly of clathrin “baskets.” The chimera C58J is a minimal construct capable of supporting both reactions; it consists of the C58 moiety of AP180, which facilitates clathrin assembly, fused with the J domain of auxilin, which recruits Hsc70 to baskets. We studied the process of disassembly by using cryo-electron microscopy to identify the initial binding site of Hsc70 on clathrin-C58J baskets at pH 6, under which conditions disassembly does not proceed further: Hsc70 interactions involve two sites: (i) its major interaction is with the sides of spars of the clathrin lattice, close to the triskelion hub and (ii) there is another interaction at a site at the N-terminal hooks of the clathrin heavy chains, presumably via the J domain of C58J. We propose that individual triskelions may be extricated from the clathrin lattice by the concerted action of up to six Hsc70 molecules, which intercalate between clathrin leg segments, prying them apart. Three Hsc70s remain bound to the dissociated triskelion, close to its trimerization hub.

One of the major endocytic pathways occurs through formation of vesicles coated with lattices of polymerized clathrin triskelions (1–3). The ability of these lattices to form curved structures deforms the membrane, possibly in concert with proteins that directly induce membrane curvature (4), leading to the formation of coated pits and ultimately their pinching off into coated vesicles. Clathrin is then released from these particles in an ATP-dependent process prior to the vesicles fusing with endosomes. In vitro, in the presence of assembly proteins (APs), clathrin assembles into membrane-free baskets whose closed clathrin lattices are similar to, albeit mostly smaller than, those that assemble on coated vesicles in vivo.

The formation and dissociation of clathrin coats is a dynamic process that is regulated by a variety of factors. Of these, the ATP-binding molecular chaperone, Hsc70, is intimately involved in uncoating (5–7). However, Hsc70 does not work alone; it requires a J domain protein such as auxilin that interacts with both the clathrin N termini and, through its J domain, with Hsc70, and presents the Hsc70 to the clathrin lattice (8). After binding to the clathrin lattice, Hsc70 dislocates triskelions in a reaction that is accompanied by ATP hydrolysis. In vitro, Hsc70 binds to clathrin-AP baskets complexed with catalytic amounts of auxilin and causes the dissociation of triskelions at neutral pH. It seemed possible that any AP binding to the clathrin N terminus and carrying the auxilin J domain would be able to facilitate uncoating by Hsc70, and this property was demonstrated for a chimera called C58J (9). This construct, consisting of C58, the 58-kDa C-terminal clathrin-binding domain of AP180, and the auxilin J domain was shown to support uncoating by Hsc70, although C58J acted stochiometrically, whereas auxilin acts catalytically.

The complex released by Hsc70 from the coated vesicle or clathrin-AP basket is thought to be composed of one clathrin triskelion, three Hsc70 molecules, and one or more APs (10). It has been reported from quick freeze/deep etch EM studies of dissociated clathrin triskelions (11, 12) that Hsc70 molecules are attached close to the trimerization hub where the C termini of the clathrin heavy chains reside. Biochemical data suggest that auxilin binds to the clathrin heavy chain N terminus and distal leg domain (13).

Clathrin baskets occur in many forms, some with defined symmetry (14, 15). This polymorphism reflects structural adaptability in vivo whereby clathrin coats may envelope vesicles of various sizes. One of the most abundant forms of basket is the D6 barrel, whose structure has been reconstructed from cryo-electron micrographs for several variants (16–18). This basket consists of a lattice of open hexagons and pentagons in which the 3-fold vertices are connected by 225-A-long spars. A clathrin triskelion is centered on each vertex. The triskelion has three legs, each with three segments (19): a (hub)proximal part, a distal part, and a terminal region representing the N terminus. Each spar is a bundle of two oppositely directed proximal leg segments and two distal segments (20). The adapter protein AP-2 (17) and auxilin (18) have been located inside the barrel, contacting the clathrin heavy chain N termini.

