The folding of many proteins depends on the assistance of chaperonins like GroEL and GroES and involves the enclosure of substrate proteins inside an internal cavity that is formed when GroES binds to GroEL in the presence of ATP. Precisely how assembly of the GroEL-GroES complex leads to substrate protein encapsulation and folding remains poorly understood. Here we use a chemically modified mutant of GroEL (EL43Py) to uncouple substrate protein encapsulation from release and folding. Although EL43Py correctly initiates a substrate protein encapsulation reaction, this mutant stalls in an intermediate allosteric state of the GroEL ring, which is essential for both GroES binding and the forced unfolding of the substrate protein. This intermediate conformation of the GroEL ring possesses simultaneously high affinity for both GroES and non-native substrate protein, thus preventing escape of the substrate protein while GroES binding and substrate protein compaction takes place. Strikingly, assembly of the folding-active GroEL-GroES complex appears to involve a strategic delay in ATP hydrolysis that is coupled to disassembly of the old, ADP-bound GroEL-GroES complex on the opposite ring.

To fold, many essential proteins require the assistance of specialized molecular chaperones known as chaperonins (1). The GroES chaperonin system of *Escherichia coli* is one of the best-studied examples of the chaperonin class of molecular chaperones (for review see Refs. 2, 3). GroEL is a tetradecamer of fourteen identical 57-kDa subunits arranged into two heptameric rings (4). Each ring contains a large, central, open cavity, and the two rings are stacked back-to-back to create a double toroid. Maximally efficient folding of most proteins that possess a strict dependence on GroEL (so-called stringent substrate proteins) requires that they be encapsulated within a closed chamber formed by a GroEL ring and the separate co-chaperonin GroES (5–8). Binding of the GroES lid over a captured substrate protein seals the GroEL cavity and releases the non-native protein into the confined space of the GroEL-GroES chamber (the cis complex). Protein folding proceeds in this isolated space for several seconds until the GroEL-GroES complex is dismantled and the substrate protein, folded or not, is ejected into free solution (5–10).

For stringent substrate proteins, ATP is required for the assembly of the GroEL-GroES cis complex and the associated steps of substrate protein encapsulation, release and folding (5, 11–13). Binding of ATP to a GroEL ring drives a large-scale rearrangement of the GroEL subunits, resulting in a dramatic elevation and rotation of the GroEL apical domains away from the central cavity (14, 15). GroES binding and substrate folding within the cis complex depend upon these ATP-induced structural rearrangements (5, 16, 17). However, despite a wealth of structural and biophysical information, precisely how assembly of the GroEL-GroES cis complex leads to substrate protein encapsulation, release, and folding remains poorly understood. Because they bind to the same part of a GroEL ring, how does GroES binding lead to substrate protein encapsulation before the non-native protein escapes into solution? In addition, how is substrate protein release and folding coordinated with ATP hydrolysis?

Large, stringent substrate proteins not only bind to sites on the GroEL apical domains that overlap with the binding sites for GroES but are also likely to fill and spill out of the open GroEL ring (14, 16, 18–22). This raises a serious issue: how does GroES locate and bind to its binding sites on a GroEL ring without the substrate protein prematurely escaping into solution? In fact, ATP must bind before GroES, and the same, highly cooperative structural rearrangements of the GroEL ring that lead to GroES binding also weaken the interaction between the substrate protein and the GroEL ring (13, 23, 24). Based on a detailed examination of changes in Trp fluorescence of an engineered GroEL mutant, it has been suggested that GroES loading and substrate encapsulation could occur prior to substrate protein release (25). In this case, however, a small and non-stringent substrate protein was employed and conformational changes in the substrate during encapsulation could not be assessed. Other work has suggested that premature substrate release is prevented by a secondary timer within the assembled GroEL-GroES complex, implying that the initiation of ATP hydrolysis and substrate release is tightly coupled (26).

Here we have examined these questions using a novel, chemically modified mutant of GroEL that has a small organic probe attached to an engineered Cys residue at the bottom of the GroEL cavity. Designated EL43Py, this modified GroEL variant displays a much higher rate of steady-state ATP hydrolysis than wild-type GroEL (wtGroEL) but is dramatically compromised in its ability to fold stringent GroEL substrate proteins. We
demonstrate that EL43Py populates, but fails to efficiently exit, a key intermediate allosteric state of the GroEL ring. We show that the ATP-driven formation of this intermediate conformation of the GroEL ring is necessary not only for GroES binding, but also for the forced unfolding of the non-native substrate protein. Premature escape of a large, non-native substrate protein from the GroEL ring is inhibited through the formation of this intermediate allosteric state, which can simultaneously bind both substrate protein and GroES with high affinity. Additionally, even though population of this allosteric state is sufficient to commit the bound ATP to hydrolysis, ATP turnover is delayed during a normal GroEL folding cycle. This slowing in ATP hydrolysis is linked to the disassembly of the ADP-bound GroEL-GroES complex on the opposite ring and ensures that GroES binding and substrate encapsulation are complete prior to the initiation of ATP hydrolysis.

**EXPERIMENTAL PROCEDURES**

**Proteins**—The GroEL variants containing single Cys residues S43C (EL43C), A529C (EL529C), A535C (EL535C), and E315C (EL315C) were created using standard site-directed mutagenesis techniques in the background of a cysteine-free version of GroEL (27). All GroEL variants were expressed and purified using a combination of previously described methods (5, 27, 28; see also supplemental “Methods”). Wild-type GroES, GroES98C, wild-type Rubisco and the various Rubisco Cys mutants were expressed and purified as previously described (5, 27, 28). Bovine rhodanese was purchased from Sigma and purified over a Sephacryl-300 column. The extent and quenching of free and chaperonin-bound fluorescent GroES was determined and quantified by PhosphorImager analysis (Amersham Biosciences Storm 860, Sunnyvale, CA) following TLC separation of free phosphate, ADP, and ATP (33, 34).

