Assembly–disassembly is coupled to the ATPase cycle of tobacco Rubisco activase

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The carbon-fixing activity of enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is regulated by Rubisco activase (Rca), a ring-forming ATPase that catalyzes inhibitor release. For higher plant Rca, the catalytic roles played by different oligomeric species have remained obscure. Here, we utilized fluorescence-correlation spectroscopy to estimate dissociation constants for the dimer–tetramer, tetramer–hexamer, hexamer–12-mer, and higher-order assembly equilibria of tobacco Rca. A comparison of oligomer composition with ATPase activity provided evidence that assemblies larger than hexamers are hydrodynamically inactive. Therefore, supramolecular aggregates may serve as storage forms at low-energy charge. We observed that the tetramer accumulates only when both substrate and product nucleotides are bound. During rapid ATP turnover, about one in six active sites was occupied by ADP, and ~36% of Rca was tetrameric. The steady-state catalytic rate reached a maximum between 0.5 and 2.5 μM Rca. In this range, significant amounts of dimers, tetramers, and hexamers coexisted, although none could fully account for the observed activity profile. Therefore, we propose that dynamic assembly–disassembly partakes in the ATPase cycle. According to this model, the association of dimers with tetramers generates a hexamer that forms a closed ring at high ATP and magnesium levels. Upon hydrolysis and product release, the toroid breaks open and dissociates into a dimer and tetramer, which may be coupled to Rubisco remodeling. Although a variant bearing the R294V substitution has been used extensively to investigate quaternary structural features of tobacco Rca, the AAA + module is extended by about 68 N-terminal residues and about 19 C-terminal residues for β-Rca and 49 C-terminal residues for α-Rca. Although the longer isoform is redox-regulated, tobacco expresses only β-Rca (383 residues, mass of 42.8 kDa). Preparations of tobacco Rca have been described as highly polydisperse in size (17), a feature that appears to be shared with all other higher plant Rcas examined to-date (18–20). In X-ray structures of the nucleotide-free AAA + domains of tobacco and Arabidopsis Rca, helical assemblies were observed that contained six subunits per turn, but with differing pitch (Fig. 1) (21, 22).

A number of Rubisco-interacting residues of Rca have been identified over the years (21, 23–25), although a detailed model for the Rca–Rubisco complex is not yet available (26). Regardless, conserved central pore residues of Rca k have been shown to be critical in Rubisco reactivation (21).

The R294V substitution has been used extensively to investigate quaternary structural features of tobacco Rca. Arg-294 is found in the AAA + sensor II domain, a region that contains several conserved arginine residues, some of which are thought to interact with the γ-phosphoryl group of bound ATP (27). Replacement of Arg-294 with a valine residue disrupts a hydrogen bond between the guanidinium group and the amide nitro-
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gen of Asn-99 from the adjacent subunit, thereby loosening subunit–subunit contacts (21). Although remote from the active site (28), introduction of the homologous substitution into cotton β-Rca appeared to abolish nucleotide binding (20). For tobacco Rca-R294V but not for WT, negative-stain electron microscopic (EM) images of closed hexameric rings were observed in the presence of ATP or ATP-γS (21). In combination with the X-ray structure, the EM images were used as a basis for the generation of a closed-ring model of the tobacco AAA+ domain (Fig. 1) (21).

Mechanistic enzymology experiments on tobacco Rca demonstrated that positive cooperativity of ATP hydrolysis (Hill coefficient nH = 1.9) requires both ATP- and ADP-bound subunits (29). In the presence of excess Mg2+, the Km,ATP was reported to be 104 μM and the KADP 37 μM, consistent with inhibition at elevated ADP content (29). Binding of a second Mg2+ was shown to activate ATP hydrolysis with a Hill coefficient nH = 2.5, suggesting a model that entails cooperative behavior between three different classes of sites (29). Regardless, several studies have reported a lack of stimulation of ATPase activity by the client protein Rubisco (21, 29). Most recently, we have demonstrated that a ratio of inhibited Rubisco/Rcaγ as high as 24:1 does not lead to accelerated ATPase activity, not even in the presence of crowding agents (29).

All experimental evidence provided to-date supports the notion that Rca’s ATPase activity is directly proportional to its Rubisco/Rca6 as high as 24:1 does not lead to accelerated ATPase activity, not even in the presence of crowding agents (29). Recent studies have reported that facile subunit exchange requires ADP accumulation to an ATP/ADP ratio of 4:1 (33), suggesting that the exchange rate depends on the distribution of loose- and tight-binding states. It appears likely that the pre-hydrolysis state is formed in hexameric assemblies, because ATP-γS has been shown to promote hexamer formation in tobacco Rca and its R294V variant (21, 32, 35), cotton β-Rca (20), as well as spinach α-Rca (32).

In 2012, we established fluorescence correlation spectroscopy (FCS) protocols to estimate the oligomeric composition of Rca as a function of subunit concentration (18). Work on cotton β-Rca demonstrated that hexamerization involves well-populated intermediate states that include dimers and tetramers (18). This assembly mechanism has recently been verified by single molecule diffusimetry (35). In a follow-up study, FCS data suggested that higher ATP/ADP ratios favor topologically closed toroidal rings of six subunits each (20), whereas ADP promotes open “lock washer”-type Rcaγ structures (Fig. 1). Such open rings are thought to serve as templates for the continuous assembly to supramolecular spirals (20), as observed in the Rca crystal structures (21, 22).

Although the available evidence suggests that the hexamer is the species responsible for coupling ATP hydrolysis to Rubisco reactivation (21, 26), the functional importance of the extreme size polydispersity of Rca has not been resolved yet. Using subunit interface mutants, the smallest oligomer capable of ATP hydrolysis was shown to consist of about three subunits (21). Likewise, analytical ultracentrifugation (AUC) suggested that the peak ATPase activity at 1 μM Rca coincides with an average assembly of about three subunits (32).

At concentrations above 1 μM, AUC demonstrated that tobacco Rca assembles continuously to larger and larger species irrespective of nucleotide status (ADP, ATP-γS, and apoprotein) (32). However, with the R294V interface mutant, ATP-γS appeared to stabilize the hexamer between 1 and 20 μM Rca (32), whereas the apo-form assembled more slowly, and ADP accelerated the formation of large aggregates (32).

Recently, the early steps of tobacco Rca assembly were monitored using single-molecule diffusimetry in an aqueous phase anti-Brownian electrokinetic (ABEL) trap (35). With this method, individual oligomers were observed directly, which allowed for the extraction of reliable Km values for the monomer–dimer and dimer–tetramer equilibria (35). Importantly, no evidence of trimeric species could be detected. Although tetramers and hexamers could not be distinguished, a strong dependence of assembly on the type of nucleotide was observed, somewhat at odds with the reported AUC data (32). Similar to the tobacco interface mutant R294V (32) and cotton β-Rca (20), ATP-γS was shown to favor the formation of tetramer/hexamer populations at the expense of dimers and larger aggregates (35). In the same work, the rate of subunit exchange between dimer and tetramer/hexamer populations was reported to be about 0.2 s−1 with Mg-ADP and 0.1 s−1 with Mg-ATP-γS (35).

