Clathrin Coat Disassembly by the Yeast Hsc70/Ssa1p and Auxilin/Swa2p Proteins Observed by Single-particle Burst Analysis Spectroscopy*

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Background: Hsc70-auxilin rapidly disassembles clathrin coats from synaptic vesicles for function in neurotransmission.

Results: Ssa1p-Swa2p cooperatively disassembles yeast clathrin into coat fragments containing multiple triskelia.

Conclusion: Single-particle analysis of yeast clathrin coat disassembly leads to the identification of a partial coat intermediate.

Significance: Discovery of a partial clathrin coat intermediate may shed light on coordinated vesicle transport events in the cell.

The role of clathrin-coated vesicles in receptor-mediated endocytosis is conserved among eukaryotes, and many of the proteins required for clathrin coat assembly and disassembly have orthologs in yeast and mammals. In yeast, dozens of proteins have been identified as regulators of the multistep reaction required for endocytosis, including those that regulate disassembly of the clathrin coat. In mammalian systems, clathrin coat disassembly has been reconstituted using neuronal clathrin baskets mixed with the purified chaperone ATPase 70-kDa heat shock cognate (Hsc70), plus a clathrin-specific co-chaperone, such as the synaptic protein auxilin. Yet, despite previous characterization of the yeast Hsc70 ortholog, Ssa1p, and the auxilin-like ortholog, Swa2p, testing mechanistic models for disassembly of nonneuronal clathrin coats has been limited by the absence of a functional reconstitution assay. Here we use single-particle burst analysis spectroscopy, in combination with fluorescence correlation spectroscopy, to follow the population dynamics of fluorescently tagged yeast clathrin baskets in the presence of purified Ssa1p and Swa2p. An advantage of this combined approach for mechanistic studies is the ability to measure, as a function of time, changes in the number and size of objects from a starting population to the reaction products. Our results indicate that Ssa1p and Swa2p cooperatively disassemble yeast clathrin baskets into fragments larger than the individual triskelia, suggesting that disassembly of clathrin-coated vesicles may proceed through a partially uncoated intermediate.

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3 The abbreviations used are: Hsc70, 70-kDa heat shock cognate; BAS, burst analysis spectroscopy; FCS, fluorescence correlation spectroscopy; Hsp, heat shock protein; NTA, nitritotriacetic acid; COPⅠ, coat protein complex I; COPⅡ, coat protein complex II.
Conventional clathrin-uncoating measurements rely on bulk biochemical assays. Clathrin coat disassembly has been reported as the appearance of clathrin triskelia in the supernatant fraction of a high speed centrifugation of fractionated brain homogenates, although baskets remain in the pellet (9, 23). Disassembly of neuronal clathrin coats has also been observed by measuring changes in average light scattering as a function of Hsc70 ATPase activity (24, 25). Recent work has suggested a cooperative disassembly model based on total internal reflection fluorescence microscopy measurements with tethered neuronal clathrin baskets reconstituted from purified components (20).

Yet despite these advances, the minimal machinery required to disassemble nonneuronal clathrin baskets, such as those in yeast, remains elusive. One possible hurdle is purification of fully active and/or complete disassembly machinery. The Hsc70, Ssa1p, with the auxilin-like cofactor, Swa2p, were shown to have ATPase activity and to bind clathrin (4, 5); however, stimulation of yeast clathrin disassembly was not detectable using a centrifugation-based assay. It is possible that, unlike in neurons, the core clathrin disassembly machinery in yeast requires additional factors. Alternatively, the basic clathrin disassembly mechanism of the yeast Hsp70-auxilin system is conserved, and the purified yeast proteins fully active, but the centrifugation assay cannot separate the products of a yeast disassembly reaction from intact clathrin baskets.

Here we reconstitute the disassembly of yeast clathrin baskets in free solution, using purified yeast Hsc70, Ssa1p, and auxilin ortholog, Swa2p. A combination of fluorescence correlation spectroscopy (FCS) and a novel technique, single-particle burst analysis spectroscopy (BAS (26)), reveals that yeast clathrin baskets are cooperatively disassembled into basket fragments that are significantly larger than individual triskelia.