Clathrin may be assembled into baskets with C58J acting as an AP, and Hsc70 can bind to these baskets at pH 6.0 without uncoating (9). This mode of binding most likely represents the first step in the uncoating pathway because this reaction proceeds upon shifting to pH 7.0. To identify the Hsc70 binding site, we have visualized clathrin-C58J baskets with and without bound Hsc70 at pH 6.0. The consistent result was the presence of Hsc70 as diffuse rings of density in the polygonal...
faces of the clathrin lattice, making closest contact with the spars close to the vertex. This placement allows Hsc70 to interact with the triskelion hub, subsequently destabilizing the interactions between leg segments to mediate uncoating.

**MATERIALS AND METHODS**

*Preparation of Proteins—C58J constructs were cloned into 6-histidine pQE30 vector, expressed, and purified as described (9). Hsc70 was prepared from bovine brains (21). Clathrin was extracted from coated vesicles using 0.5 M Tris, pH 7.5, as described (20). Clathrin-C58J baskets were made by overnight dialysis against 0.1 M MES, 1 mM dithiothreitol, pH 6.5. After centrifugation for 1 h at 150,000 × g, the pellet was suspended in buffer A consisting of 20 mM MES, pH 6.0, 2 mM magnesium acetate, 25 mM KCl, 10 mM (NH4)2SO4, 1 mM dithiothreitol. The concentration of triskelions in clathrin-C58J baskets was determined from absorbance after solubilizing the baskets with 0.5 M Tris, pH 8.0. The binding of Hsc70 to clathrin-C58J baskets was performed in buffer A by incubating either 0.9 M triskelions in the presence of 1 mM ATP and an ATP-regenerating system consisting of 30 units/ml creatine phosphokinase and 15 mM phosphocreatine.

*ATP-regenerating system* consisting of 30 units/ml creatine phosphokinase and 15 mM phosphocreatine.

*Binding of Hsc70 to Clathrin Baskets*—Hsc70 with 0.3 M (stoichiometric) or 3 M (excess) Hsc70 with 0.3 μM triskelions in the presence of 1 mM ATP and an ATP-regenerating system consisting of 30 units/ml creatine phosphokinase and 15 mM phosphocreatine.

*Cryo-electron Microscopy—* Three μl samples at 1–3 mg/ml protein were applied for 10 min to grids bearing holey carbon films, blotted, quenched, and observed in a CM200-field emission gun (FEG) electron microscope (FEI, Mahwah, NJ) equipped with a model 626 cryo-holder (Gatan, Pleasanton, CA). Grids were searched at low magnification using a CCD camera, and micrographs were recorded on film, using low dose procedures and a magnification of 38,000 ×.

*Image Processing—* Negatives were scanned with a SCAI scanner (Z/I Imaging, Huntsville, AL) and binned 3-fold to give 5.53 Å pixels. Micrographs typically contained 100–300 usable particles, which were extracted with the program X3D preprocess (22) or the program bshow (23). Defocus values determined in bshow ranged from 1.3 to 7.0 μm (first CTF zeros at 21–48 Å). CTF effects were corrected by simply flipping the phases in alternate lobes of the CTF with the program bctf (23).

Reconstructions were calculated by an iterative projection matching approach. For an initial model of the clathrin-C58J D6 barrel, a stick model was constructed, with a vertex-to-vertex length of 225 Å (3). Particle orientations and origins were determined with the programs PFT (24) and EMAN (25). Final orientations were determined with PFT2 (a modified version of EMPFT interfacing with Bsoft), and reconstructions were done with the program preconstruct (23). This final model was used as starting model to determine particle image orientations for Hsc70-loaded baskets, whose final reconstruction was made after several rounds of orientation determination.

The hand of the D6 basket was determined by the method of Belnap et al. (26). Images of the same particles were recorded at tilt angles of 0° and −5°, and a reconstruction was computed from the 0° images. Projections corresponding to the −5° views were computed from maps with both of the possible hands for each particle, using their Euler angles and the known position of the tilt axis. These projections were then compared with the −5° EM images by calculating the cross-correlation coefficient in each case.

**RESULTS AND DISCUSSION**

Our goal was to observe assembly-regulating proteins bound to clathrin baskets by cryo-EM. In two previous studies of this kind, with AP2 (17) or auxilin (18), the interpretability of the results was limited by the presence of thick-walled inner shells in which individual subunits could not be distinguished. In an attempt to avoid this problem, we decided to use C58J as both an assembly protein and a J domain protein. C58J is relatively small, compared with AP180 and AP-2. Moreover, it is likely to be a good model for auxilin activity in the initial steps of uncoating because the two proteins behave identically in inducing the binding of Hsc70 to baskets, with or without APs, at pH 6.0 (9).

