The unusual dynamics of parasite actin result from isodesmic polymerization

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Previous reports have indicated that parasite actins are short and inherently unstable, despite being required for motility. Here we re-examine the polymerization properties of actin in Toxoplasma gondii, unexpectedly finding that it exhibits isodesmic polymerization in contrast to the conventional nucleation–elongation process of all previously studied actins from both eukaryotes and bacteria. Polymerization kinetics of actin in T. gondii lacks both a lag phase and critical concentration, normally characteristic of actins. Unique among actins, the kinetics of assembly can be fit with a single set of rate constants for all subunit interactions, without need for separate nucleation and elongation rates. This isodesmic model accurately predicts the assembly, disassembly and the size distribution of actin filaments in T. gondii in vitro, providing a mechanistic explanation for actin dynamics in vivo. Our findings expand the repertoire of mechanisms by which actin polymerization is governed and offer clues about the evolution of self-assembling, stabilized protein polymers.
Actin shares a common fold with a superfamily of proteins that includes sugar kinases, Hsp70, and actin-related proteins, including bacterial homologues of actin (that is, MreB, ParM and so on)\(^1\). Among this group, actins characteristically cycle between monomeric and filamentous forms using a nucleation–elongation mechanism for polymerization, which supports structure, mechanics and motility in cells\(^2\). Apicomplexan parasites, such as Toxoplasma and Plasmodium, are ancient, divergent eukaryotes that also require actin polymerization for motility\(^3,4\) and active invasion of host cells\(^5\). Despite this requirement, the vast majority of parasite actins remains unpolymerized \textit{in vivo}\(^6–8\) and purified parasite actins form very short filaments \textit{in vitro}\(^9–10\). Actin in \textit{T. gondii} (TgACTI) is only \(~80\%\) identical to mammalian or yeast actin (ScACT)\(^6\) and differences in the molecular interactions responsible for inter-strand filament contacts contribute to the instability of parasite actins\(^11\). It has remained enigmatic how the seemingly inefficient polymerization and transient nature of actin filaments support motility in these organisms.

Conventional actins, as well as the actin-related protein Arp1, undergo cooperative assembly to polymerize head-to-tail into two inter-twined helical strands that form a filament\(^1\). Polymerization occurs by a nucleation–elongation mechanism in which filaments elongate rapidly from nuclei that assemble slowly and only if the actin concentration exceeds a characteristic critical concentration (Cc)\(^13\). Polymerization from monomeric subunits displays a slow nucleation step because of the instability of dimer and trimer intermediates, resulting in a lag phase in the time course of polymerization\(^14\). In contrast to a nucleation–elongation mechanism, protein polymers can theoretically assemble by an isodesmic mechanism, which is non-cooperative and does not require a nucleus to initiate polymerization\(^15,16\). During isodesmic polymerization, all monomer–polymer interactions occur with equal affinity and the amounts of both monomer and polymer increase with total protein concentration, in contrast to cooperative assembly, where the monomer concentration plateaus upon reaching the Cc\(^15\).

All well-studied actins, from yeast, amoeba and animal cells\(^12\), as well as from bacterial actin-like homologues\(^17,18\), display nucleation–elongation polymerization, which is generally regarded as a conserved property of actins.

Given the unusual nature of parasite actins, we challenged the assumption that a standard nucleation–elongation mechanism could account for actin polymerization in more distant eukaryotic species such as \textit{T. gondii}. Our findings indicate that TgACTI actin assembles by a process that is largely isodesmic, thus revealing a novel means of assembly for a cytoskeletal protein polymer. These findings have implications for the regulation of assembly and turnover of filaments that help explain the unusual actin dynamics observed \textit{in vivo}.