**EXPERIMENTAL PROCEDURES**

**Strains and Media—**Bacterial strains, DH5-α and Rosetta BL21(DE3) (Novagen), were grown in Luria-Bertani medium supplemented with ampicillin and/or chloramphenicol, as indicated. *S. cerevisiae* haploid strains, W303b, HRY54 CHC1-GFP which carries a chromosomal GFP-tagged variant of the yeast clathrin heavy chain gene CHC1 (yGL206C; Invitrogen), chc1-ts (27), and NY13 (as a wild-type control; a gift from P. Novick) were grown in yeast extract-peptone-dextrose medium supplemented with (NH₄)₂SO₄ to 0.5 M, and loaded onto a phenyl-Sepharose HP hydrophobic interaction column (GE Healthcare) equilibrated with HIC buffer A: 50 mM NaPO₄, pH 8.0, 500 mM NaCl, 20 mM imidazole, 10 mM 2-mercaptoethanol, and Ssa1p was eluted in a step gradient at 20% Ni-NTA buffer B (50 mM NaPO₄, pH 8.0, 500 mM NaCl, 500 mM imidazole, 10 mM 2-mercaptoethanol). Fractions containing Ssa1p (detected by Coomassie-stained SDS-PAGE) were dialyzed into 25 mM Tris-Cl, pH 7.4, 50 mM KCl, 0.5 mM EDTA, and 2 mM DTT. Diaxylized Ssa1p was loaded onto a Mono Q column (GE Healthcare) equilibrated with Q-buffer A (50 mM Tris-Cl, pH 7.4, 0.5 mM EDTA, 2 mM DTT). Impurities were separated over a linear gradient of Q-buffer B (50 mM Tris-Cl, pH 7.4, 0.5 mM EDTA, 2 mM NaCl, 2 mM DTT). Ssa1p fractions were concentrated and loaded onto a HiLoad Superdex 200 gel filtration column (GE Healthcare) equilibrated and run in gel filtration buffer (25 mM Tris-Cl, pH 7.4, 100 mM KCl, 0.5 mM EDTA, and 2 mM DTT). The same protocol was used to purify His₆-Ydj1p (hereafter “Ydj1p”), except pET15b-YDJ1 was transformed into Rosetta BL21(DE3) cells, PMSF was used as the protease inhibitor, and one passage through the microfluidizer was sufficient for lysis. For His₆-Swa2p (hereafter “Swa2p”), pET21a-SWA2-transformed Rosetta BL21(DE3) cells were induced at 22 °C, A₆₀₀nm = 0.8 with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cell lysis and affinity chromatography were performed as for Ydj1p. Ni-NTA fractions containing Swa2p were pooled, supplemented with (NH₄)₂SO₄ to 0.5 M, and loaded onto a phenyl-Sepharose HP hydrophobic interaction column (GE Healthcare) equilibrated with HIC buffer A: 50 mM Tris-Cl, pH 7.4, 0.5 mM EDTA, 0.5 M (NH₄)₂SO₄, and 2 mM DTT. The column was washed with 10 column volumes of HIC-buffer B, and Swa2p was eluted by stepping up to 100% HIC-buffer B (the same buffer without ammonium sulfate). Swa2p fractions were concentrated, diluted 5-fold with Q-buffer A, loaded onto a Mono Q column equilibrated with Q-buffer A and eluted in a linear gradient of Q-buffer B at 20% buffer A. Swa2p fractions were concentrated, loaded onto a HiLoad Superdex 200 column (GE Healthcare), and eluted in gel filtration column buffer. All lysis and purification steps were kept at 4 °C.

**ATPase Assay—**The rate of ATP hydrolysis by Ssa1p was measured using [γ-³²P]ATP in a radioactive phosphate release assay (29).