Here, we have used FCS to demonstrate that tobacco Rca aggregates of more than six subunits do not turn over ATP and may therefore serve as storage forms in the stroma. We found that both substrate and product nucleotides are required to stimulate tetramer accumulation and that hydrolytic activity does not coincide with one specific oligomeric species. Our data are most consistent with a model that couples hexameric ATPase activity with dissociation into dimers and tetramers.
We propose that the mechanical force generated in this way may be coupled to the conformational adjustment of Rubisco’s active sites.

**Results**

**Determination of microscopic equilibrium constants for the continuous assembly of tobacco Rca**

To elucidate the relationship between ATPase activity and assembly, we have used FCS methods to monitor the self-association of tobacco Rca from monomeric species to large supramolecular complexes. The methods employed here closely follow previously established methods used to monitor the assembly of cotton β-Rca (18, 20). To label tobacco Rca with a fluorescent label, the S379C substitution was introduced near the C terminus and covalently labeled with AlexaFluor-546 by means of maleimide chemistry. Analysis of labeled Rca-S379C by HPLC and absorbance provided molar dye-to-protein ratios of 1.0 ± 0.09, in line with the incorporation of a single fluorophore per Rca subunit. Control experiments on the parent protein provided molar dye-to-protein ratios of 0.01, indicating that attachment of the dye is specific to S379C. Because neither ATPase nor Rubisco reactivation activities were affected by this mutation (33), *Nicotiana tabacum* (Nt)-Rca-S379C is loosely referred to as WT protein.

As described previously (18), fluorescence fluctuations were monitored using confocal optics, and the autocorrelation function was utilized to calculate an apparent diffusion coefficient ($D_{app}$), which represents the average diffusion of all particles in the observation volume (36). A series of FCS measurements were carried out as a function of Rca subunit concentration in the presence of different nucleotides (2 mM ATP, ADP, ATP-γS, ATP-βS/ADP = 4, ATP-ALF$_{α}$, or apoprotein, i.e. no nucleotide). In all experiments, the concentration of labeled Rca was kept constant at 50 nM, and the concentration of label-free Rca ranged from 0 to 60 μM.

To estimate individual $K_d$ values from the concentration-dependent $D_{app}$ values, we adapted a self-assembly model used in previous work (18, 20). The model involves a dimeric intermediate as originally observed by gel filtration (19) and recently confirmed by single-molecule diffusometry (35). The assembly of species larger than hexamers is assumed to proceed in a helical fashion, with subunit arrangements similar to those observed in Rca crystals (Fig. 1) (21, 22). Therefore, we are explicitly modeling steps involving the oligomeric stoichiometries 1 → 2 → 4 → 6 → 12 → 18 → ... → 6n, where n is allowed to grow indefinitely (Fig. 1). In close analogy to our previous work (18), $K_d^{1–2}$, $K_d^{2–4}$, and $K_d^{3–6}$ are described by individual parameters. However, in this work, the formation of supramolecular helices is explicitly modeled as the continuous association of hexameric units (Fig. 1). In this updated assembly model, each hexamer addition involves the same enthalpy change (37). The equilibrium constant describing the first step of this indefinite assembly process, $K_d^{6–12}$, is allowed to differ from the equilibrium constant $K_d^{cont}$ describing subsequent additions of hexamers, i.e. $K_d^{12–18} = K_d^{18–24} = K_d^{6n – 6(n + 1)} = ... = K_d^{cont}$, with $n \geq 2$ (for a full description, see Equations 1–14).

Figure 1. Model for the stepwise assembly of higher plant Rca. The solvent-accessible surface of the AAA$^+$ domain of tobacco Rca is represented in blue, with individual subunits colored light and dark blue. The closed-ring hexamer (PDB code 3ZW6) may reactivate Rubisco, whereas the open hexamer (lock-washer) may serve as a template for continuous assembly to form helical filaments (PDB code 3T15). In the bottom row, a dodecamer (two helical turns, side view) and an 18-mer (three helical turns, side view) are shown. Rubisco from spinach (PDB code 1RCX) is shown in its inhibited form (large subunits green, small subunits gray, and inhibitor orange). Based on the results presented in this work, areas shaded green depict active forms of Rca, and areas shaded red depict inactive forms of Rca. Images were prepared using the program PyMOL.
With this approach, the number of variables fitted manually was reduced to a total of four, $K_d^{2-4}, K_d^{4-6}, K_d^{6-12},$ and $K_d^{\text{cont}}$.

Extensive FCS simulations indicated that for each nucleotide condition, the numerical product $K_d^{2-4} \times K_d^{4-6}$ remains constant for all $K_d$ combinations that provide a good fit to the data. This observation suggests that $K_d^{2-4}$ and $K_d^{4-6}$ are highly correlated (Table 1). A very large $K_d^{2-4}$ works well in combination with a very small $K_d^{4-6}$, providing the minimum tetramer content consistent with the experiment (Table 2). However, a very small $K_d^{2-4}$ in combination with a very large $K_d^{4-6}$ yields a poor fit to the data, thereby setting a limit to the amount of tetramer that may accumulate. To determine the maximum tetramer content consistent with the data, iterative modeling was used to determine the lower limit for $K_d^{2-4}$ and the upper limit for $K_d^{4-6}$ (Table 2). It is worth noting that in the presence of ATP and ATP-γS, the lower limits of the respective $K_d$ values, 9.5 and 0.3 μM, are similar to the ABEL trap values of 5.7 and 0.2 μM (35), thereby validating our procedure. The remaining two variables, $K_d^{6-12}$ and $K_d^{\text{cont}}$, were found to be uncorrelated to other $K_d$ values and could therefore be determined more precisely (Table 1).

Although a standard deviation is available for the experimental $D_{\text{app}}$ values (Fig. 2A), the manual fitting procedure is not amenable to the extraction of errors on individual $K_d$ values. Instead, a range of appropriate values was determined for the product $K_d^{2-4} \times K_d^{4-6}$, which in turn was used to determine a range of appropriate values for $K_d^{2-4}$ and $K_d^{4-6}$ (Table 1). Errors on $K_d^{6-12}$ and $K_d^{\text{cont}}$ were estimated by setting $K_d^{2-4}$ and $K_d^{4-6}$ to their respective mid-points and then modifying $K_d^{6-12}$ and $K_d^{\text{cont}}$ such that the simulated curves traced the top and bottom ends of the experimental error bars (Table 1).