\section*{Results}

\textbf{TgACTI exhibits unusual polymerization kinetics.} We began by re-examining the polymerization of TgACTI by light scattering, using recombinant protein purified from baculovirus as described previously\(^11\). Polymerization was very limited at low protein concentrations but increased steadily over time at higher concentrations (Fig. 1a). Notably, the time course displayed no lag in the initiation of TgACTI polymerization at any of the concentrations tested (Fig. 1a). A previous study of the polymerization kinetics of TgACTI, as determined by tryptophan quenching, reported a lag phase but only at very low concentrations of protein\(^5\). Indeed, a lag phase is not exclusive to a nucleation–elongation mechanism, because this feature can also be observed for an isodesmic process when \(k_{\text{off}} > k_{\text{on}}\)\(^18\). When we examined the initial time of polymerization by light scattering over a wide range of concentrations, no lag phase was present (Fig. 1a, right). Instead, the rate of polymerization increased in proportion to the concentration of protein, and continued to rise over the 2-h time frame (Fig. 1a). In contrast, actin from \textit{Saccharomyces cerevisiae}, ScACT, expressed in baculovirus and

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\caption{Concentration-dependent polymerization of TgACTI versus ScACT. (a) Light scattering (arbitrary units, a.u.) versus concentrations (\(\mu\text{M}\)) of TgACTI. Plot at right shows the initial time course expanded to highlight the lack of lag phase. (b) Light scattering (arbitrary units, a.u.) versus concentrations (\(\mu\text{M}\)) of ScACT. Initial plots show evidence of lag (expanded plot at right) and plateau at later time points. (c) Fluorescence microscopy of polymerized TgACTI visualized with 0.33 \(\mu\text{M}\) Alexa 488-phalloidin \(\pm\) equimolar amounts of unlabelled Ph (1:1). Scale bar, 5 \(\mu\text{M}\). (d) Quick-freeze, deep-etch electron micrographs of ScACT (5 \(\mu\text{M}\)) versus TgACTI (40 \(\mu\text{M}\)) polymerized in F-buffer. Scale bars, 100 nm. (e) Lengths of actin filaments measured \textit{in vitro} for ScACT and TgACTI as in d, or for TgACTI \textit{in vivo} as reported previously\(^5\). Left y axis, ScACT; right y axis, TgACTI. Means \(\pm\) s.d. (f) Circular dichroism measurements of purified ScACT versus TgACTI, 0.4 \(\mu\text{M}\). All panels are representative experiments of three or more similar experiments.}
\end{figure}
purified in parallel, showed the typical lag, elongation and plateau phases characteristic of conventional acts (Fig. 1b; refs 13,14).

The differences between yeast and TgACTI polymerization were apparent, despite the fact that light scattering is not directly proportional to filament mass because the light-scattering signal is also influenced by bundling. With some other methods, such as fluorescence of pyrene–actin, the signal is proportional only to filament mass19. In the pyrene–actin method, actin is labelled with pyrene on a conserved cysteine residue. TgACTI has this conserved cysteine (Cys375), and we were able to label Cys375 with pyrene. However, we found that pyrene–TgACTI did not show enhanced fluorescence when polymerization was induced (C. Ma, unpublished). Although the effects of bundling on the signal meant that we were not able to directly model the assembly kinetics from the light-scattering traces, dramatic differences were evident between the traditional nucleation–elongation process exhibited by ScACT and the unconventional process exhibited by TgACTI.

Although TgACTI showed evidence of polymerization by light scattering, it did not form conventional length filaments detectable by fluorescence phalloidin (Ph) staining unless incubated with equimolar concentrations of Ph (that is, at 5 μM; Fig. 1c), consistent with previous reports11. This requirement was overcome at higher protein concentrations (that is, 25–40 μM), allowing visualization of bundles of long filaments that were detected by adding trace amounts of labelled Ph and fluorescence microscopy (Fig. 1c). When a high concentration of TgACTI was polymerized in F-buffer and examined by quick-freeze, deep-etch electron microscopy (EM), occasional filament bundles were surrounded by many small heterogeneously sized oligomers and short filaments ranging from 25 to 100 nm, which are similar to previous in vivo measurements8 (Fig. 1d,e). In contrast, ScACT formed much longer filaments (that is, 500–1,500 nm) with a typical right-handed helical pattern of two protofilaments, which also occasionally formed bundles (Fig. 1d,e). The short filaments formed by TgACTI had a conventional double-stranded structure but showed less evidence of a helical pattern when compared with ScACT (Fig. 1d). The failure of TgACTI to form filaments at lower concentrations was not because of a general defect in folding because circular dichroism measurements indicated a similar secondary structure compared with that of ScACT (Fig. 1f).