**Isolation of GFP-labeled Clathrin Baskets from Yeast—**A 2-liter culture of HRY54 CHC1-GFP *S. cerevisiae* was grown to A₆₀₀nm = 0.6 in YPD at 30 °C. Yeast cells were suspended in 500 ml of spheroplast solution (10 mM Tris-Cl, pH 7.5, 10 mM CaCl₂, 0.8 mM sorbitol, 2 mM DTT, and 50 μg/ml of Zymolase 100-T (US Biologicals)) and incubated at 30 °C until the A₆₀₀nm indicated a 40% drop in optical density upon dilution with water. Spheroplasts were centrifuged for 20 min at 230 × g. The pellet was washed in 50 mM MES, pH 6.5, 0.5 mM MgCl₂, 0.8 mM sorbitol, 1 mM EGTA, and 0.2 mM DTT and lysed by Dounce homogenization in lysis buffer (50 mM MES, pH 6.5, 0.5 mM antipain, 1 μg/ml aprotinin, 30 μM leupeptin, 30 μM chymostatin, 20 μM pepstatin A). DNase I (25–40 μl of 100 units/μl) and 5 mM MgCl₂ were added, and yeast cells were lysed by three passages through a microfluidizer (Watts Fluidair, Inc., Kittery, ME). Lysates were clarified by centrifugation for 45 min at 100,000 × g. The supernatant fraction was loaded onto a Ni-NTA column (Qiagen) equilibrated with Ni-NTA buffer A (50 mM NaPO₄, pH 8.0, 500 mM NaCl, 20 mM imidazole, 10 mM 2-mercaptoethanol), and Ssa1p was eluted in a step gradient at 20% Ni-NTA buffer B (50 mM NaPO₄, pH 8.0, 500 mM NaCl, 500 mM imidazole, 10 mM 2-mercaptoethanol). Fractions containing Ssa1p (detected by Coomassie-stained SDS-PAGE) were dialyzed into 25 mM Tris-Cl, pH 7.4, 50 mM KCl, 0.5 mM EDTA, and 2 mM DTT. Diaxylized Ssa1p was loaded onto a Mono Q column (GE Healthcare) equilibrated with Q-buffer A (50 mM Tris-Cl, pH 7.4, 0.5 mM EDTA, 2 mM DTT). Impurities were separated over a linear gradient of Q-buffer B (50 mM Tris-Cl, pH 7.4, 0.5 mM EDTA, 2 mM NaCl, 2 mM DTT). Ssa1p fractions were concentrated and loaded onto a HiLoad Superdex 200 gel filtration column (GE Healthcare) equilibrated and run in gel filtration buffer (25 mM Tris-Cl, pH 7.4, 100 mM KCl, 0.5 mM EDTA, and 2 mM DTT). The same protocol was used to purify His₆-Ydj1p (hereafter “Ydj1p”), except pET15b-YDJ1 was transformed into Rosetta BL21(DE3) cells, PMSF was used as the protease inhibitor, and one passage through the microfluidizer was sufficient for lysis. For His₆-Swa2p (hereafter “Swa2p”), pET21a-SWA2-transformed Rosetta BL21(DE3) cells were induced at 22 °C, A₆₀₀nm = 0.8 with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cell lysis and affinity chromatography were performed as for Ydj1p. Ni-NTA fractions containing Swa2p were pooled, supplemented with (NH₄)₂SO₄ to 0.5 M, and loaded onto a phenyl-Sepharose HP hydrophobic interaction column (GE Healthcare) equilibrated with HIC buffer A: 50 mM Tris-Cl, pH 7.4, 0.5 mM EDTA, 0.5 M (NH₄)₂SO₄, and 2 mM DTT. The column was washed with 10 column volumes of HIC-buffer A, and Swa2p was eluted by stepping up to 100% HIC-buffer B (the same buffer without ammonium sulfate). Swa2p fractions were concentrated, diluted 5-fold with Q-buffer A, loaded onto a Mono Q column equilibrated with Q-buffer A and eluted in a linear gradient of Q-buffer B at 20% buffer A. Swa2p fractions were concentrated, loaded onto a HiLoad Superdex 200 column (GE Healthcare), and eluted in gel filtration column buffer. All lysis and purification steps were kept at 4 °C.
MgCl₂, 1 mM NaN₃, 1 mM EGTA, 0.2 mM DTT, 1 mM PMSF, 10 μM antipain, 1 μg/ml aprotinin, 30 μM leupeptin, 30 μM chymostatin, 20 μM pepstatin A). After centrifugation for 5 min at 3000 × g, the supernatant fraction was centrifuged at 9000 × g in a SLA-1500 rotor for 10 min at 4 °C. The resulting supernatant fraction was centrifuged at 100,000 × g in a Ti45 rotor for 90 min at 4 °C. The 100,000 × g pellet, enriched in small vesicles, was resuspended by Dounce homogenization in 2 ml of lysis buffer. This pellet homogenate was loaded onto an S1000 column (GE Healthcare) equilibrated and run in lysis buffer without protease inhibitors. Fractions containing GFP-labeled baskets were identified from the emission spectra of peak fractions using an excitation wavelength of 488 nm on a spectrofluorometer (PTI). Additionally, peak fractions were assayed for the presence of Chc1p-GFP by Western blot analysis, using anti-EGFP as the primary antibody (BD Biosciences).