To visually illustrate Rca assembly, the fractional contribution of each oligomer was plotted as a function of Rca subunit concentration (Fig. 2B, aggregates larger than 42-mers for clarity). Here, fractional contribution is defined as the fraction of subunits found in each oligomeric state, as described by Equation 1. In Equation 1, $\alpha_n$ equals the fractional concentration; $n$ is the oligomeric stoichiometry; $C_n$ is the concentration of oligomers with $n$ subunits, and $C_0$ is the total subunit concentration (see Equation S4).

$$\alpha_n = \frac{C_n}{C_0} \quad \text{(Eq. 1)}$$

To calculate the curves in Fig. 2B, the mid-point for the range of $K_d^{2-4}$ and $K_d^{4-6}$ values was used. To illustrate the rise of supramolecular aggregates with more than one helical turn, the numerical sum of all contributions arising from species larger than hexamers was also plotted (Fig. 2C).

In the presence of a single nucleotide, the tetramer does not accumulate

A comparison of the assembly mechanism of Nt-Rca-S379C under different nucleotide conditions demonstrates that in the absence of nucleotide (apo), in the presence of ADP, and in the presence of ATP-γS, the tetramer does not accumulate to any extent (Fig. 3). For these conditions, the maximum tetramer content is estimated to be 3.1, 1.6, and 7.8%, respectively (Table 2).

Assembly of the apoprotein proceeds primarily via monomers, dimers, and hexamers, species that are nearly equally populated at 0.74 μM. At 2.5 μM, the hexamer exhibits a maximum of 53%, and at higher concentrations, larger aggregates form (Fig. 3, A and B). These samples contained residual amounts of ADP between 0.5 and 60 μM, increasing with Rca concentration. Therefore, active sites remain essentially nucleotide-free at low protein concentration, based on the $K_d^{\text{ADP}}$ value of 37 μM (29).
Upon addition of 2 mM ADP (Fig. 3, C and D), the equilibria are shifted to slightly higher concentrations. At 1.5 μM Rca, monomers, dimers, and hexamers are nearly equally populated; the hexamer peaks are at 3.5 μM with a 48% contribution, and continuous assembly is observed at higher concentrations.

In the presence of 2 mM ATP-γS, a small amount of tetramer accumulates, reaching a maximum of ~7.8% at 1 μM Rca (Fig. 3, E and F). This effect could be due to the 6.6% ADP contamination of commercial ATP-γS preparations, as assemblies harboring both ATP-γS and ADP may stabilize tetrameric states (see below). Because ATP-γS is not hydrolyzed by tobacco Rca (contrary to cotton β-Rca [20]), the ATP-γS/ADP ratio of ~14 remains constant during FCS data acquisition (Table 3).

Regardless, the hexamer dominates over a broad concentration range at the expense of both dimers and larger aggregates, reaching a peak of 78.3% at 9.4 μM Rca (Fig. 3F). The elevated K_d values (Table 1) suggests that the ATP-γS–bound hexamer is less likely to support supramolecular helix propagation than the ADP-bound hexamer. Therefore, closed toroidal assemblies may be favored over open lock washer-type structures that can template helix nucleation (20).

**ATP hydrolysis appears to stimulate tetramer formation**

The addition of 2 mM ATP at the onset of FCS data collection appears to strongly stimulate tetramer formation (Fig. 4, A and B, and Table 2). At its peak position of 2.6 μM Rca, the tetramer represents 36.3% of all particles. Interestingly, at a concentration of 3.5 μM, tetramers and hexamers appear to be equally populated, and at 7.4 μM, the hexamer peaks with 40.7% contribution. Because ATP will be hydrolyzed during the 10-min data acquisition time of each FCS measurement, the extracted K_d values may be considered pseudo-equilibrium constants. Parallel experiments were carried out to monitor the accumulation of ATP with time. Upon averaging over the 10-min time course, the ATP/ADP ratios were estimated to be 6.3 ± 1.8, 2.4 ± 0.5, and 1.0 ± 0.1 at 1.5, 5, and 15 μM Rca, respectively. Based on these values, ATP was calculated to represent 14–30% of total nucleotide between 1.5 and 5 μM Rca, the concentration range with high tetramer content (Fig. 4B). These values provide an average ATP/ADP ratio of 4.3 ± 1.5 (Table 3), suggesting that tetramer accumulation may result from the presence of both ATP and ADP. Above 20 μM Rca, larger aggregates become dominant, as seen with ADP alone.

**Mix of substrate and product nucleotides induces the accumulation of tetrarmers**

To investigate tetramer accumulation in relation to ADP content, additional FCS experiments were carried out in the presence of 80% ATP-γS and 20% ADP. Under these conditions, the tetramer reaches 26% at 2.6 μM Rca, significantly above the ~8% observed with ATP-γS alone but below the 36% observed under ATP hydrolysis conditions (Fig. 4, C and D). Therefore, it appears that a mix of substrate and product nucleotides may be necessary to increase the tetramer content, whereas either alone prevents the tetramer from accumulating (Table 3).
Significant tetramer content is also observed in the presence of ADP/H18528AlFx, a coordination complex that mimics the transition state for hydrolysis. The tetramer reaches a maximum of 30% at 1.1/HisRca, similar to the ATP and the 80% ATP/His20%ADP conditions (Fig. 4, E and F, and Table 2). The ADP/H18528AlFx complex is generated in the protein’s active site by the addition of excess ADP, NaF, and AlCl3 to the protein solution. The biphasic behavior of the thermal denaturation profiles (see below) supports the notion that Rca assemblies harbor both ADP/H18528AlFx and ADP alone, mimicking assemblies bearing a mix of substrate and product nucleotides.

Assemblies larger than hexamers are catalytically inactive

To better understand the relationship between oligomer size and ATPase activity, the rate of steady-state ATP hydrolysis was monitored using an enzyme-coupled NADH-dependent assay (33). Assays were carried out at concentrations between 0.2 and 20 µM Nt-Rca-S379C, a range that was demonstrated to lie within the linear response of the assay. The average per-subunit hydrolytic rate was shown to increase at low Rca concentrations and to reach a plateau between 0.5 and 2.5 µM Rca, followed by a sharp decrease at higher protein concentrations (Fig. 5A). On a linear scale, the drop in activity above 2.5 µM Rca displayed a distinctly convex shape (Fig. 5B).

To facilitate visual comparison, the concentration-dependent ATP turnover rates were superimposed onto the FCS assembly data obtained under ATP hydrolysis conditions (Fig. 5, A and B). Between 7.5 and 20 µM Rca, aggregates larger than hexamers accumulate rapidly, whereas turnover rates decrease steeply, suggesting that large species may lack ATPase activity. Therefore, a comparison of the rate of activity loss with the rate of aggregate formation was carried out. A linear fit of normalized data yielded respective slopes of \(-0.023\) and \(-0.023\) (Fig. 6A), providing strong support for the notion that oligomeric species larger than hexamers are catalytically inactive. Similar arguments can be made to confirm that monomers are catalytically inactive, in line with an active site that is located at the interface of two subunits.