**Asymmetric GroEL-GroES Complex Formation and Protease Protection**—The binding of Rubisco to the trans ring of a GroEL-GroES complex was conducted by first creating an asymmetric GroEL-ADP-GroES complex (see supplemental “Methods”). For protease protection experiments, the asymmetric GroEL-ADP-GroES complex was mixed (100 nM GroEL and 350 nM GroES) with acrid-urea-denatured fluorescein-labeled Rubisco (Rub454F, 100 nM). The sample was then supplemented with either buffer or ATP (1 mM) and rapidly mixed (within ~5 s) with hexokinase (0.05 unit/μl) and glucose (20 mM). Protease K (0.05 μg/ml) was added to each sample, aliquots at each time point were removed, and the protease was deactivated with phenylmethylsulfonyl fluoride (0.2 mg/ml). Samples were then run on SDS-PAGE, and the level of undegested Rubisco was determined by quantifying the amount of intact and fluorescent Rubisco using a Storm 860 system. For the protease experiments shown in Fig. 3C, GroES binding was blocked at different times following the addition of ATP (1 mM) with excess SR1 (600 nM). In all cases, the ATP was depleted after 5 s with hexokinase and glucose. Samples were supplemented with proteinase K (0.9 μg/ml) for 2 min, and then separated and quantified.

**Gel Filtration**—Analytical gel filtration of chaperonin complexes was conducted with a Superose 6 (GE Healthcare Systems) column connected to a high-performance liquid chromatograph (Waters, Milford, MA) configured with an in-line fluorescence detector. All component mixing was performed in 50 mM Hepes (pH 7.6), 10 mM KOAc, 5 mM Mg(OAc)2, 2 mM dithiothreitol (Buffer A). For experiments examining the disassociation rate of GroES, the column running buffer was 50 mM Hepes, pH 7.6, 100 mM KOAc, 5 mM Mg(OAc)2, 200 μM ADP, 2 mM dithiothreitol. For both wtGroEL and EL43Py, excess unlabeled GroES (4 μM) was added to mixtures of each chaperonin containing fluorescently labeled GroES and ATP after 2 min of steady-state cycling. At various times, the ATP was rapidly quenched with hexokinase and glucose, and the samples were loaded onto the Superose gel-filtration column. The amount of free and chaperonin-bound fluorescent GroES was then measured.

**ADP Release**—ADP bullets with radiolabeled ADP in the cis ring were made by mixing GroEL (7 μM), GroES (14 μM), and radiolabeled ATP (245 μM, and 20 μCi of [α-32P]ATP) in Buffer A. The mix was incubated for 10 min at 25 °C to form ADP bullets (see supplemental “Methods”). The ADP bullets were then diluted to 100 nM and mixed with denatured Rubisco (100 nM) and incubated at 25 °C for 10 min to allow non-native Rubisco binding to the trans ring. Cold ATP (1 mM) or buffer was then added, and at varying times the solution was applied to a Zeba micro spin desalting column (Pierce) and centrifuged at maximum speed (16 × 103 relative centrifugal force) in a tabletop microcentrifuge for 4 s. The sample flow-through was analyzed with a scintillation counter (Wallac).

**Stopped-flow Fluorescence and Data Analysis**—Stopped-flow experiments were performed essentially as previously described (29), using an SFM-400 rapid mixing unit (BioLogic,
EL43Py is a chemically modified GroEL variant that folds proteins more slowly than wtGroEL (Fig. 1). A, the structure of the GroEL-GroES complex (14) is shown in cross-section with amino acid position 43 highlighted in yellow and the last crystallographically resolved amino acid at position 526 highlighted in red. The figure was prepared with PyMOL (DeLano, W. L. (2002) The PyMOL molecular graphics system. DeLano Scientific, San Carlos, CA). B, the structures of the reactive dyes N-(1-pyrene)maleimide (pyrene) and 5-(2-acetoamidothethyl)aminonaphthalene 1-sulfonate (AEDANS) are shown conjugated to the thiol side chain of Cys. C, the refolding of Rubisco by wild-type GroEL (wtGroEL), a single Cys mutant of GroEL (EL43C), pyrene-labeled EL43C (EL43Py), a second pyrene-labeled GroEL mutant (EL529Py), and an AEDANS-labeled GroEL mutant (EL43Ed) are shown. D, the refolding of rhodanese by the same GroEL variants is shown. Rubisco and rhodanese were denatured in acid-urea and mixed (100 nM) with the indicated GroEL variant (500 nM), GroES (1 μM), and ATP (2 mM). Error bars represent the standard deviation of n = 3 experimental replicates. Unless otherwise stated, all concentrations listed are post-mixing.

RESULTS

EL43Py Is a Fully Productive, but Slow, Protein-folding Machine—We recently carried out a targeted screen for amino acid positions at the base of the GroEL cavity that could be used to attach small fluorescent probes. We introduced a series of conservative Cys mutations into a cysteine-less GroEL background (27) at locations around the bottom of the GroEL cavity (Fig. 1A). Labeling one of these GroEL mutants (S43C) with the organic fluorophore N-1-pyrenemaleimide (Fig. 1B) serendipitously resulted in a GroEL variant (EL43Py) that demonstrated unexpected alterations in assisted protein folding. While EL43Py productively folds substrate proteins like Rubisco (Fig. 1C) and rhodanese (Fig. 1D), it does so much more slowly than wtGroEL.