**Calibration of S1000 Column**—Twenty-nm-diameter red and 100-nm yellow fluorescent beads were purchased from Molecular Probes, and 44-nm green fluorescent beads were purchased from Duke Scientific. To prevent sticking to the gel filtration matrix, beads were sonicated in 1 ml of 5 mg/ml BSA, 50 mM MES, pH 6.5, 0.5 mM MgCl₂, 1 mM NaN₃, 1 mM EGTA, and 0.2 mM DTT. The bead suspension was applied to an S1000 column equilibrated and run in 50 mM MES, pH 6.5, 0.5 mM MgCl₂, 1 mM NaN₃, 1 mM EGTA, and 0.2 mM DTT. After elution, each fraction was assayed by fluorometry. To simplify the location of peaks on the composite chromatogram, samples of different bead size were processed individually.

**Electron Microscopy of GFP-labeled Clathrin Baskets**—Assembled and disassembled clathrin basket samples were prepared by mixing 5 μl of purified clathrin baskets in 100 mM Hepes at pH 7.0, 10 mM MgCl₂, and 2 mM DTT in the absence and presence of 2.5 mM ATP, respectively. Samples were fixed with 10% glutaraldehyde immediately before adsorption onto fresh, 200-mesh thin-film carbon with nitrocellulose glow-discharged grids for 2 min, washed twice with distilled water for 30 s each, and stained with 1% aqueous uranyl acetate. These negative stained samples were observed at 80 kV under a Zeiss EM109. An acousto-optical tunable filter (NEOS model 6404010W) enabled color selection. For all experiments, a single-mode fiber (26). An acousto-optical tunable filter (NEOS model 640410W) enabled color selection. For all experiments, a N.A. 1.4 objective was used. We constructed a modular pinhole (50 μm) and dual-color filter assembly that attached to one of the side access ports of the microscope. The output of this modular assembly was coupled to a photomultiplier tube detector (PerkinElmer Life Sciences SPCM-ARQ-12-FC) via multimode fiber optics. The instrument point-spread function was measured by scanning through 100-nm beads to confirm the accuracy of Gaussian-Lorentzian model assumptions.

For all measurements, the 488-nm laser line excitation was chosen, and emission was collected with a 525/50-nm bandpass filter. The laser power was 80 μW. The beam waist diameter in the flow direction was confirmed, from standard FCS measurements of tetramethylrhodamine (data not shown), to be 0.4 μm for the green channel and 0.45 for the red channel. The photomultiplier tube output was time-stamped via a Flex01–12D hardware correlator (Correlator.com) connected to a standard PC via a USB-II communication link.