High ATPase activity coincides with high dimer, tetramer, and hexamer content

Further comparison of the activity data with the oligomeric composition of tobacco Rca suggests that ATP hydrolysis may arise from three different species: dimers, tetramers, and hexamers (Fig. 5, A and B). The per-subunit turnover rate rises to a plateau between 0.5 and 2.5 µM Rca, a concentration range that is dominated by the tetramer, but it also contains significant amounts of dimers and hexamers. Because the tetramer peaks at 2.6 µM Rca, it is unlikely that this oligomer is the sole species responsible for ATP hydrolysis. Clearly, the activity curve does not follow the tetramer curve over the entire concentration range (Fig. 7A, green curve). For example, between 2.5 and 20 µM Rca, the tetramer concentration decreases by 47%, whereas turnover rates drop by 76%. For similar reasons, neither the

Table 3

| FCS condition | ATP-γS | ATP-γS + ADP | ATP turnover |
|---------------|--------|-------------|-------------|
| Wildtype      | ATP-γS/ADP 14 | 4.0 | ~4.3* |
|               | Tetramer % 7.8 | 26    | 36        |
| Interface mutant | ATP-γS/ADP 14 | 3.0 | 45       |
|               | Tetramer % 12 |              | 45        |

* The estimated average ratio is between 1.5 and 5 µM Rca.

Figure 3. FCS experiments on WT Rca in the presence of a single nucleotide. The FCS data (black) and simulations (red) are shown in the top row, and the corresponding oligomeric compositions are shown in the bottom row. A and B, in the absence of nucleotide (apo-form); C and D, in the presence of ADP; E and F, in the presence of ATP-γS. For clarity, the data in Fig. 2A are reproduced in C, and the data in Fig. 2C are reproduced in D.
dimer nor the hexamer alone can account for the observed activity profile (Fig. 7A, red and blue curves). Although the dimer content appears to roughly follow the activity curve along the down-slope, the peak positions do not coincide (Fig. 7A, red curve).

We next considered models that combine the turnover rates of dimers, tetramers, and hexamers, under the premise that each species operates independently (Fig. 7A, purple and orange curves). When assuming that all subunits turn over at the same rate regardless of oligomer type (activity ratio...
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1:1:1, subunits-in-dimers/subunits-in-tetramers/subunits-in-hexamers), a normalized summation of calculated activities provided a poor fit to the data (Fig. 7A, purple curve). Therefore, we examined whether the oligomer stoichiometry modulates the turnover rate by testing a large number of different activity ratios (e.g. 0.5:1:0.2). Only one condition led to a somewhat improved fit to the experimental data, a condition that assigns tetramer subunits half the turnover rate of dimer subunits (subunit activity ratio 1:0:5:0) (Fig. 7A, orange curve). At this ratio, dimers and tetramers would turn over at the same rate, whereas all hexamers would be inactive. However, the fit to measured turnover rates remains unsatisfactory. In addition, assigning zero activity to the hexamer seems less than plausible, given Rca’s propensity to hexamerize with ATP-

Subunit exchange may play a role in the ATPase cycle

As an alternative model, we considered that oligomeric association–dissociation processes may play a role in the catalytic cycle. Because the accumulation of the tetramer appears to result from the accumulation of ADP (Fig. 4), it is feasible that the tetramer itself is generated by the process of ATP hydrolysis. If so, the hexamer would be the primary species carrying out hydrolysis. This idea is supported by the finding that ATP-yS promotes hexamer formation (Fig. 3, E and F), while also stabilizing the pre-hydrolysis state (33). The emerging picture suggests that the energy released by ATP hydrolysis may be utilized to break hexameric toroids open. Once a “lock-washer” conformation is obtained, the dissociation of a dimeric unit would be facilitated (Fig. 8). In this way, hydrolytic events within Rca6 assemblies would naturally lead to the formation of Rca4. Taken together, the data support a dynamic assembly–disassembly model with a catalytic cycle that consists of assembly of dimers and tetramers to form hexamers, hydrolysis of ATP, cleavage of toroidal rings, and the release of dimers and tetramers (Fig. 8).

ADP production appears to be a prerequisite for steady-state turnover

When monitoring the ATPase activity of tobacco Rca as a function of time, an initial lag period was observed. Turnover rates appear to increase slowly, until a steady state with maximal velocity is attained. Higher protein concentrations provide shorter lag periods, e.g., a 1-min lag at 20 \( \mu \)M Rca and a 5-min lag at 1.0 \( \mu \)M Rca. It is unlikely that the lag period results from slow inhibitor release, because the amount of ADP or ATP-

Nucleotide occupancy under rapid turnover and equilibrium conditions

The ADP-occupied fraction of sites can be estimated from the average nucleotide concentrations during FCS data acquisition. At 1.5 \( \mu \)M Rca, the tetramer content is near its maximum of ~36% (Fig. 5A), and on average, about 14% of nucleotide consists of ADP (ATP/ADP = 6.3 ± 1.8, see above). Using this ratio and the published \( K_{d,ADP} \) value of 37 \( \mu \)M and \( K_{m,ATP} \) value of 104 \( \mu \)M (29), active-site occupancy was calculated according to Equation 2.

\[
\frac{k_s}{k_{cat}} = \frac{[ES]}{E_{total}} = \frac{[S]^n}{K_m + \frac{[I] \times K_m}{K_i} + [S]^n}
\] (Eq. 2)

In Equation 2, S = ATP, I = ADP, ES equals the Michaelis complex, \( E_{total} \) the total Rca subunit concentration, \( k_s \) the observed turnover rate, and \( k_{cat} \) the turnover rate under substrate-saturating conditions. Cooperativity is indicated by the Hill coefficient \( n_{H} \), denoted by \( n \). For tobacco Rca, we have reported weak cooperativity in ATP hydrolysis in the presence of small amounts of ADP (29). When using \( n_{H} = 2 \) in Equation 2, 17% of binding-competent sites are predicted to be occupied by ADP. Therefore, it appears that one out of six available sites must carry ADP to promote tetramer accumulation, a condition that provides high-turnover rates (Fig. 5A).
Because ATP-γS is not hydrolyzed by tobacco Rca, ADP occupancy can also be estimated under equilibrium conditions. 80% ATP-γS and 20% ADP cause an increase in tetramer accumulation from ～8 to ～26% (Table 3). To evaluate the binding equilibria under these conditions, the dissociation constant for ATP-γS must be known. Therefore, turnover rates of 2 μM Nt-Rca were measured in the presence of ATP-γS, with concentrations ranging from 0 to 125 μM (Fig. S1). Using Equation 2, the $K_d^{ATP-γS}$ was extracted to be 16.2 μM. By setting $K_{on} = K_d^{ATP-γS}$ and $K_i = K_d^{ADP}$ in Equation 2, the fraction of ADP-occupied sites was estimated to be about 11% ($n_{ADP} = 1$). This fraction is somewhat lower than the 17% estimated under ATP turnover conditions, providing a rationale for the reduced tetramer content (～26% versus ～36%).