We reasoned that a modified chaperonin like EL43Py might represent a powerful new tool for studying the mechanism of GroEL-mediated protein folding, especially given the ongoing debate about the importance of the GroEL C-terminal tails, which are located in a similar region at the base of the GroEL cavity (35–37). To establish the utility of EL43Py, we therefore examined whether the folding deficiency of EL43Py can be explained by a trivial and inhibitory interaction between exogenous dyes localized at the bottom of the GroEL cavity and non-native folding intermediates. We employed two strategies for this analysis: 1) conjugation of different, modestly hydrophobic dyes to the same EL43C position and 2) development of additional GroEL variants with labeled Cys residues at nearby but distinct positions within the GroEL cavity. When we attached a range of chemically and physically dissimilar substituents to position 43, most showed little or no alteration in assisted protein folding (see supplemental “Methods”). For example, modification of the 43C position with the moderately hydrophobic dye AEDANS (Fig. 1B), results in a GroEL variant (EL43Ed) that displays no significant alteration in Rubisco folding (Fig. 1C). More importantly, conjugation of pyrene to unique Cys residues at positions 529 and 535 results in chaperonins (EL529Py and EL535Py) that, while possessing pyrene dye rings at the base of the GroEL cavity similar to EL43Py, show no significant perturbation in Rubisco folding (Fig. 1C and supplemental Fig. S1). EL43Ed and EL529Py do display a somewhat reduced capacity to refold rhodanese (Fig. 1D), suggesting that the presence of the dyes can have a modest influence on the folding of certain proteins. However, this effect is relatively small in comparison to the more dramatic effects observed with EL43Py. Overall, these observations strongly suggest that the perturbed folding behavior displayed by EL43Py is not primarily caused by inhibitory interactions between the pyrene dyes and a substrate protein.

EL43Py Executes a Full Hydrolytic Reaction Cycle More Quickly Than GroEL—Because efficient protein folding by GroEL depends upon a carefully coordinated, ATP-driven reaction cycle (Fig. 2), we next examined how EL43Py binds and hydrolyzes ATP. Unexpectedly, EL43Py demonstrates a substantially elevated rate of steady-state ATP hydrolysis, both in
The elevated rate of steady-state ATP hydrolysis by EL43Py implies that the rate-limiting step of the EL43Py reaction cycle is different from that exhibited by wtGroEL (Fig. 2). To establish the nature of this difference, we first examined the intrinsic, single-turnover rate of ATP within the EL43Py-GroES cis complex. Using a rapid mixing, quench-flow apparatus, we directly measured the rate of ATP turnover for both the EL43Py-GroES and wtGroEL-GroES complex (Fig. 3B), employing only enough ATP to support one round of GroES binding and ATP hydrolysis (Fig. 2, Phase I). Remarkably, the $k_{cat}$ values observed for the EL43Py and GroEL cis complexes are essentially identical (0.11–0.13 s$^{-1}$ per active subunit) and are well matched to the values obtained from the steady-state hydrolysis data in supplemental Fig. S3, as well as measurements from other studies (5, 28, 38). Thus, the increase in steady-state ATP turnover by EL43Py cannot be due to a stimulation of intrinsic ATP hydrolysis within the cis complex.

We next examined the pre-steady-state ATP hydrolysis kinetics of EL43Py and wtGroEL, in the presence of GroES, and at levels of ATP sufficient to allow multiple rounds of GroES association and release (Fig. 3C). With wtGroEL, we observe a burst of ATP hydrolysis followed by a linear, steady-state reaction phase. The burst is a direct consequence of the rapid formation of a committed, GroEL-ATP-GroES cis complex (Fig. 2, Phase I), followed by the slow decay of the post-hydrolysis GroEL-ADP-GroES complex upon ATP binding to the trans ring and initiation of the steady-state cycle (Fig. 2, Phase II (9, 27, 38)). The pre-steady-state kinetics of ATP hydrolysis by EL43Py are distinct from wtGroEL. Although EL43Py also displays a burst of ATP hydrolysis that is followed by a linear steady-state reaction, the amplitude of the burst from EL43Py is smaller than that observed with wtGroEL while the linear phase is significantly faster (Fig. 3C). In the context of a simple two-step kinetic model that describes the pre-steady-state hydrolysis burst of GroEL (see supplemental “Methods”), these observations suggest that the rate-limiting transition that controls cis complex disassembly (Fig. 2, rate-limiting step) proceeds much more quickly with EL43Py than wtGroEL. This conclusion further predicts that EL43Py should release GroES more rapidly than wtGroEL in the course of a steady-state reaction cycle. We tested this prediction using an assay capable of tracking the dissociation rate of GroES during the course of a steady-state GroEL hydrolysis reaction (Fig. 3D). In strong sup-

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The GroEL ATPase cycle in the presence of GroES and absence of substrate protein is shown. The hydrolytic reaction is divided into two phases, where the first phase (Phase I) illustrates the pre-steady-state behavior of the system when apo-GroEL is first mixed with ATP and GroES. An apo-GroEL ring first fills cooperatively with ATP, followed by rapid binding of GroES (2, 3). The resulting asymmetric GroEL-ATP-GroES complex (‘ATP bullet’) hydrolyzes the bound ATP with an intrinsic turnover rate of 0.12 s$^{-1}$, yielding an asymmetric GroEL-ADP-GroES complex (an ‘ADP bullet’ (38)). When ATP is limiting, the reaction stops at the ADP bullet following a single hydrolytic turnover. In the presence of excess ATP, the binding of ATP to the open trans ring of the ADP bullet initiates the disassembly of the cis complex, leading to release of GroES and ADP (5, 9, 27). Nearly simultaneous binding of another GroES heptamer (indicated by the brackets) results in the formation of a new cis complex, regenerating the ADP bullet upon hydrolysis of the ATP within the cis complex (5, 9, 27). The steady-state cycling between ATP and ADP bullets constitutes the second phase of the reaction (Phase II), and disassembly of the ADP-bound cis complex is the rate-limiting step of this cycle in the absence of substrate protein (27, 38). Under in vivo conditions, the GroEl-GroES system persists almost exclusively in the steady-state cycle (Phase II).