Inefficient sedimentation of heterogeneous TgACTI filaments. When conventional acts undergo polymerization, nearly all of the polymer assumes the form of long filaments that sediment efficiently at 100,000g (ref. 20). Previous studies using low concentrations (that is, below 5 μM) of TgACTI have reported that sedimentation requires higher g forces than conventional acts (that is, 350,000g for 1 h; ref. 8). Given our findings for concentration dependence of TgACTI polymerization above, we examined the influence of protein concentration on the efficiency of sedimentation. When 5 μM TgACTI was polymerized in F-buffer and centrifuged at 100,000g, ~30% of the protein appeared in the pellet (Fig. 2a, top). Addition of Ph at an equimolar ratio caused a greater fraction of TgACTI to shift to the pellet (~60%; Fig. 2a, top). In contrast, centrifugation at 350,000g was sufficient to pellet the majority of TgACTI, both in the presence and absence of Ph (~85–90%; Fig. 2a, top). At higher protein concentrations (that is, 30 μM), ~37% of TgACTI pelleted on centrifugation at 100,000g (Fig. 2a, middle). Addition of Ph and centrifugation at 100,000g, or centrifugation at 350,000g, was sufficient to sediment nearly all of the TgACTI (~90%; Fig. 2a, middle). In comparison, >90% of ScACT, expressed and purified in the same manner as TgACTI, was found in the pellet after incubation in F-buffer, regardless of the centrifugation speed or the presence of Ph (Fig. 2a, bottom). These findings indicate that although TgACTI undergoes dose-dependent polymerization, it forms oligomers that sediment far less efficiently than conventional acts.

To evaluate the size distribution of polymerized TgACTI, samples were subjected to density gradient centrifugation. When 5 μM TgACTI incubated in non-polymerizing G-buffer was analysed by sucrose gradient sedimentation, the majority of protein was found near the top of the gradient (Fig. 2b, top). At higher protein concentrations (that is, 30 μM) in G-buffer, TgACTI sedimented more rapidly, appearing farther down the gradient (Fig. 2b, middle). Incubation of 5 or 30 μM TgACTI in polymerizing F-buffer shifted the protein distribution further down the gradient, although the majority of protein was still found in lighter fractions under both conditions (Fig. 2b). Following the addition of Ph, ~15% of the 5 μM actin was shifted to the pellet, whereas the majority of 30 μM actin sedimented into the lower fractions (Fig. 2b). In contrast to the heterogeneous behaviour of TgACTI, sucrose gradient sedimentation of ScACT produced two discrete populations at opposite ends of the gradient. In G-buffer, the majority of ScACT was found in light fractions (S<4.4 standard for BSA), likely corresponding to monomers and a mixture of dimers (Fig. 2b, bottom). However, in F-buffer, the majority of ScACT was found in the pellet, consistent with polymerization into long filaments (Fig. 2b, bottom). To estimate the size of TgACTI polymers, we estimated the proportion of actin found in the pellet after F-buffer incubation and high-speed centrifugation at 100,000 or 350,000g (data from Fig. 2a). These estimated amounts of pelleted actin were plotted against the cumulative fractions of the sucrose gradients (Fig. 2b, dashed line, solid line, respectively) to estimate the relative size of the filaments. From this comparison, it was evident that TgACTI filaments are not long, like those of yeast, which sediment rapidly. In contrast, TgACTI filaments are much shorter, and their length distribution is more heterogeneous.