**R294V interface mutant serves to validate assembly-mediated regulation**

To assess the validity of the proposed catalytic cycle, the interface mutant Nt-Rca–R294V/S379C was subjected to the same FCS procedures as WT (Fig. 9), and its oligomeric composition was compared with its ATPase activity (Fig. 5, C and D). Although the FCS data were much noisier than for WT, plausible $K_d$ values could be extracted for each step of the assembly process (Table 1). By and large, these values are similar to those determined for the WT protein, especially in the presence of ADP, ATP-γS, and ATP. With ATP-γS, the maximum hexamer content is nearly identical to that of WT (79%, Fig. 9F), whereas ADP(AlF$_3$) more than doubles the hexamer content, providing 71% at 17 μM Rca (Table 2 and Fig. 9, I and J). As with WT, significant tetramer accumulation occurs only if both substrate and product nucleotides are present, such as during ATP turnover or in the presence of ADP(AlF$_3$) (Fig. 9 and Tables 2 and 3).

As before, concentration-dependent initial rate measurements were carried out, and the per-subunit turnover rate was superimposed onto the oligomeric distribution obtained in the presence of ATP (Fig. 5, C and D). Turnover was found to be maximal between 0.8 and 2.5 μM (Fig. 5, C and D). The slope of activity loss (～0.020) and the slope of aggregate (~Rca$_6$) formation (+0.021) were found to be nearly identical (Fig. 6B) upon normalization, mimicking the results obtained for WT almost exactly. These data provide independent support for the conclusion that species larger than hexamers are catalytically inactive, and they suggest that activity is highest when a substantial amount of dimers, tetramers, and hexamers coexist.

**Faster turnover rates may be coupled to higher tetramer content**

Compared with WT, a consistent elevation of ATPase activity was observed over the entire concentration range tested (0.2 to 20 μM Rca), with an average increase of 13.5 ± 13.1% ($n = 13$). Despite the large error, this effect is corroborated by a decrease in the ATP/ADP ratio, as determined in parallel experiments mimicking the FCS conditions with ATP. The ATP/ADP ratio was measured to be 4.8 ± 1.3, 1.2 ± 0.2, and 0.8 ± 0.2 at 1.5, 5, and 15 μM Rca, respectively, consistently lower than those measured for WT (6.3 ± 1.8, 2.4 ± 0.5, and 1.0 ± 0.1). The interface mutant appears to yield a larger amount of tetramer than WT (Fig. 9, G and H), with a maximum of 45% at 2.4 μM compared with 36% for WT (Tables 2
and 3). The increase in tetramer could be the result of accelerated turnover, if ATP hydrolysis breaks the hexameric ring (Fig. 8).

**The interface mutant can access a state of elevated thermal stability**

To assess any modifications in thermostability, the midpoint for protein denaturation $T_m^{\text{app}}$ (apparent melting temperature) was determined by means of the Thermofluor assay (19). The protein concentration was kept at 5 μM, with buffer conditions identical to FCS. Despite the similarities in assembly, the thermal profile of the interface mutant differs substantially from WT. Although the R294V substitution destabilizes apo-Rca by ~9 °C, ligand stabilization by ADP and ATP is similar for both proteins (Table 4). Interestingly, ATP-γS and ADP-AlF$_4^-$ strongly stabilize the interface mutant relative to WT (21–25 °C versus 4–9 °C), reaching a $T_m^{\text{app}}$ value of 50.9 and 54.6 °C for the two analogs.

ADP-AlF$_4^-$ stabilizes the hexameric state over a broad concentration range (Table 2 and Fig. 9, I and J), suggesting that the H-bond disruption facilitates structural adjustments to form a thermally stabilized hexameric state. It appears that a tight-binding pre-hydrolysis conformation (33) may be more accessible to the interface mutant than to WT.

**In its apo-form, the interface mutant is assembly-compromised**

In the absence of nucleotide, the R294V substitution destabilizes the dimer (Fig. 9, A and B), as indicated by a $K_d^{1 \rightarrow 2}$ elevated from 0.7 to 25 μM (Table 2). Because the monomer dominates below 1 μM, this condition is the only condition for which a reliable $K_d^{1 \rightarrow 2}$ value could be extracted from the FCS data. The thermal destabilization of the apo-form (Table 4) provides a rationale for its sluggish assembly and tendency to aggregate.

**Discussion**

In this work, we identified oligomeric forms of tobacco Rca that participate in ATPase activity, and we characterized Rca assembly mechanisms that down-regulate the ATPase activity. Rca-mediated ATP hydrolysis is a prerequisite for Rubisco activation and has been shown to be directly proportional to the activation rate (17, 29, 30). Several studies have demonstrated that the rate of hydrolysis is not stimulated by inhibited Rubisco complexes (21, 29). Although Rubisco activation is thought to involve hexameric rings (6, 7, 21), the structure of the bound state has not been characterized yet. Because of Rca’s polydispersity, the relationship between quaternary structure and function is not fully understood (17, 21, 26, 30).

The FCS work presented here provides a stepwise mechanism for continuous Rca assembly and is complementary to our recently published study based on single-molecule diffusometry using an ABEL trap (35). In the ABEL trap work, early equilibria (1 → 2 → (4 + 6)) were particularly well-resolved, although tetramers and hexamers could not be distinguished. In contrast, this work provides critical information about tetramer–hexamer equilibria and the accumulation of large aggregates. Therefore, the results presented here allow for the construction of models for Rubisco regulation based on assembly–disassembly processes. To aid in the extraction of

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**Table 4**

| Nucleotide condition | Wildtype °C | Interface mutant °C |
|----------------------|------------|---------------------|
| ADP                  | 45.1 ± 0.5 | 37.4 ± 1.1          |
| ATP-γS               | 42.7 ± 1.0 | 50.9 ± 1.6          |
| Apo                  | 38.2 ± 0.9 | 29.7 ± 2.2          |
| ATP                  | 44.2 ± 0.3 | 35.9 ± 1.2          |
| ADP-AlF$_4^-$        | 47.0 ± 0.4 | 54.6 ± 0.8          |
Functional oligomers of tobacco Rca

Under active ATP hydrolysis conditions, the concentration-dependent activity profile suggests that dimers, tetramers, and hexamers contribute to steady-state turnover (Fig. 5). None of these oligomers by themselves can fully account for the observed activity. Therefore, we propose a dynamic assembly–disassembly model for Rca activity. Our model for the catalytic cycle involves the association of dimers with tetramers to form hexameric species that are topologically closed when ATP is high (Fig. 8, step 1). The hexameric toroids are able to bind to inhibited Rubisco complexes (Fig. 8, step 2) and enter the prehydrolysis state (33). Once ATP hydrolysis has occurred, product dissociation causes the closed ring to break open (Fig. 8, step 3), a motion that leads to the mechanical remodeling of Rubisco sites. Subsequent breakage of the Rca ring into a dimer and tetramer and dissociation from Rubisco allows the cycle to be repeated (Fig. 8, steps 4 and 5).