- Phase I: ATP binding occurs to only one ring of the tetradecamer complex (38–40), EL43Py displays a positive cooperative response to ATP that is very similar to that exhibited by wtGroEL (supplemental Fig. S3). At low ATP concentrations, where ATP binding occurs to only one ring of the tetradecamer complex (38–40), EL43Py displays a positive cooperative transition that is very similar to wtGroEL. EL43Py also displays changes in ATP hydrolysis at higher concentrations of ATP that are similar to wtGroEL (supplemental Fig. S3), whereas EL43Py displays a consistently higher steady-state rate of ATP turnover at high ATP concentrations. Overall, these observations suggest that ATP binding, and the attendant structural changes that lead to hydrolysis, are similar in EL43Py and wtGroEL, but that EL43Py completes a cycle of ATP hydrolysis (Fig. 2, Phase II) more quickly than wtGroEL.

- Phase II: The pre-steady-state kinetics of ATP hydrolysis by EL43Py are distinct from wtGroEL. Although EL43Py also displays a burst of ATP hydrolysis that is followed by a linear steady-state reaction, the amplitude of the burst from EL43Py is smaller than that observed with wtGroEL while the linear phase is significantly faster (Fig. 3C). In the context of a simple two-step kinetic model that describes the pre-steady-state hydrolysis burst of GroEL (see supplemental “Methods”), these observations suggest that the rate-limiting transition that controls cis complex disassembly (Fig. 2, rate-limiting step) proceeds much more quickly with EL43Py than wtGroEL. This conclusion further predicts that EL43Py should release GroES more rapidly than wtGroEL in the course of a steady-state reaction cycle. We tested this prediction using an assay capable of tracking the dissociation rate of GroES during the course of a steady-state GroEL hydrolysis reaction (Fig. 3D). In strong sup-
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port of our model, the rate of GroES release from EL43Py is 2.6-fold faster than that observed with wtGroEL, an increase that is very similar to the 2.3-fold increase in the steady-state hydrolysis rate of EL43Py in the presence of GroES.

EL43Py Fully Supports Protein Capture and Encapsulation Beneath GroES—EL43Py thus displays two key characteristics: 1) a considerably faster hydrolytic cycle and 2) a substantially slower rate of assisted protein folding. The behavior of EL43Py could thus be interpreted as satisfying a key prediction of one model of GroEL action, in which the primary stimulatory action provided by GroEL derives from substrate protein confinement within the GroEL-GroES cavity. A faster reaction cycle in this model should result in a reduced cis-cavity lifetime and therefore slower folding. The strength of this conclusion, however, rests upon whether EL43Py: 1) captures non-native substrate protein, 2) encapsulates the substrate protein beneath GroES, and 3) releases the substrate protein into the GroEL-GroES cavity as efficiently as wtGroEL. We therefore examined whether EL43Py correctly binds non-native substrate protein. Because the open trans ring of a GroEL-ADP-GroES complex (an "ADP bullet") captures the vast majority of the non-native substrate proteins of GroEL in vivo (8, 27, 41), we tested whether an EL43Py ADP bullet can capture non-native Rubisco. Based on both gel filtration and protease protection assays (Fig. 4A, 4B, and 4C), EL43Py displays no observable defect in its ability to bind non-native Rubisco. Additionally, the extent of binding-driven, passive unfolding of the Rubisco monomer (29) is the same for both EL43Py and wtGroEL (Fig. 4B). EL43Py thus displays no deficiency in substrate protein binding, and the average conformational states of the bound substrate protein on an EL43Py and wtGroEL ring are similar.

We next examined the capacity of an EL43Py ring to support non-native substrate protein encapsulation beneath GroES. Fluorescently labeled, non-native Rubisco bound to the trans ring of an ADP bullet, made from either wtGroEL or EL43Py, is highly susceptible to rapid proteolysis (Fig. 4C) (31). However, when additional GroES and ATP are added, followed by rapid enzymatic depletion of the ATP to prevent cycling and allow only a single round of GroES binding to the substrate-occupied trans ring, the non-native Rubisco becomes resistant to proteolysis (Fig. 4C). Notably, EL43Py appears to be as efficient in GroES capture and Rubisco encapsulation as wtGroEL (Fig. 4D). Based upon these and other protease protection experiments (see supplemental "Methods"), as well as complementary gel filtration measurements (data not shown), GroES encapsulation of non-native Rubisco on the trans ring of EL43Py bullets is 80–95% efficient within the 45- to 60-s window of these measurements.

EL43Py Fails to Release Substrate Protein into the GroEL-GroES Cavity—We next examined the ability of EL43Py to release non-native Rubisco in the presence of ATP and GroES. If EL43Py executes normal cycles of substrate protein binding and release (9, 10), the addition of excess wtGroEL to an EL43Py reaction should out-compete the EL43Py and facilitate normal folding. Strikingly, the addition of a large excess of wtGroEL to an EL43Py-Rubisco folding reaction does not result in normal Rubisco folding (Fig. 5A). This observation suggests that EL43Py does not correctly execute a normal GroEL cycle of substrate protein binding and release.