To provide a better estimate of the sizes of actin oligomers formed by TgACTI, we examined protein samples by dynamic light scattering and used an autocorrelation function to estimate hydrodynamic radius (Rh), mass and polydispersity. ScACT suspended in G-buffer was detected primarily as a single peak, with a Rh of 3.6 nm and approximate mass of 67 kDa (Table 1). This size estimate possibly represents an average of monomer (estimated Rh 3.0) (ref. 21) and dimer forms (estimated Rh 3.8–4.1 for low-pitch and high-pitch dimer, respectively, as estimated using Stokes–Einstein theory). Addition of F-buffer to ScACT shifted the size to the upper end of the spectrum that was detectable by the instrument (that is, >109 kDa), consistent with assembly of actin into long polymers (Table 1). The behaviour of TgACTI in G-buffer indicated a much larger average Rh and mass than that predicted for monomer, consistent with a range of small oligomers from 2 to 5 subunits at 1 μM that shifted to 5–10 subunits at 5 μM (Table 1, Supplementary Table S1). Addition of F-buffer shifted the size of TgACTI to an intermediate range with an average size of ~7,000 kDa, and a much broader polydispersity, consistent with a variety of oligomers from 35 to 500 subunits but without the extremely large polymers seen in ScACT (Table 1, Supplementary Table S1). The estimated size of TgACTI oligomers formed in G-buffer is consistent with globular aggregates previously reported by EM8,11, and the size distribution in F-buffer corresponds to observations of filaments observed by EM above, and reported previously8,11. The length distribution of TgACTI filaments depended on the concentration of TgACTI in a continuous manner, with longer filaments at higher concentrations of

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protein. In contrast, the length distribution of ScACT filaments displayed an abrupt transition to a population of long filaments when the protein concentration exceeded the Cc (Fig. 1d, Table 1, Supplementary Table S1). These different behaviours of filament length with respect to protein concentrations are consistent with predictions based on isodesmic versus nucleation–elongation modes of assembly.

**TgACTI polymerizes by an isodesmic process.** Having established that TgACTI polymerizes more robustly at higher concentrations than those used in previous analyses, we revisited the question of the existence of a Cc. We examined the amount of protein in the supernatant and pellet after incubation for 1 h at room temperature in F-buffer (pH 8.0) and centrifugation for 1 h at 100,000 \( g \). If TgACTI underwent nucleation–elongation polymerization similar to conventional actins, the concentration of protein in the supernatant would be expected to plateau at the Cc\(^\text{13,14}\). Strikingly, this was not observed. Rather, the amount of protein in the supernatant continued to increase up to 100 \( \mu \text{M} \) TgACTI, the highest concentration tested (Fig. 3a).

**Figure 2 | TgACTI forms small heterogeneous polymers.** (a) TgACTI (5 \( \mu \text{M} \) top versus 30 \( \mu \text{M} \) middle) was polymerized in F-buffer for 1 h at room temperature ± equimolar Ph and centrifuged at 100,000 or 350,000g for 1 h at room temperature. Pellet (P) or precipitated supernatant (S) fractions were resolved on a 12% SDS-polyacrylamide gel, stained with SYPRO-Ruby and quantified by phosphorimager analysis. ScACT (5 \( \mu \text{M} \), bottom) was analysed in parallel. Mean ± s.d., \( n = 3 \) or more experiments combined in graphs; representative gels are shown. (b) Sedimentation of TgACTI (5 \( \mu \text{M} \) top, 30 \( \mu \text{M} \) middle) in G-buffer (G, blue), F-buffer (F, red) or F-buffer with equimolar Ph (F+, green) by sucrose density centrifugation. ScACT (5 \( \mu \text{M} \)) was analysed in a parallel gradient. Dashed and solid black lines correspond to the proportion of TgACTI that would be found in the 100,000 and 350,000g pellets under standard sedimentation conditions as in a. Size standards are denoted with black arrows. All panels are representative of three or more similar experiments.