**Uncoating of GFP-labeled Clathrin Baskets**—Uncoating reactions with Ssa1p and Swa2p were carried out in 100 mM Hepes at pH 7.0, 10 mM MgCl₂, 2.5 mM ATP, 2 mM DTT, and an ATP regeneration system consisting of 20 mM phosphocreatine and 30 units/ml creatine kinase. Twenty-microliter reactions were mixed with Chc1p-GFP baskets at approximately picomolar concentraton, when referenced to the concentration of 20-nm fluorospheres, and ATP was added immediately before the reaction was placed on a coverslip, positioned on top of the objective. Coverslips were cleaned then blocked with 10 mg/ml BSA for 30 min, rinsed in water, and dried before use. For FCS, measurements were taken of the freely diffusing particles on the coverslip; for BAS (26), the coverslips were rotated at 300–500 μm/s measured at the excitation volume using a custom torque motor assembly affixed to the objective. The rotation speed was calibrated using a precision reticle.

As a reference, triskelia were isolated nonenzymatically (“artificially disassembled”), by adding 2 M Tris, pH 7.0, to the clathrin basket-enriched fraction to a final concentration of 0.5 M (30). The sample was incubated 1 h at 4 °C and then centrifuged for 1 h at 168,000 × g. The supernatant fraction was analyzed using BAS and FCS with the laser power set to 150 μW.

**Kinetic Analysis**—Average uncoating kinetics were analyzed using a custom program written in MATLAB. The time trace of the single-particle fluorescence burst events gathered over the
The course of the uncoating reaction was broken into 70-s bins. Because the dynamics of the uncoating reaction were most faithfully tracked by the large burst events in each bin and not the fluorescence background, we isolated these events by applying a threshold cut to examine the upper amplitude quartile of bursts in each time bin. The results were robust to the exact choice of the amplitude threshold. The very largest event in each bin was also removed. The mean amplitude of the remaining events in each bin was then used to represent the average fluorescence burst size in that time window.

The resulting value for each bin was plotted using OriginLab Pro, and curves were fitted to single-exponential equations. Rate plots were normalized by dividing the curves by the average value of the starting fluorescent baskets obtained via a similar analysis. Rate constants were determined by fitting the curves to Equation 1,

\[ y = y_0 + Ae^{-kt_{\text{obs}}} \]  

(Eq. 1)

where \( k_{\text{obs}} \) is the observed rate constant. The rate constants were then plotted and fit to the Hill plot, Equation 2.

\[ k_{\text{obs}} = \frac{k_{\text{max}}[\text{Ssa}]}{K_{1/2} + [\text{Ssa}]} \]  

(Eq. 2)

The Hill equation fit was weighted by the error on the rate constants calculated from the single-exponential fits to the rate plots.
RESULTS

Biochemical research using purified neuronal proteins has demonstrated that clathrin coats (henceforth referred to as “baskets,” when membrane is absent), Hsc70, auxilin, and ATP comprise the minimal system to reconstitute clathrin uncoating in vitro (4, 8, 10, 14). Furthermore, a recent mechanistic study of individual baskets has revealed a cooperative uncoating mechanism under single turnover conditions, with Hsc70 and auxilin prebound to baskets tethered to a coverslip (20). Although genetic evidence from S. cerevisiae has confirmed that the Hsc70 ortholog, Ssa1p, and the auxilin ortholog, Swa2p, are necessary for clathrin uncoating (4, 5, 22), it has not been determined whether these proteins are sufficient for yeast clathrin coat disassembly in a reconstituted system. To examine the mechanism of clathrin uncoating in yeast, we analyzed the single-particle dynamics of clathrin coat disassembly, in free solution, reconstituted from purified components.

Enrichment of Clathrin Baskets from S. cerevisiae—To detect clathrin baskets, we used a GFP-tagged variant of the yeast clathrin heavy chain gene, CHC1 (HRY54 CHC1-GFP; yGL206C from the GFP-tagged library; Invitrogen). Initially, we were concerned that Chc1p tagged at the C terminus (Fig. 1a) could be defective for formation and/or disassembly of clathrin coats because the C terminus of the heavy chain is located at the vertex of the triskelion (Fig. 1b), near known binding sites of auxilin and Hsc70 (20). Furthermore, a C-terminal clathrin heavy chain mutation, or truncation, resulted in defective coat disassembly (18, 20). Two observations suggest that HRY54 CHC1-GFP forms functional CCVs, in vivo. First, localization of Chc1p-GFP in yeast is punctate at the late Golgi apparatus (31), consistent with functional clathrin coat structures. Second, HRY54 CHC1-GFP does not display a growth defect such as that seen for a chc1-ts allele known to be defective in clathrin coat assembly (Ref. 27 and Fig. 1c).