To more directly examine substrate protein release with EL43Py, we developed a single round encapsulation and release assay based on a fluorescently labeled Rubisco variant whose conformational state can be monitored by fluorescence reso-
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nance energy transfer (FRET, Fig. 5B) (29, 31). Release of the Rubisco monomer into the enclosed GroEL-GroES cavity is observable as a distinctive FRET signature in single round encapsulation experiments (29, 31). Following encapsulation and compaction of the non-native Rubisco intermediate on the trans ring of a wtGroEL bullet complex, the release and folding of the Rubisco monomer can be detected as an increase in distance (decrease in FRET efficiency) between the labeled domains of the monomer as it matures inside the wtGroEL-GroES complex (Fig. 5, B and C). When the same experiment is conducted with EL43Py, by contrast, even complete GroES binding does not lead to substrate release and folding, as shown by the persistently high FRET signal observed when Rubisco is enclosed in the EL43Py-GroES complex (Fig. 5C). This result is consistent with a failure of EL43Py to release the vast majority of the non-native Rubisco from the cavity walls despite full substrate encapsulation following ATP and GroES binding.

EL43Py Only Slowly Executes a Key ATP-driven Conformational Change—The ATP-driven conformational shift of the EL43Py ring thus appears to stall at a critical point following GroES binding. Completion of this step seems to be central to the ability of GroEL to trigger protein release and folding. Previous kinetic studies suggested that an ATP-saturated GroEL ring populates an intermediate conformation that simultaneously binds substrate protein and GroES (25). EL43Py could thus provide additional evidence for the existence of this state, as well as offer the opportunity to trap and study this critical and otherwise fleeting intermediate conformation in detail. We therefore sought to determine whether the allosteric transitions of an EL43Py ring differ from those of wtGroEL. Because we previously demonstrated that the conformation of a bound Rubisco monomer is linked to the conformational state of the GroEL ring (29, 31), we employed labeled Rubisco in a FRET-based assay to examine the conformational shift of the wtGroEL and EL43Py rings (Fig. 6A). Upon ATP and GroES binding to the Rubisco-occupied trans ring of a wild-type ADP bullet, we observed an initial decrease in
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The GroES binding and substrate encapsulation on an EL43Py trans ring requires more time to reach completion than does GroES binding to a wtGroEL trans ring (Fig. S4). Second, experiments that directly probe the conformational shift of the chaperonin ring by following changes in pyrene fluorescence suggest that the ATP-driven allosteric transition of the EL43Py ring is slowed (supplemental Fig. S5). Importantly, pyrene fluorescence has previously been used to map the allosteric transitions of GroEL (38). At low pyrene substitution ratios, where alterations in assisted protein folding and ATPase rate cannot be detected (supplemental Fig. S1), ATP-induced changes in pyrene fluorescence are very rapid. However, these same fluorescence changes become significantly slower in the fully labeled EL43Py (supplemental Fig. S5). Additionally, a rise in pyrene fluorescence observed at low pyrene substitutions, most likely reflecting a subsequent structural transition of the GroEL ring, appears to fail completely at high pyrene substitution (supplemental Fig. S5).

ATP Hydrolysis by a Newly Formed GroEL-GroES Complex on a trans Ring Is Delayed—Our observation of slow GroES binding to the EL43Py trans ring raises an important question: if a GroEL ring commits to hydrolysis immediately after binding ATP, why does substrate protein encapsulation with EL43Py succeed at all? For GroES to bind to a GroEL ring and encapsulate a large substrate protein like Rubisco, the ring must remain filled with ATP (2, 3). Thus, if ATP hydrolysis proceeds unimpeded, and the rate of GroES binding is slowed sufficiently, then GroES binding should fail as the EL43Py ring prematurely depletes its store of ATP. However, despite a substantial slowing in the rate of GroES binding, the EL43Py trans ring

The shallower FRET decrease seen with EL43Py could, importantly, be the result of a slowing in the forced conformational expansion of Rubisco on the EL43Py ring. With wtGroEL, the initial unfolding phase, and its associated decrease in FRET, occurs much faster than GroES binding, which causes an increase in FRET. The two steps are kinetically separable and therefore fully observable with wtGroEL. In the case of EL43Py, however, if the step that leads to substrate unfolding slows and proceeds at a rate that is similar to the rate that GroES binds, then the average change in FRET efficiency will be much lower, because the drop in FRET caused by unfolding in a subset of the population would be simultaneously canceled by the binding of GroES to another subset. If correct, this implies that the ATP-driven conformational transition of the GroEL ring that results in forced protein unfolding precedes, and is required for, GroES binding. To test these ideas, we conducted a series of experiments where the consequences of ATP binding alone to the substrate-occupied trans ring can be observed (Fig. 6D). For these experiments, we prevented substrate protein encapsulation by adding a large excess of a single ring version of GroEL (SR1) as a GroES trap (8). SR1 efficiently binds both ATP and GroES but cannot release the bound GroES under the conditions of this experiment. In contrast to the simple, fast change observed with wtGroEL, the FRET signal measured with EL43Py displays a biphasic decrease upon ATP binding (Fig. 6D). The most rapid phase occurs at a rate very similar to wtGroEL (t1/2 = 0.2 s), whereas the slowest phase, constituting ~65% of the total observed amplitude, proceeds much more slowly (t1/2 = 13.9 s). These observations strongly suggest that roughly two-thirds of the EL43Py bullet population only slowly completes the ATP-driven allosteric transition that unfolds the substrate protein and permits GroES binding. Two additional observations are consistent with this conclusion. First, GroES binding and substrate encapsulation on an EL43Py trans ring requires more time to reach completion than does GroES binding to a wtGroEL trans ring (Fig. S4).