**Table 1 | Dynamic light scattering of actins in solution.**

| Sample | G-buffer | F-buffer |
|--------|----------|----------|
|         | Rh (nm)\(^\text{I}\) Molecular weight\(^\text{I}\) PD (%) Int (%) | Rh (nm)\(^\text{I}\) Molecular weight\(^\text{I}\) PD (%) Int (%) |
| TgACTI  | 4.7 128 12 81.6 | ND ND ND ND |
| 1\( \mu \text{M} \) | 7.2 344 22 89 | 26.3 7,015 33.0 87 |
| TgACTI  | 3.6 67 27 81 | 3,656 731,106,000 0 92 |
| 5\( \mu \text{M} \) | | |
| ScACT  | | |
| 1\( \mu \text{M} \) | | |

Int, intensity of scattered light; ND, not determined; PD, polydispersity; Rh, hydrodynamic radius; TgACTI, actin in *T. gondii*.  
\(^\text{I}\)Estimate of Rh.  
\(^\text{I}\)Estimated from Rh, given in kDa.
TgACTI was polymerized in KMEI buffer (pH 7.2; Fig. 3b), indicating that this behaviour is not attributed to some unusual salt or pH preference. In contrast, polymerization of ScACT under similar conditions showed a marked plateau in the concentration of the supernatant and a clear Cc below which polymer was not detected (Fig. 3c). Purified TgACTI was also polymerized for 20 h, and the steady-state levels of polymerized for 20 h, and the steady-state levels of polymerization over the full range of actin concentrations (Fig. 4a). The isodesmic model predicts rate constants of $k_{on} = 5.9 \times 10^{-5} \, \mu M^{-1} \, s^{-1}$ and $k_{off} = 4.0 \times 10^{-4} \, s^{-1}$, both remarkably low in comparison with the rate constants for elongation of conventional actin, for which the on-rate constant is typically $\sim 10 \, \mu M^{-1} \, s^{-1}$ (refs 12,22). The rate constants for TgACTI give a binding affinity of 6.8 \, \mu M, consistent with the observed low rate of TgACTI polymerization at low concentrations and increased abundance of filaments with increasing concentration. Simulation of the polymerization kinetics using the isodesmic model generated curves that closely matched the proportion of TgACTI in the supernatant versus pellet at both 1 and 20 h (curves in Fig. 3a,c). Structural modelling and molecular dynamics support a role for an isodesmic mechanism in explaining the behaviour of TgACTI. Molecular dynamics simulation of muscle versus TgACTI filaments confirms that much weaker lateral interactions are formed between protofilaments (Fig. 4d), consistent with previously identified residues that affect lateral contacts and filament stability11 (Fig. 4c).

Although this simple isodesmic model predicts the polymerization behaviour of TgACTI, across a range of concentrations, it does not address the depolymerization behaviour of filaments once they form. To examine this directly, we polymerized TgACTI at 20 \, \mu M in F-buffer overnight, then diluted it by

![Image](image-url)
fourfold, a concentration below the threshold where most of the actins form polymers of sufficient mass to sediment at 100,000 g (Fig. 3a). We observed a very gradual but significant decline in light scattering after dilution, which supports the conclusion that polymers of TgACTI have a slow off-rate. Again, the isodesmic model accurately fits the observations (Fig. 3f) but as there is no Cc, dilution results in loss of >30% of the polymer. In contrast, when ScACTI were diluted under similar conditions, they underwent more modest depolymerization and reached a new steady state where the free monomer concentration again equaled the Cc (Fig. 3f). ScACTI depolymerization was accurately described by a nucleation–elongation model (Fig. 3f), which predicts only minor loss of polymer (that is, ∼5%). Collectively, these observations fit the expectation that conventional actins are robust to depolymerization because they are naturally buffered by their Cc, whereas TgACTI respond more dramatically to alterations in concentration, thus resulting in filaments that are less stable.