Clathrin baskets were obtained by cell fractionation, using differential centrifugation and size exclusion chromatography (4, 5). Small vesicles and GFP-tagged clathrin baskets, enriched in the pellet of a 100,000 × g centrifugation step, were further fractionated on a S1000 gel filtration column, resulting in a large, Chc1p-GFP-positive peak (Fig. 2, a–c). Fractions at the leading edge of the peak contain spherical clathrin structures, particles of an average diameter of ~25 nm by negative stain electron microscopy (Fig. 2, d and e). In solution, the baskets are similar in size to 40-nm spheres, by FCS (Fig. 2f), consistent with their elution position from the S1000 column, as well as the expected size of small, closed clathrin baskets composed of 28–36 clathrin triskelia (32). The assembled clathrin structures did not appear to stain with lipophilic dyes (data not shown), suggesting that the majority of the clathrin does not coat membranous vesicles. The spherical particles are absent from

FIGURE 3. Disassembly of fluorescent clathrin baskets by Ssa1p and Swa2p. a and b, samples prepared for negative stain electron microscopy and incubated at 23 °C for 10 min with 5 μM Ssa1p and 50 nM Swa2p, either in the absence (a) or the presence (b) of 2.5 mM ATP. Representative micrographs from each sample are shown. Scale bar, 100 nm. c, stimulation of Ssa1p ATPase by Ydj1p or Swa2p. ATPase activity was measured using [γ-32P]ATP and thin layer chromatography and quantitated by densitometry. The basal rate of ATP hydrolysis catalyzed by 1 μM Ssa1p (0.043 μM/min, green) was stimulated by addition of 2 μM Ydj1p (0.174 μM/min, red), or 2 μM Swa2p (0.167 μM/min, blue). Error bars represent the S.E. of three experiments. d, FCS curves of a fluorescent clathrin basket sample prior to (black) and following the addition Ssa1p, Swa2p and ATP (blue) compared with the FCS curve of artificially disassembled triskelia, isolated from baskets incubated at elevated pH and ionic strength (magenta). FCS curves of pure GFP (red) and fluorescein (green) are also shown. Enzymatic disassembly conditions are similar to a and b, except that the reaction was incubated for 30 min at 23 °C.
Chc1p-GFP-positive fractions that elute later from the S1000 gel filtration column, which are likely to contain basket fragments and unassembled clathrin triskelia.

We next examined the size distribution of the baskets using BAS (26). For this measurement, each clathrin basket produces a single burst as it advectively flows through the detection volume of a custom confocal microscope. Quantitative analysis of the fluorescence burst distribution allows, in principle, extraction of the actual size distribution of the assembled baskets, provided the unitary fluorescence of an appropriate standard is known (26). As a calibration standard, we employed purified EGFP (33). During advective flow, single-molecule EGFP is near the sensitivity limit of the instrument used for these measurements. Determination of the basket size by BAS was therefore only possible to within a factor of 2, but is consistent with the purified yeast baskets being composed of ~30 triskelia.

Reconstitution of Uncoating with the Chaperone Complex Ssa1p and Swa2p—Using the Chc1p-GFP baskets obtained from yeast, we next tested whether the yeast Hsc70 Ssa1p and auxilin Swa2p, constitute the minimal uncoating machinery (Fig. 3). Recombinant Swa2p was also tested for ATPase activation of Ssa1p, using as a control, purified recombinant Ydj1p, another yeast DnaJ homolog known to stimulate Ssa1p ATPase activity (34). Disassembly of Chc1p-GFP baskets was confirmed by negative stain electron microscopy and FCS (Fig. 3, a and b).