*Cryo-micrographs of Baskets with and without Hsc70—* Clathrin-C58J baskets were assembled at pH 6.0 and examined by cryo-EM (Fig. 1A). As expected, these populations were polymorphic, exhibiting a variety of sizes and shapes. Initial binding experiments in which equimolar amounts of Hsc70 were added yielded baskets that, by visual criteria, differed little from those without Hsc70 (data not shown). When the experiments were repeated using a 3-fold excess of Hsc70 (Fig. 1B), the shells of the baskets appeared to have been systematically thickened. The vertices and spars that are often visible as punctate features and striations in cryo-micrographs of Hsc70-free baskets were blurred. However, the periphery appeared to be as sharply defined in both cases. These observations suggest that Hsc70 binds to clathrin shells but protrudes little if at all beyond their outer margins.

*Image Reconstruction and Determination of Hand—* The D6 barrel is a relatively abundant assembly form that has been analyzed in previous studies, and we also focused on these particles. Composed of 36 triskelions, the barrel has 12 pentagonal and 8 hexagonal faces, and exhibits 6-2-2 point group symmetry. To calculate three-dimensional reconstructions, we followed an iterative projection matching approach. To start the calculation, we generated a stick model. As data, we selected all particles of approximately the right size, anticipating that particles of similar size but different symmetry or with imperfect D6 symmetry would fail to achieve high correlation coefficients during the subsequent analysis and would be screened out for that reason.

**FIG. 1.** Cryo-electron micrographs of clathrin baskets prepared in the presence of C58J (A) and C58J (B) with excess Hsc70 added. Galleries of D6 barrels are shown at higher magnification in the bottom strips.
Although the initial model did not have any handedness, handedness gradually became evident after several rounds of reconstruction. However, there remained an ambiguity as to the absolute hand, i.e. both the model obtained and its mirror image were equally compatible with the data. To resolve this ambiguity, we employed a method that involves recording tilt pair micrographs (26). Tilting the models (both enantiomorphs) through the same angle produces differing projections that may be matched with the experimental tilted image, with better agreement for the correct hand. The correlation coefficients obtained for the correct versus incorrect hand were 0.35 (± 0.12) and 0.10 (± 0.07), respectively (n = 107). This result confirmed the hand that has been assigned in previous studies, although we are not aware of this property having been determined before.

As noted above, micrographs of clathrin-C58J baskets to which equimolar Hsc70 had been added differed little from those without Hsc70. Nevertheless, reconstruction produced a model with faint rings of density inset in its polygonal faces. Repeated rounds of erasing these densities before feeding the map into the next cycle yielded a reconstruction very similar to the basket with no Hsc70 (the latter data are not shown), with no densities in the polygonal faces. We infer that our filtering procedure selected particles with little or no Hsc70 from a mixed population. The assumption that Hsc70 binding to clathrin is not saturated under these conditions is consistent with earlier binding studies (12) showing that three Hsc70s bind per clathrin triskelion. Because this map is of slightly higher resolution, we used it as our reference structure, lacking Hsc70 (Fig. 2A).

Location of C58J in the Clathrin Basket—Baskets assembled under the conditions used are estimated to contain one C58J molecule/clathrin triskelion (9). C58J, like AP180 (27), binds to the N terminus of clathrin, which contributes prominent hook-like features that cluster in rings and are connected to the outer framework of the clathrin shell (Fig. 2, A and C). Docked into the hooks of our density map, the atomic model for this portion of clathrin (28) was well accommodated but left unoccupied density that we assign to (part of) C58J (arrows).