### Discussion

Collectively, our findings reveal that TgACTI slowly polymerizes to form a heterogeneous mixture of relatively short filaments, resulting in a length distribution remarkably different from that of conventional actins. This discovery explains previous differences from conventional actins and offers insight into the behaviour of parasite actin in vivo. First, nucleation is not rate limiting, so that T. gondii does not need nucleation-promoting factors. Indeed, homologues of actin-nucleating proteins, such as Arp2/3 complex, have not been identified within apicomplexan genomes. Second, an isodesmic mechanism results in a distribution of small oligomers, which explains why TgACTI only sediments efficiently at higher g force. Our findings also explain why long TgACTI filaments have not been observed in parasites by any method, including EM, fluorescence imaging of GFP–TgACTI and Ph staining. Other parasite actin-binding proteins are also adapted to this difference in kinetics; for example, actin-depolymerizing factor (ADF) acts primarily as a sequestering agent while exhibiting minimal severing activity, which is consistent with absence of stable filaments. As predicted by an isodesmic model, EM showed a distribution of heterogeneously sized small oligomers and short filaments ranging in size from 25 to 100 nm in length (Fig. 1e, excluding bundles that may form by annealing of individual filaments). This size distribution is similar to filaments that form in vivo during gliding motility (Fig. 1e and ref. 8).

Previous studies have also shown that T. gondii has a streamlined repertoire of actin-binding proteins including ADF and profilin. The ADF of T. gondii is somewhat unusual in functioning largely in sequestering of G-actin; the concentration of G-actin is ∼35 μM in the cytosol, whereas that of TgACTI is ∼40 μM (ref. 25). Combined with the affinity of TgADF for TgACTI (that is, 0.8 μM), it is likely that the majority of TgADF is bound to TgACTI. In this regard, profilin has also been shown to function in sequestering TgACTI (~38 μM cytosolic concentration and a dissociation binding constant of ~5 μM) (ref. 26). Collectively, ADF and profilin should then combine to keep the concentration of free TgACTI monomer very low (that is, below 5 μM), a level that would only allow for the filaments to be short and of variable length. The robust control of free TgACTI levels, and hence the distribution of filament lengths, would appear to be of critical importance based on the observation that disruption of the genes encoding TgADF or profilin leads to profound defects in cell motility and cell invasion. Hence, isodesmic polymerization is better suited than nucleation–condensation for the assembly of short filaments and oligomers, which function in parasite motility rather than the long, stable filaments that represent the functioning form of F-actin in yeast and mammalian cells.

Importantly, the fact that TgACTI undergoes isodesmic assembly does not make any a priori predictions about the directionality of assembly or the structure of the filament. Directionality during filament growth is determined by the on- and off-rate constants at the two ends of the filament, which are not required to be the same in an isodesmic process. Likewise, the net rate of assembly/disassembly may differ at the two ends, although such a difference would require energy, probably provided by ATP hydrolysis as is true for actins in general. Determining the directionality of assembly is an important future goal that awaits methods for single-molecule imaging of TgACTI polymerization.

An isodesmic polymerization process also does not imply that the structure of the TgACTI filament includes only a single protofilament. The reactions from monomer to dimer, dimer to...
trimer and so forth must have similar binding constants. The structural basis of this biochemical process is an open question that will require atomic-level structural and mutational analysis for proper resolution. At this point, we know that TgACTI filaments viewed by EM appear to display two protofilaments; however, the resolution was not sufficient to reveal whether the protofilaments are in a helical conformation. The filament may be in a relaxed conformation because of an intrinsically lower contribution of intra-strand interactions to filament stability, consistent with previous reports showing that TgACTI contains natural substitutions in residues that normally stabilize lateral interactions between the two protofilaments. Thus, the subunit interactions that dominate during filament assembly may be arranged in a one-start helix. Bacterial actin-like proteins also form a variety of filament types ranging from single-stranded protofilaments (that is, MreB) to both right- and left-handed helices comprising two protofilaments. Collectively, these findings indicate that actin can adopt a wider range of conformations and assembly processes than those reflected by conventional animal cells.