Our dynamic assembly–disassembly model is consistent with a recent report stating that maximal ATPase activity coincides with an average particle size of about three subunits (32). Our model is also consistent with the earlier finding that units as small as trimers may be ATPase-active (21). We find that maximal activity is achieved between 0.5 and 2.5 μM Rca (Fig. 5), a range in which monomers, dimers, tetramers, and hexamers coexist at ratios that could provide an average molecular weight of a trimer. Because FCS allows for the extraction of specific oligomeric states rather than averages, we argue here that trimeric species are only indicated as an average particle size of heterogeneous mixtures but do not play a role in catalysis. In line with this notion, trimers were not observed by single-molecule diffusometry (35).

Inactive aggregates may serve as a biological storage mechanism

Furthermore, we demonstrate that the rate of activity loss matches the rate of aggregate formation almost exactly (Fig. 6), indicating that assemblies larger than hexamers do not catalyze ATP hydrolysis. This result is based on the interpretation of high-concentration FCS data in terms of a continuous assembly model. The $D_{app}$ values measured at higher protein concentrations are consistent with the formation of helical filaments that contain variable numbers of hexamers (Fig. 28).

Therefore, the emerging model for the biological regulation of Rca includes the aggregation to fibrillar structures (Fig. 1), as nucleated by open lock washer-type ring structures (29). For nonredox-sensitive Rcas such as tobacco, aggregation may be favored when the ATP/ADP ratio and the Mg²⁺ ion concentration drop (29). These conditions would be prevalent in the dark, when the energy charge of the stroma is low (Fig. 1, area shaded red). Rca fibers may serve as a storage mechanism when hydrolytic activity is not beneficial to the cell, as described for the fibrillation of red-type Rcas in the absence of the allosteric activator RuBP (38). In response to rising Mg²⁺ and ATP, larger aggregates may dissociate to species of six or fewer subunits, thereby facilitating diffusion through the highly crowded environment of the stroma. Because Rubisco outnumbers Rca substantially (Rubisco/Rca₆ ~ 10:1) (30), binding to Rubisco would outcompete self-association, especially when closed rings prevail over open ones.

Although the interaction of green-type Rca with Rubisco remains uncharacterized, our data support the notion that the prehydrolysis state of Rca₆ interacts with Rubisco. We and others have demonstrated that the presence of inhibited Rubisco complexes does not affect the rate of steady-state ATP turnover (29). In the test tube, the relatively dilute Rca concentrations (up to 20 μM) suggest that the association of dimers with tetramers (Fig. 8, step 1) could be rate-limiting, such that stimulation by Rubisco would not be observed. However, it is difficult to predict the nature of the rate-determining step under physiological conditions in the chloroplast stroma, where Rca concentrations are thought to be 160–480 μM and Rubisco concentrations to be about 500 μM (1, 30).

Fraction of active sites occupied by ADP in relation to tetramer accumulation

The lag period observed during time scans of ATPase activity suggests that a fraction of active sites must be occupied by ADP to achieve high turnover rates. During this priming period, the production of ADP may be essential in establishing steady-state oligomer concentrations. We propose that the accumulation of tetrameric species requires destabilization of the hexameric toroid by ADP, and our calculations show that ADP must occupy at least one out of six sites for rapid ATP turnover (Fig. 8). Therefore, a plausible mechanistic model is one in which active turnover generates tetramers by increasing the ADP occupancy of hexamers. This model is in line with our previous work on ADP-mediated allosteric activation of ATPase activity (29) and ADP-mediated quaternary reorganization (33). In combination, the data suggest that small amounts of ADP are critical in generating an oligomer distribution that optimizes the turnover rate.

We find that at the high turnover rates observed at 1.5 μM Rca, the tetramer concentration reaches its maximum of about 36%. However, under equilibrium conditions in the presence of 80% ATP·γS and 20% ADP, the tetramer accumulates to only 26%, consistent with the notion that fewer hexamers bear ADP. Indeed, only about one-tenth of available sites are estimated to carry ADP under these conditions. These observations support a strong connection between oligomeric reorganization, tetramer accumulation, and rapid turnover.

R294V mutation facilitates access to a tight-binding pre-hydrolysis state

Replacement of Arg-294 with a valine residue disrupts a hydrogen bond between adjacent subunits, possibly destabilizing the spiral subunit arrangement observed in the X-ray struc-
**Functional oligomers of tobacco Rca**

...ture (21). This variant, referred to as interface mutant, has been shown to stabilize ATP-γS-bound closed-ring hexamers (21). Interestingly, in the presence of ATP or ADP, the interface mutant is highly thermostable, similar to spinach β-Rca (36–37 °C versus 34–35 °C) (33). Here, we demonstrate that substantial thermal stabilization is achieved by substrate analogs, again reminiscent of spinach Rca (33). Surprisingly, for the interface mutant, the most significant stabilization is observed with ADP-AlF₄⁻ (Tₘpp = 54.6 °C), although ADP(AIF₄⁻) has been shown to destabilize cotton β-Rca (20) and displays only a minimal effect on WT tobacco Rca (Table 4). Therefore, it appears that conformational flexibility plays a role in the ability of Rca variants to form a highly stabilized inhibitor complex (33). The destabilizing effect of the R294V mutation may allow for structural adjustments that facilitate formation of a locked complex. It is likely that this complex resembles a pre-hydrolysis state sampled immediately prior to the transition state for phosphodiester bond cleavage (33). The large degree of thermal stabilization suggests that the pre-hydrolysis state involves closed hexameric rings (Figs. 1 and 8).

**Materials and methods**

**Subcloning and site-directed mutagenesis**

The gene coding for tobacco Rca (383 residues) was transferred from the pET23a(+) vector to the pHUE vector as described for So-β-Rca (33). The pHUE expression system, a generous gift from S. Whitney (Australian National University, Canberra, Australia), allows for the N-terminal fusion of Rca to His₆-tagged ubiquitin, which is cleavable using a His₆-tagged deubiquitylating enzyme (39, 40). To derivatize the protein with a fluorescent label, the S379C substitution was introduced, and to generate the interface mutant, the R294V substitution was introduced, each by using the QuickChange site-directed mutagenesis kit (Agilent). All sequences were verified by DNA sequencing.

**Protein expression and purification**

*Escherichia coli* strain BL21(DE3) was transformed with the pHUE expression plasmid; liquid cultures were grown as described previously (33). All purification steps were carried out in the presence of 0.5 mM ADP. The first step involved affinity chromatography on a HisTrap HP nickel-nitrilotriacetic acid (Ni-NTA) column. Rca-containing fractions were pooled, concentrated using a Centriprep concentrator (Millipore, Milford, MA), buffer-exchanged into 50 mM HEPES-NaOH, pH 7.5, 250 mM KCl, 0.5 mM ADP, 5 mM MgCl₂, and 10% glycerol using PD-10 columns (GE Healthcare), and then further concentrated to about 20 mg/ml. Aliquots were flash-frozen and stored at −80 °C. Protein concentrations were determined using the Bradford method with BSA as a standard. Typical yields were 3–5 mg of homogeneous Rca/liter of liquid culture.