The disassembly of Chc1p-GFP baskets was readily observable at the single-particle level in the presence of Ssa1p, Swa2p, and ATP. As shown in Fig. 4, the number of fully assembled baskets decreases rapidly following addition of Ssa1p, Swa2p, and ATP, whereas none of the proteins alone had an affect on the fluorescence burst pattern. Additionally, basket disassembly requires the clathrin-specific auxilin ortholog, Swa2p; Ydj1p did not induce Ssa1p disassembly of Chc1p-GFP baskets (Fig. 5). The average disassembly kinetics of the Chc1p-GFP baskets are well described by a single-exponential rate law, with an observed rate constant that responds in a dose-dependent manner to changes in the Ssa1p concentration (Fig. 6). A maximum rate of ~0.017 s⁻¹, with a half-time of <1 min, was observed at Ssa1p concentrations ~10 μM. Importantly, the observed changes in fluorescence burst intensity are not due to photobleaching or to changes in the quantum yield of CHC-GFP fusion protein. The total GFP fluorescence prior to and following basket disassembly by Ssa1p and Swa2p is the same within experimental error (Fig. 6b).

Analysis of Ssa1p-Swa2p Uncoating Kinetics Using Burst Analysis Spectroscopy—The single-particle disassembly kinetics of the Chc1p-GFP baskets are well described by a single-exponential rate law, with an observed rate constant that responds in a dose-dependent manner to changes in the Ssa1p concentration (Fig. 6). A maximum rate of ~0.017 s⁻¹, with a half-time of <1 min, was observed at Ssa1p concentrations ~10 μM. Importantly, the observed changes in fluorescence burst intensity are not due to photobleaching or to changes in the quantum yield of CHC-GFP fusion protein. The total GFP fluorescence prior to and following basket disassembly by Ssa1p and Swa2p is the same within experimental error (Fig. 6b).
changes in the clathrin basket size and number over ~ 20 min (z axis, Fig. 7), two distinct subpopulations of clathrin objects are revealed. The fully assembled baskets observed at early time points (dark and light blue, Fig. 7) disappear at later time points, coincident with appearance of a large number of small particles (green, yellow, orange, Fig. 7). Strikingly, particles of intermediate size are not significantly populated during basket disassembly. This behavior strongly suggests a two state-like transition between the fully assembled baskets and a less assembled state, which is highly consistent with a cooperative model for Ssa1p-Swa2p-mediated clathrin uncoating.

Notably, the size of the disassembly products from the Ssa1p-Swa2p reaction is substantially larger than that expected for individual triskelia, suggesting that, under these conditions, the Che1p-GFP baskets break apart cooperatively into groups of 5–7 triskelia (Fig. 7). The absence of a large population of free triskelia at the end of the disassembly reaction is also supported by the fact that the average diffusive behavior of the Ssa1p-Swa2p disassembly products is intermediate between that of the starting baskets and clathrin products that were artificially disassembled by incubation of the baskets at a higher pH and

FIGURE 6. Disassembly rate is proportional to Ssa1p concentration. a, the disassembly of fluorescent clathrin baskets was measured by fluorescence burst detection at different concentrations of Ssa1p. All reactions contained 50 nM Swa2p and 2.5 mM ATP. The increase in disassembly rate is observable as an increasingly rapid loss in the large fluorescent bursts (baskets) as a function of time after Ssa1p addition. b, the disassembly of fluorescent clathrin baskets has no observable effect on the fluorescence quantum yield of the GFP probe. The total emitted GFP fluorescence from a sample of clathrin baskets is shown following a 10-min incubation at 23 °C with 5 μM Ssa1p and 50 nM Swa2p in the absence of ATP (“assembled”). The total fluorescence of a matched sample (5 μM Ssa1p and 50 nM Swa2p), after a 10-min incubation at 23 °C with 2.5 mM ATP, is also shown (“disassembled”). c, for rate measurement, the raw fluorescence burst data for each reaction was binned (30-s bins), and the average intensity of the top quartile of bursts within each bin was calculated and plotted as a function of time. Solid lines show single-exponential fits to the decay of the binned average intensity. d, observed rate constants extracted from the average intensity decay at each Ssa1p concentration are shown. The change in the apparent average disassembly rate constant was fit to the Hill equation yielding a $k_{\text{max}}$ of 33.5 ± 9 s$^{-1}$, $K_{1/2} = 5.1 ± 2$ μM, and $n_H$ of 1.7 ± 0.6. Error bars indicate 1 S.D.