the presence of either wtGroEL or EL43Py (250 nM) in both cases, with 500 nM wtGroEL (1.25 M). Folding was initiated by the addition of ATP (2 mM) and excess GroES (3 μM, EL43Py rescue). The refolding curves of Rubisco (100 nM) in the presence of either wtGroEL or EL43Py (250 nM) are shown for reference. B, schematic of a FRET experiment designed to examine conformational changes in Rubisco following a single round of GroES binding to the trans ring of an ADP bullet. Fluorescently labeled, non-native Rubisco (29, 31) was first bound to the trans ring of an ADP bullet in the presence of excess GroES. Subsequent addition of ATP permits GroES binding and Rubisco encapsulation. To prevent cycling and permit long time observation of the complex, the excess ATP was quenched with hexokinase and glucose (Hex/Glc) ~ 4 s after the addition of ATP. C, the change in FRET efficiency was monitored as a function of time following GroES binding to the Rubisco-occupied trans ring of wtGroEL and EL43Py ADP bullets. Rubisco-bound ADP bullet complexes in the presence of excess GroES were manually mixed (60 nM) with ATP (1 mM) in a standard fluorometer cuvette with magnetic stirring. Excess ATP was then quenched with hexokinase and glucose to prevent cycling. This mixing sequence precludes observation within the first ~8 s of the experiment.


FIGURE 6. EL43Py only slowly completes the ATP-driven transition that causes forced substrate unfolding. A, schematic of a stopped-flow FRET experiment designed to examine conformational changes in non-native Rubisco upon encapsulation beneath GroES on the trans ring of an ADP bullet (31). B, ATP and GroES binding results in the forced unfolding and compaction of the Rubisco monomer. Rubisco-bound ADP bullet complexes in the presence of excess GroES were rapidly mixed in a stopped flow (60 nM) with ATP (1 mM). Each FRET trace is the average of n = 10 replicates of matched experimental pairs, calculated from donor-only (Rubisco 454-D) and donor-acceptor (Rubisco 454-D/58-A) samples. The inset shows the FRET change over the first second of data. The wtGroEL data shown are from Ref. 31 and are re-plotted here for reference. C, schematic of a stopped-flow FRET experiment designed to follow conformational changes in a substrate protein upon the binding of ATP alone to the trans ring (31). For this experiment, an excess of the single ring GroEL variant SR1 is used as a GroES trap (8). D, the allosteric transition triggered by ATP binding that causes forced unfolding proceeds slowly on an EL43Py trans ring. Stopped-flow FRET experiments were conducted in essentially the same manner as in B, except that SR1 was present in a 20-fold excess (1.2 μM). The inset shows the FRET change over the first second of data. The decrease in FRET efficiency, reflecting an increase in the intra-probe distance, was fit to a double exponential rate law for EL43Py with rate constants of \( k_{\text{off}} = 2.9 \pm 0.4 \text{ s}^{-1} \) and \( k_{\text{slow}} = 0.05 \pm 0.02 \text{ s}^{-1} \) for EL43Py. The wtGroEL data shown are from Ref. 31 and are re-plotted here for reference.

displays no loss in its ability to bind GroES (Figs. 4 and supplemental Fig. S4). This observation implies that ATP hydrolysis by a GroEL trans ring is inhibited or slowed until assembly of a new cis ternary complex is complete. The observation that the stretched conformation of the Rubisco monomer on an ATP-bound, wtGroEL trans ring is stable for 7–10 s (Fig. 6D) is also consistent with retarded ATP hydrolysis. Ten seconds is enough time for the majority of the bound ATP to hydrolyze to ADP at the intrinsic turnover rate of a GroEL ring (Fig. 3B). Hydrolysis should lead to a relaxation of the ring and a change in Rubisco conformation. By contrast, the stretched Rubisco conformation stably persists for several seconds, suggesting that the rate of ATP hydrolysis by the trans ring is reduced. We tested this prediction by examining the pre-steady-state kinetics of ATP hydrolysis by the ADP bullet trans ring (Fig. 7). To push the ADP bullet through the rate-limiting transition of the GroEL cycle (27), we saturated the trans ring with non-native Rubisco prior to the addition of ATP. Upon the addition of ATP, we observe a near total loss of the pre-steady-state burst of ATP hydrolysis from the ADP bullet trans ring (Fig. 7C). For apo-GroEL, the burst of hydrolysis is caused by slow decay of the post-hydrolysis GroEL-ADP-GroES complex following the faster step of phosphodiester bond cleavage (Fig. 2) (9, 27, 38). However, the nearly linear pre-steady-state behavior seen with the ADP bullet strongly suggests that ATP hydrolysis by the ADP bullet trans ring is, at a minimum, dramatically slowed in comparison to hydrolysis by an apo-GroEL ring.