![Reconstructions of clathrin baskets prepared in the presence of C58J (A and C) and C58J with an excess of Hsc70 (B and D). The diffuse densities (blue) in B and D are inferred to be Hsc70, showing the closest approach to the clathrin lattice near the vertices (arrows).](image1)

![The atomic structure of the clathrin heavy chain N terminus and part of the distal domain (28) built into a pentagonal face of the reconstruction of the clathrin-C58J basket. The irregular densities between the N termini and extensions from them are possibly parts of C58J (arrows).](image2)
these accessory molecules, on whose surface the clathrin-interacting domains were exposed. This interpretation is supported by the absence of such an inner shell from our density maps (Figs. 3 and 4), correlating with the absence of the remainder of AP-2 and auxilin, respectively. It follows that the C58 domains organize the N termini into rings and in this role are not dependent on interactions among the remaining portions of APs or auxilin, i.e. the inner shells of density seen in the earlier reconstructions.

Interaction of Hsc70 with Clathrin-C58J Baskets—The reconstruction obtained in the binding experiment with a 3-fold excess of Hsc70 had stronger rings in each polygonal face (blue in Fig. 2, B and D). The density in these rings, around the 5-fold axis and the local and global 6-fold axes, is not differentiated into discreet features that we might equate with Hsc70 molecules. Moreover, this density is lower (~50%) than that of clathrin. This property accounts for the fact that the rings do not appear to make contact with the clathrin shell, when represented by isodensity surface rendering (as in Fig. 2B). However, the Hsc70-associated density is evident upon comparing gray level sections through the respective maps (Fig. 4). This density appears to coat each spar on either side (arrows in Fig. 4, A and B), making closest contact near the hub (arrows in Figs. 2B and 4, C and D). The Hsc70 molecules also contact well defined underlying density (arrows in Fig. 4, F and G) that we associate with C58J molecules bound to clathrin N termini (see above). Given the role of J domains in Hsc70 recruitment, it is probably this interaction that is visualized. It appears, then, that Hsc70 binding involves two interactions: (i) it binds to a J domain associated with the N terminus of one (or two) clathrin heavy chain N terminus.

![Diagram](image-url)
other clathrin molecules. Because there are insufficient C58Js to accommodate all the Hsc70s (see below), we infer that, after their recruitment, some Hsc70s relax or are displaced from the first interaction while retaining interaction or adjusting their interaction with the clathrin lattice.

How to explain the observed Hsc70-associated density as continuous rings? In principle, such a distribution might arise from steric interference whereby an Hsc70 molecule, once bound, blocks binding at an adjacent position. If binding sites were to be occupied at random, the resulting averaged density distribution would show an apparent fusing of molecules at adjacent, mutually exclusive, positions and reduced density compared with clathrin. However, because Hsc70 is a globular monomer of 6–8 nm in diameter (11), there should be enough space in the polygonal faces for full occupancy, and our resolution is well sufficient to resolve subunits of this size. Alternative explanations are that there is more than one binding site on the side of a spar or the attachment site is mobile. There is, in fact, prior evidence for multiple binding sites for Hsc70 on clathrin (30), and if two distinct and mutually exclusive sites were to be partially occupied, the observed smearing of Hsc70-related density would be expected to occur.

Although we cannot rule out the possibility that occupancy of Hsc70 molecules was reduced somewhat when samples were prepared for cryo-EM, the most plausible explanation for the lower level of Hsc-related density compared with clathrin density is the presence of two or more mutually interfering Hsc70 binding sites. Notwithstanding this complication, the data sufficed to identify the predominant points of contact with the clathrin lattice (Fig. 4, arrows).

Resolution—The resolution of the three density maps was assessed in terms of the Fourier shell correlation coefficient (FSC) criterion (Table I). To facilitate comparison with earlier studies, we list resolutions for three thresholds. The most conservative threshold is 0.5. By this standard, the resolution of our clathrin-C58J barrel is 28 Å, which compares with 36 Å achieved for the clathrin-auxilin barrel (18). The least conservative criterion corresponds to the FSC remaining above three times the theoretical noise limit (adjusted for 12-fold symmetry): according to it, the resolution of clathrin-C58J barrel is 23 Å, which is comparable with the 21 Å of the clathrin-AP2 map (17).

Global Distortion of the Basket upon Hsc70 Binding—To facilitate localization of Hsc70, we attempted to generate different maps. However, the results were not easily interpretable. On closer examination it turned out that the basket with Hsc70 bound has a slightly different axial ratio, arising from a slight shortening (by ~20 Å), with an increase in width of ~10 Å, i.e. Hsc70 binding effects a global distortion of the basket lattice. Accordingly, we based our localization on side-by-side comparison of the Hsc70-bound structure and the control.