Actin and related proteins have evolved to form higher order assemblies that perform mechanical work and impart structural rigidity. Conventional eukaryotic actins use cooperative assembly to generate filaments with strong inter-strand contacts. Prokaryotic actins such as the bacterial actin homologue MreB form both single-stranded helices and linear double-stranded filaments, yet polymerization occurs by a nucleation–elongation mechanism with a Ca of ~0.9 μM (ref. 33). In contrast, TgACTI represents a different evolutionary strategy, utilizing an isodesmic process to form a double-stranded filament that lacks a cooperative mechanism of assembly and features weak inter-strand filament interactions. Our findings predict that other unconventional actins may also undergo a similar process of isodesmic polymerization. For example, Plasmodium ACTI shares many of the properties of TgACTI, as do actins in many plants and protozoan parasites. In all these cases, actins exhibit unusual polymerization kinetics and remain primarily un polymerized in vivo. Collectively, these observations are consistent with actin having adapted different modes of polymerization to support a diverse array of structural and motile processes in cells.

### Methods

**Actin expression and purification.** TgACTI or ScACT were expressed and purified from baculovirus, as described previously. In brief, His-tagged actins were purified using Ni-NTA agarose (Invitrogen) and stored in G-actin buffer (5 mM Tris–Cl, pH 8.0, 0.2 mM CaCl₂, 0.2 mM ATP) containing 0.3 mM dithiothreitol with 100 μM succrose. Purified actins were quantified as described, stored at 4 °C and used within 2–3 days. Before use, purified actins were clarified by centrifugation at 100,000 g for 30 min using a TL100 rotor and a Beckman Optima TL ultracentrifuge. To examine the effects of Ph on polymerization, samples were incubated in F-buffer (pH 8.0) ± equimolar unlabelled Ph (Molecular Probes) for 1 h at room temperature and centrifuged at 100,000 g for 1 h at room temperature. Acetone-precipitated supernatants and pellets were resolved on 12% SDS–PAGE gels, stained with Sypro Ruby (Molecular Probes), visualized using an FLS-5000 phosphorimager (Fujifilm Medical Systems) and quantified using Image Gauge v4.23. To establish the extent of polymerization at steady state, samples were polymerized in F-buffer (pH 8.0) for 20 h at room temperature and sedimented at 100,000 g. The ratio of the pellet/total actin at 20 h was plotted using a sigmoidal dose response (Prism, GraphPad).

**Density centrifugation.** Purified recombinant actins were incubated with 1 mM EGTA and 30 μM MgCl₂ for 10 min (to replace bound Ca²⁺ with Mg²⁺). Actins were incubated in G-buffer, F-buffer (pH 8.0) or F-buffer supplemented with equimolar Ph (Molecular Probes) for 1 h at room temperature. Samples were loaded on the top of 11 ml 5–40% continuous sucrose gradients, made in the corresponding buffer using an Auto Densi-Flow Gradient Fractionator (LabConco, Kansas City, MO, USA) and centrifuged at 100,000g, 4°C for 2 h using a SW41 rotor. da/dt was monitored at 200–250 nm using a Jasco J-810 Spectrometer.
Estimation of hydrodynamic radii. To estimate the hydrodynamic radii of different-sized actin oligomers, we used the Oda et al.29 actin filament structure and created structures for the monomer, low-pitch and high-pitch dimers, trimers and so on. Using HYDROPRO41, we calculated the translational diffusion contact at 20 °C and used this value to determine the corresponding Rh based on Stokes–Einstein theory.

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Author contributions

K.M.S., C.I.M., D.H.F., K.D., J.A.C., D.S. and L.D.S. conceived and designed the experiments; K.M.S. and C.I.M. performed the wet lab experiments; K.D. and D.S. performed the simulations, molecular dynamics and modelling; K.M.S., C.I.M., D.H.F., K.D., J.A.C., D.S. and L.D.S. analysed the data; and K.M.S., J.A.C., D.S. and L.D.S. wrote the paper.

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