**DISCUSSION**

Using a novel GroEL variant, we have examined the sequence of events that lead to substrate protein release and folding inside the GroEL-GroES cis cavity (Fig. 8). We have shown that a chemically modified variant of GroEL, EL43Py, binds non-native substrate protein, ATP, and GroES and initiates a substrate protein encapsulation reaction. EL43Py fails, however, to efficiently complete the steps of the process needed to trigger substrate protein release and folding, allowing us to examine a key but poorly understood allosteric state of the GroEL ring. EL43Py executes a series of non-productive and nearly futile GroES binding and release cycles that only rarely result in substrate protein release and folding. The failure of the EL43Py ring to populate the folding-competent conformation of the cis ring, in addition, bypasses the slowest step in the GroEL reaction cycle (GroEL release from a folding competent, ADP-bound asymmetric complex (27, 38)), resulting in a faster chaperonin cycle and an elevated rate of steady-state ATP turnover (Figs. 3 and 8). The perturbations exhibited by EL43Py further allowed us to identify a central and previously unrecognized role for ADP dissociation in controlling the progression of the GroEL reaction cycle.

**Chemical Modification and Altered Allostery in GroEL**—Our results with EL43Py highlight the utility of tethering small molecules to a target protein as a method for probing complex allosteric systems. The site-specific chemical attachment of exogenous functional groups offers a much wider array of physical and chemical moieties than afforded by standard mutagenesis. Indeed, this basic idea has been developed over the last several years into a rational drug discovery strategy that has yielded a set of novel small molecules that allosterically modulate the activity of several caspases (42–44). Although the creation of EL43Py was serendipitous, this modified chaperonin has nonetheless provided several very important insights into the complex allostery of GroEL. We do not currently understand how the addition of particular substituents at position 43 causes the observed alterations of the allosteric transitions of GroEL. The dye moiety at position 43 is relatively distant from the GroEL ATPase site, and the GroEL equatorial domains overall display only small conformational shifts upon binding ATP (14, 45). However, the loop that contains position 43 does
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Substrate Protein Encapsulation by GroES—The observation that GroES binding can be uncoupled from substrate protein release suggests that at least two intermediate allosteric states of the ATP-saturated GroEL-GroES complex are necessary to trigger protein folding (Fig. 8). The binding of ATP initiates the movement of the GroEL apical domains, permitting GroES binding and substrate protein encapsulation. However, this initial movement is not sufficient to drive substrate protein release. Following GroES binding, a second structural transition of the ATP-loaded GroEL ring takes place, resulting in tight GroES binding and ejection of the substrate protein into the GroEL-GroES cavity. Although the structural details of these transitions remain unclear, our observations with EL43Py are consistent with a recent kinetic analysis of a GroEL mutant containing a strategically engineered engineered Trp mutation (25).

In this study, we found that the encapsulation of a substrate protein is associated with both forced unfolding and compaction of the non-native folding intermediate (29, 31). However, these conformational changes could not be unambiguously assigned to specific conformational states of the GroEL machine. The alterations introduced by EL43Py make these assignments possible. The ATP-driven forced unfolding of the Rubisco monomer on both wtGroEL and EL43Py rings occurs faster than GroES binds. Additionally, completion of this step is necessary for GroES binding and substrate encapsulation (Fig. 6 and supplemental Fig. S4). Using the notation suggested by Clift et al. (25), our observations suggest that forced substrate unfolding is caused by the transition between the R1 and R2 states of the GroEL ring (Fig. 8). We also observe that the Rubisco monomer is compacted upon GroES binding to both wtGroEL and EL43Py. However, because the Rubisco intermediate is not released into the cis cavity on an EL43Py ring, the observed Rubisco conformational change display a shift (~5 Å) when a GroEL ring moves between the cis and trans conformations (supplemental Fig. S6). Additionally, this loop bridges two strands of a β-sheet that forms part of an important intra-ring equatorial interface (14, 45). This intrasubunit β-sheet displays a 4- to 5-Å shift in the trans ring when the cis ring is occupied by ATP in comparison to ADP (45). If these structural changes are important to the allosteric control of GroEL’s reaction cycle, it seems reasonable that exogenous dye modification at position 43 could slow or inhibit aspects of this conformational transition. Indeed, several different GroEL variants with modifications of the C-terminal flexible tails, which localize to a similar part of the GroEL cavity, also induce substantial perturbations in assisted protein folding and ATP turnover (35–37). In the end, a more detailed structural analysis will be necessary to fully address how modifications at position 43 result in the altered folding and ATPase behavior we observe.