Binding Stoichiometries—Like AP180 (31), C58J binds with a stoichiometry of one molecule/clathrin triskelion, in contrast to auxilin, which binds with a stoichiometry of three molecules/clathrin triskelion. The parts of both auxilin and AP180 that bind to clathrin appear to be extended with little regular secondary structure (13, 32). In particular, the C58 domain of AP180 has 12 DLL sequence motifs that could link multiple N termini (32). Such an arrangement could explain the somewhat variable appearance of different hooks (Figs. 2, A and C, and 3).

The J domain in C58J (~100 amino acids) has a four-helix structure (33), but it may not be clearly visible in our maps because it occurs as only one copy/three N termini: Because C58J binds at a stoichiometry of one/triskelion, whereas three Hsc70 molecules bind per triskelion (9), at most one of three Hsc70 molecules can remain bound to C58J. The other Hsc70 molecules are presumably bound to the clathrin. There are, in fact, indications that Hsc70 can remain associated with the clathrin lattice after relinquishing its interaction with a J domain. One is the stoichiometry data summarized above for saturating binding of C58J and Hsc70. Another is the catalytic role played by auxilin in Hsc70 recruitment (8).
Uncoating—The inferred location of the major Hsc70 binding site places it close to the triskelion hub (Figs. 2B and 4). It is plausible that when uncoating proceeds, e.g. by shifting to neutral pH in this in vitro system, the Hsc70 molecules adjust their interactions, each remaining associated with a proximal leg segment emanating from that hub and prying it apart from the other three leg segments in that spar. Similar interactions at other adjacent hubs would facilitate this disengagement by releasing the distal leg segments, although the Hsc70 molecules responsible would remain with other triskelions. In any event, the three Hsc70 molecules associated with a given triskelion hub would remain there where they have been visualized on Hsc70-dissociated triskelions (11). Extrac tion of triskelions from the lattice is facilitated by the fact that the interactions between the clathrin leg segments are rather weak (34, 35) and should be further promoted by the prior release of adjacent triskelions, leaving fewer interactions to disrupt. Given the weakness of individual interactions, fewer than six Hsc70 molecules may suffice to extract a triskelion.

In this context, the release of an individual triskelion-Hsc70-ADP complex with C58J bound to it is predicted by the observation that individual clathrin triskelions can be dissociated from clathrin baskets made with low ratios of C58J to C58 (9). Extraction of individual triskelions could also explain the exchange of individual clathrin triskelions that occurs as the vesicle (36).

Fig. 5 shows a scheme of such an uncoating pathway employing Hsc70 and a J domain protein. After recruitment of Hsc70 to the clathrin coat, hydrolysis of ATP would then lead to dissociation of triskelions from the lattice by the mechanism outlined above. Then the Hsc70-ADPs bound to the hubs of dissociated triskelions would rebind ATP and be released and recycled. Alternatively, they might remain bound if auxilin and Hsc70 were involved in cha rpening the dissociated clathrin triskelions, as suggested by studies showing that, in the absence of auxilin, clathrin aggregates in the cytosol and is unable to form coated pits. Finally, after the coated pit reforms, auxilin and Hsc70 at the clathrin hubs would be involved in the exchange of individual clathrin triskelions that occurs as the coated pit invaginates and is pinched off to form a coated vesicle (36).

Addendum—While this paper was under review, a paper appeared reporting a cryo-EM structure for the D6 clathrin basket with a J domain-containing fragment of auxilin bound to it (37). The auxilin fragment was localized above the hooks of the clathrin heavy chain N termini. These authors speculated that Hsc70 might bind underneath the trimerization hub to a C-terminal sequence of the clathrin heavy chain. Our observation is that Hsc70 binds to the sides of the spars, preferentially near the hubs but not underneath them. Interestingly, these authors reported a global distortion of the basket upon binding the auxilin fragment comparable with the one that we have observed between the C58J-bound state and the C58J+Hsc70-bound state.

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