FIGURE 7. BAS measurements indicate cooperative disassembly of yeast clathrin baskets. The size distribution of fluorescent clathrin baskets was examined during a disassembly reaction with BAS. Basket disassembly was conducted under conditions identical to those used in Fig. 4, except at a lower concentration of Ssa1p (625 nM), to slow the reaction enough for multiple BAS measurements over the course of the disassembly reaction. The fluorescence bursts were recorded for 25 min, and the raw data were then divided into 4-min bins, each of which was subjected to BAS analysis. The distribution of fluorescence species for each bin is shown.
Single-particle Analysis of Yeast Clathrin Disassembly

**FIGURE 8. Four models for the disassembly of a clathrin coat.** Sequential disassembly models predict a range of intermediate states. a and b, either single clathrin triskelion is individually and sequentially released from a basket in a noncooperative fashion (a), or disassembly occurs by the sequential release of fragments composed of multiple triskelia (b). c and d, cooperative disassembly models predict two stages, assembled and disassembled baskets, with no intermediates. The products of cooperative disassembly could be individual triskelia (c), or basket fragments larger than individual triskelia (d).

A surprising outcome of the results presented here is the discovery of a clathrin coat intermediate. The fact that Ssa1p-Swa2p does not disassemble coats all the way to individual triskelia may have physiological significance. In the cell, patches of coat fragments are observed at “hot spots,” which efficiently nucleate new coated vesicles (37–39). Coat fragments might also be incorporated into larger sized transport carriers, as has been observed for COPI- and COPII-coated carriers between the endoplasmic reticulum and Golgi apparatus (40).

The identification of a partial uncoating intermediate may also have important implications for the coordination of intracellular vesicle trafficking. Recent studies of various vesicle trafficking steps have uncovered a requirement for direct interactions between specific vesicle coat proteins and proteins of
vesicle tethering complexes. Families of vesicle-tethering complexes are specialized for accurate docking and fusion of a variety of intracellular vesicles with specific target membranes (41). COPI-coated vesicles derived from the Golgi apparatus are tethered to the ER through a COPI coat-protein interaction with the vesicle tether p115, in mammalian cells (42) and the Ds1p vesicle-tethering complex, in yeast (43). Similarly, tethering of ER-derived COPII vesicles requires a COPII coat-protein interaction with the transport protein particle (TRAPP) tethering complex on the Golgi apparatus (44). For clathrin-coated vesicles, there is evidence to suggest that coats are disassembled soon after vesicles are formed, to permit vesicle-target membrane fusion (3, 11). However, there is also evidence that before fusion, vesicle tethering requires an interaction between clathrin coat components and the homotypic fusion and vacuole protein sorting (HOPS) tethering complex at the yeast vacuolar compartment (45). Thus, identification of a partial uncoating intermediate could offer a solution that satisfies both requirements, allowing vesicles to retain important information for accurate docking and fusion.

The ultimate disassembly of clathrin coats from fragments to individual triskelia may require additional factors. Among the candidates are the nucleotide exchange factors of the Hsp110 family. Hsp110 proteins have recently been shown to activate Hsp70-Hsp40 molecular chaperone systems in the disassembly of amorphous protein aggregates (46). Although Hsc70 and auxilin are fully sufficient for generating triskelia from neuronal clathrin baskets, Hsp110 has been shown to substantially accelerate neuronal clathrin disassembly in a light scattering assay (47). It remains to be seen what impact yeast Hsp110s or other regulatory factors, have on disassembly of yeast clathrin coats.

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