**Protein labeling with Alexa dye**

To specifically target the engineered cysteine residue of tobacco Rca-S379C, the labeling reaction was carried out in the presence of ATP-γS. Protein was labeled with AlexaFluor-546 C5-maleimide (λₘₐₓ-ex = 554 nm; λₘₐₓ-em = 570 nm; mass = 1,034 Da) (Molecular Probes, Inc.) in 300 μl of buffer containing 50 mM HEPES, pH 7.2, 125 mM KCl, 10 mM MgCl₂, and 5 mM ATP-γS. The Rca subunit concentration was kept at 100 μM and the dye concentration at 200 μM. The reaction was allowed to proceed for 4 h at 4 °C. To remove free dye, 300 μl of saturated ammonium sulfate was added incrementally, and the sample was incubated at 4 °C for an additional 30 min. Subsequently, the sample was pelleted by micro-centrifugation in an Eppendorf tube, resuspended in 180 μl of buffer containing 50 mM HEPES, pH 7.5, 250 mM KCl, 10% glycerol, 5 mM MgCl₂, and 0.5 mM ADP, and desalted by passing it twice over a gel-filtration spin column pre-equilibrated with the same buffer (Sephadex G-50—fine, 2-ml bed volume, and centrifugation at 400 × g for 2 min). Desalted samples were aliquoted, flash-frozen, and stored at −80 °C.

**Analysis of Rca preparations**

Labeled and label-free protein preparations were analyzed by reverse-phase HPLC (Agilent Technologies 1260 Infinity Quaternary LC system and Agilent 1260 Series Diode-Array detector) on a C18 analytical column (Vydac, 218TP, 5 μm, 250 × 4.6 mm) using a linear water/acetonitrile gradient with 0.1% TFA. The absorbance of the sample was monitored at A₂₅₀, A₂₈₀, and A₅₅₀ for AlexaFluor-546. Labeled Rca eluted as a single peak. Free dye could not be detected in any of the samples.

Labeling efficiencies were determined from absorbance spectra (Shimadzu UV2401-PC spectrophotometer) following published procedures (33), using the extinction coefficient of AlexaFluor-546 (ε₅₅₄ = 104,000 M⁻¹·cm⁻¹) and correction factors published by the manufacturer (Molecular Probes, Inc). Labeled and label-free protein preparations were analyzed by MALDI-MS (Applied Biosystems/MDI SCIEX 4800 MALDI-TOF/TOF analyzer) using sinapinic acid as matrix and following published procedures (33). The experimentally determined mass for label-free Rca-S379C was 42,786 Da (theoretical mass 42,764 Da), whereas that of AlexaFluor-546–labeled Rca-S379C was 43,797 Da (theoretical mass 43,798 Da). The experimentally determined mass for label-free Rca-S379C/R294V was 42,594 Da (theoretical mass 42,564 Da), whereas that of AlexaFluor-546 labeled Rca-S379C/R294V was 43,637 Da (theoretical mass 43,741 Da). The error is estimated to be about 100 Da.

In control experiments, tobacco Rca lacking an engineered C-terminal cysteine residue was subjected to the same labeling procedure followed by MALDI analysis. The experimental mass was determined to be 42,852 Da (theoretical mass 42,748 Da), indicating that no label was attached. Therefore, the labeling method specifically targets the C terminus, but not any of the five endogenous cysteine residues, in line with previous findings (18, 33).

**Sample preparation for fluorescence fluctuation measurements**

Labeled and label-free Rca samples were thawed on ice for 10 min (20 μl of 553 μM label-free Rca and 5 μl of 69 μM labeled Rca). For labeled Rca, a 1 μM working solution (69 μl) was...
prepared by diluting with buffer containing 50 mM HEPES-NaOH, pH 7.5, 250 mM KCl, 5 mM MgCl₂, 10% glycerol, and 2 mM of either ATP-γS, ADP, ATP, ADP(AlF₃), or a mixture of nucleotides (80% ATP-γS and 20% ADP). For apoprotein preparations, nucleotides were omitted. For label-free Rca, a working solution (20–600 μL) of either 1, 10, or 100 μM protein, or no dilution, was prepared using the same buffer. Working solutions were prepared fresh at the time of use and kept on ice for no more than 2 h. For FCS data collection, the two working solutions were mixed at appropriate ratios to obtain the desired final protein concentration, while keeping the labeled component fixed at 0.05 μM. Protein concentrations between 0.05 and 60 μM were analyzed by FCS.

Prior to measurement, samples were incubated on ice for 10 min to allow for subunit equilibration. Subsequently, a 35-μL sample was placed into the perfusion chamber, and the FCS decay was measured 20 times for 30 s each, giving a total acquisition time of 10 min. This procedure was repeated for all protein concentrations and all nucleotide conditions. For FCS measurements in the presence of ADP(AlF₃), the sample was first diluted into buffer without nucleotide (50 mM HEPES-NaOH, pH 7.5, 200 mM KCl, 5 mM MgCl₂, and 10% glycerol), and then 2 mM ADP and 50 mM NaF were added. Subsequently, the sample was allowed to equilibrate at room temperature for 10 min, followed by the addition of 2 mM AlCl₃ and another 10-min incubation on ice prior to FCS data collection.

To prevent systematic errors, samples were measured from low to high protein concentration, from high to low, and from intermediate to high, and then to low concentrations. At each concentration, three different samples were assayed while varying the incubation period. Additionally, a control experiment was conducted to ensure that the attachment of AlexaFluor-546 did not affect the self-assembly process. To this end, tobacco Rca-S379C was labeled with trimethylrhodamine (TMR) dye exactly as described above, and FCS data were collected in the presence of ADP.

**FCS instrumental setup and data collection**

FCS measurements were carried out using a home-built confocal optical setup (18). Excitation was achieved with a Compass 215M-10 532-nm CW laser (Coherent GmbH, Germany) attenuated to 60 microwatts to minimize triplet dynamics. The output of the laser was expanded, collimated, and directed via a dichroic lens into an Olympus PlanApo ×100/1.4NA oil objective. Samples were placed into perfusion chambers pre-treated with BSA to minimize Rca adsorption onto the coverglass (see below). Fluorescence from samples was collected via the same objective, separated from excitation light through the dichroic filter, and reflected into a 50-μm pinhole. The emission was then focused into an avalanche photodiode detector (Perkin-Elmer Life Sciences Optoelectronics, SPCM-AQR14). A band-pass filter was used before the detector to minimize stray light (Omega 3RD560-620). The recorded fluorescence signal was autocorrelated in real time using an ALV5000/EPP USB-25 correlator (ALV GmbH, Germany).

The confocal parameters of the observation volume were determined by measuring a 20 nm carboxylic acid of tetramethylrhodamine (TAMRA) free dye solution (λmax-em = 574 nm; λmax-ex = 548 nm; λmax-em = 574 nm; mass = 430 Da) (Molecular Probes, Inc.) with a known diffusion coefficient (420 μm² s⁻¹) (41). The average decay was fitted using the autocorrelation function (Equation 3) to extract the values of r₀ and z₀. We note that z₀ ≫ r₀ in our setup, so Equation 3 is only marginally sensitive to the value of z₀. For each protein sample, 20 autocorrelation traces were collected (30 s each). Average photon count rates were about 50–90 kHz.

$$G(\tau) = G_0 \left(1 + \frac{4D\tau}{r_0^2}\right)^{-1} \left(1 + \frac{4D\tau}{z_0^2}\right)^7$$

(Eq. 3)

**Extraction of apparent diffusion coefficients and identification of monomeric species**

The 20 raw autocorrelation curves were analyzed individually to remove traces consistent with aggregated material. If more than 25% of traces indicated aggregation, a fresh sample was prepared and data collection repeated. For each 10-min measurement, FCS decays were averaged and fitted manually to the autocorrelation function (Equation 3) to extract the apparent diffusion coefficient (Dapp). All Dapp values were normalized with respect to the diffusion coefficient of the monomer (Dₘ). To determine Dₘ, the Dapp value measured at 0.05 μM Rca was corrected for contributions from dimeric species using the published Kᵢ⁻² (35). Dapp/Dₘ values were plotted as a function of protein concentration and compared with a scale showing the expected ratios Dᵢ/Dₘ = k⁻¹/² for the pure oligomeric forms, where k denotes the oligomeric stoichiometry (Figs. 2–4 and 9).

To confirm the presence of predominantly monomeric species at low concentration, TMR-labeled Rca-S379C was employed in buffer containing 50 mM HEPES-NaOH, pH 7.5, 250 mM KCl, 5 mM MgCl₂, 10% glycerol, and 2 mM ADP. The total number of observed particles was determined by FCS at different protein concentrations ranging from 0.05 to 0.3 μM Rca. To this end, the inverse of the amplitude was extracted after fitting the decay curves to the autocorrelation function. The total number of particles increased linearly with increasing concentrations, providing support for predominantly monomeric species in this concentration range. In addition, the brightness per particle was determined for free TMR (ε₁), and TMR-labeled Rca-S379C (ε) by dividing the average count rate by the number of particles. The ε/ε₁ ratio was calculated to be ~0.8 over this concentration range, consistent with about 1 dye per particle, as expected for single subunits. Furthermore, Dapp values were determined to be constant over this concentration range, again consistent with predominantly monomeric species.

**Mathematical modeling to extract Kᵢ values**

To analyze the concentration-dependent FCS data in terms of microscopic equilibrium constants for Rca assembly, a mathematical model developed previously was used with some modifications (18). Following the formalism introduced by Kanno et al. (36), the total autocorrelation function was expressed in terms of the concentrations of all oligomeric species present at each Rca concentration, while taking into account the ratio of labeled and unlabeled Rca. A complete mathematical descrip-
tion of the model is provided in the [supporting information](#). Similar to our previous work, $K_d^{2-4}$, $K_d^{2-4}$, and $K_d^{2-4}$ were described by individual parameters. However, in this work, assembly from hexamers to dodecamers was modeled explicitly ($K_d^{n-12}$), whereas the assembly of larger aggregates was described by a continuous assembly model. The continuous assembly model is characterized by a unique equilibrium constant ($K_d^{eq}$) that describes the dissociation of a $6n$-mer into a hexamer and a $6(n-1)$-mer ($n > 2$).

The diffusion coefficients $D_k$ for the oligomers with $k = 1, 2, 4,$ and $6$ subunits were estimated using the software WinHYDROPRO (42) using PDB files created by extracting $k$ sub-units from the helical structure of tobacco Rca (PDB code 3T15) (21). The diffusion coefficients of the higher oligomers were assumed to decrease in proportion to the cube root of the number of hexameric units in the assembly. $D_k/D_{60} = n^{1/3}$

For manual fitting, a set of $K_d$ values was first assumed and used to calculate the concentrations of all oligomers at each Rca concentration. The oligomer concentrations were then used to calculate theoretical autocorrelation functions, from which $D_{app}$ values were obtained for each Rca concentration. Calculated $D_{app}/D_k$ values were then compared with the experimental data. If agreement was deemed acceptable, the set of equilibrium constants was considered to be consistent with the experimental data. If not, equilibrium constants were modified, and the process was repeated iteratively until agreement with the experiment was satisfactory.

Thermofluor assays

Thermofluor assays were carried out to estimate the apparent mid-point for protein denaturation $T_{app}$. The protocol used was exactly as described previously (19), with a premix containing 0.25 mg/ml protein.

Continuous ATPase assays based on ADP production (NADH assay)

The ATPase activity of Rca was monitored as a function of subunit concentration using the NADH assay as described previously (33). The working solution contained 100 mM Tricine, pH 8.0, 20 mM KCl, 5 mM DTT, 5 mM MgCl$_2$, 2 mM phosphoenolpyruvate, 0.3 mM NADH, 1.3–2.2 units of pyruvate kinase, and 2–3.1 units of lactate dehydrogenase (Sigma). Samples of Rca at different protein concentrations were mixed with buffer containing 50 mM HEPES, pH 7.5, 5 mM MgCl$_2$, 250 mM KCl, 10% glycerol. To allow for comparison with FCS results, the dilution scheme was similar to that used in the FCS experiments. After dilution, 25 μl of each Rca sample was mixed with 373 μl of assay mix in the cuvette, providing final Rca concentrations between 0.25 and 20 μM. NADH oxidation rates were determined for the steepest part of the curve over a 36-s window. All velocities were measured a minimum of five times. An ADP standard curve was utilized to demonstrate that up to 500 μM ADP provided a linear response.

End-point ATPase assay based on ADP production (NADH assay)

To determine the total amount of ADP produced at specific time points during a 10-min FCS experiment, an appropriate amount of Rca stock was mixed with buffer containing 50 mM HEPES, pH 7.5, 5 mM MgCl$_2$, 250 mM KCl, 10% glycerol, and 2 mM ATP (or 2 mM ATP-γS), followed by incubation at room temperature. At specific time points, 20 μl was withdrawn and added to a cuvette containing 380 μl of working solution containing 100 mM Tricine, pH 8.0, 20 mM KCl, 5 mM DTT, 5 mM MgCl$_2$, 2 mM phosphoenolpyruvate, 0.3 mM NADH, 1.3–2.2 units pyruvate kinase, and 2–3.1 units of lactate dehydrogenase (Sigma). The $A_{340}$ was monitored for 100 s, and the ADP content was calculated by using the total change in absorbance.

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