FIGURE 7. ATP hydrolysis by a newly formed GroEL-GroES complex on a trans ring is delayed by the slow release of ADP from the opposite ring. The steps involved in the pre-steady-state hydrolysis of ATP by apo-GroEL (A) and the trans ring of an ADP bullet (B) are illustrated. In both cases, the pre-steady-state steps are followed by the same steady-state cycle. C, the pre-steady-state kinetics of ATP hydrolysis by apo-GroEL and the GroEL ADP bullet trans ring are shown. In both cases, GroEL samples (1 μM) were mixed with 1 μM acid-urea-denatured Rubisco and incubated for 10 min at room temperature prior to the addition of radiolabeled ATP (250 μM). GroES was present at a total concentration of 3.5 μM for both reactions. Error bars show the standard deviation of n = 3 experimental replicates. The rates of the linear, steady-state phase of the hydrolysis reactions are very similar (~0.02 s⁻¹ per active subunit). However, in comparison to the apo-GroEL sample, the pre-steady-state burst for the ADP bullet sample is dramatically reduced and only just detectable. D, the rate of GroES release from an ADP bullet cis complex was monitored with a previously described FRET assay employing fluorescently labeled GroEL and GroES (27). Non-native Rubisco was first bound to the trans ring of ADP bullets formed from donor-labeled GroEL and acceptor-labeled GroES. The Rubisco-bound ADP bullets (50 nM) were then mixed with a 20-fold excess of unlabeled GroES (4 μM) in a stopped-flow in the absence (+ dRub and – ATP) or presence (+ dRub and + ATP) of 500 μM ATP. Dissociation of the labeled GroES was monitored as a loss in FRET efficiency. The FRET traces shown represent the average of n = 14 experimental replicates of matched donor-only and donor-acceptor experiments. The rate of ADP release from the cis complex was monitored using radiolabeled ADP (iso32P)ADP. The trans ring of wtGroEL ADP bullets containing 32P-labeled ADP in the cis cavity was saturated with non-native Rubisco and then briefly supplemented with either buffer (+ dRub and – ATP) or 1 mM ATP (+ dRub and + ATP). The sample was then applied to a rapid spin column that separated free from bound ADP in 4 s. Scintillation counting was employed to monitor the amount of GroEL-bound, radiolabeled ADP at each time point. Error bars show the standard deviation of n = 3 experimental replicates.
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FIGURE 8. The stages of the protein folding trigger of GroEL. A simplified reaction cycle is shown (left), illustrating the sequential progression of allosteric states (R1, R2, and R3) of the ATP-saturated GroEL cis ring required to trigger protein unfolding. Initial binding of ATP leads to population of the R1 state. The R1 state was not directly observed in the current work but has been previously described (25). The transition from the R1 to the R2 state, involving the directed movement of the GroEL apical domains, is responsible for forced substrate unfolding (1). The movement of the apical domains also permits GroES binding and the subsequent compaction of the non-native protein (2). The R2 state simultaneously binds the substrate protein and GroES (2 and 3). Following GroES binding to the R2 state, the GroEL-GroES complex undergoes an additional transition to the R3 state, which releases the substrate protein into the stable GroEL-GroES cavity and initiates folding. ATP hydrolysis in the cis ring is delayed by an event on the opposite GroEL ring, most likely ADP release, allowing GroES binding to reach completion. The stages of substrate, ATP, and GroES binding for EL43Py are similar to wtGroEL. However, the EL43Py ring only rarely populates the R3 state, usually stalling in R2 (3, left arrow). Because the bound ATP is committed to hydrolysis, and these EL43Py rings usually fail to populate the folding-competent allosteric state of the GroEL ring (R3), the slowest step of the GroEL steady-state ATPase cycle (GroES cis complex) is usually bypassed by EL43Py, resulting in faster steady-state ATP hydrolysis and much less efficient folding.

must occur as a direct result of simple GroES binding before the protein is released. The compaction of the Rubisco monomer appears, therefore, to reflect something akin to an actual compression event and not a relaxation or “snapping back” of an expanded substrate protein upon its release into the cis cavity.

Synchronizing ATP Hydrolysis and Protein Release—Our study of EL43Py also sheds light on how GroEL’s folding trigger is coordinated with the overall progression of the chaperonin ATPase cycle. It has been suggested that the transition from the R2 to the R3 state of the ATP-saturated GroEL ring is responsible for the release of the substrate into the enclosed GroEL-GroES cavity (25). Our observations with EL43Py are consistent with this model and indicate that EL43Py stalls in the R2 state, rarely making the transition to the R3 state. Strikingly, despite failing to populate the R3 state, ATP hydrolysis by an EL43Py ring appears to proceed normally (Fig. 3B). This observation suggests that the cis ring ATP commits to phosphodiester bond cleavage prior to population of the R3 state. Our observations with EL43Py are thus not consistent with the GroEL cycle being controlled by a double timer as has been suggested (26). The two-timer model was formulated as a way to explain how GroES binding to an open GroEL ring could occur without premature substrate protein release. This model proposes that ATP hydrolysis is not possible until the R3 transition is complete and the substrate protein is released into the GroEL-GroES cavity. However, EL43Py rarely populates the R3 state but hydrolyzes ATP with single-turnover kinetics that are identical to wtGroEL (Fig. 3B), suggesting that commitment to ATP hydrolysis is achieved prior to formation of the R3 state of the ring, most likely in either the R1 or R2 states.

Nonetheless, we do find strong evidence that the initiation of ATP hydrolysis by a newly formed cis complex is subject to a key delay during a steady-state reaction cycle. The ability of the EL43Py trans ring to complete GroES binding, despite a slowing of the transition that makes this binding reaction possible (Figs. 4, 6, and S4), suggests that ATP hydrolysis is substantially slowed on a trans ring that is in the process of forming a new cis complex. The dramatic reduction in the pre-steady-state burst of ATP hydrolysis by a wtGroEL trans ring also strongly supports this conclusion (Fig. 7).

How could such a slowdown in hydrolysis be encoded? In the course of a typical GroEL reaction cycle, the assembly of a new GroEL-GroES complex on a trans ring is linked to the ejection of GroES and ADP from the other ring, because the cis cavity left over from the previous phase of the cycle is dismantled (27). Binding of both ATP and non-native substrate protein to the trans ring causes GroES to be released from the other GroEL ring very rapidly (27). Additionally, ADP bound to one GroEL ring has been shown to be an effective non-competitive inhibitor of ATP hydrolysis on the other ring (46). Thus if ADP release is delayed behind GroES and substrate protein release by even a few seconds, ADP release could function as a simple and effective throttle, slowing the rate of ATP hydrolysis by the other ring. Under our experimental conditions, we observe this effect as a dramatic slowing of ATP hydrolysis by a newly formed cis complex (Fig. 7C). At higher and more physiologic ADP concentrations, where non-competitive inhibition of ATP hydrolysis is stronger (46), ADP could function more like a true hydrolysis delay gate.

To test this model, we examined the rate of both GroES and ADP release from an ADP bullet under the same conditions (Fig. 7D). Using a previously described FRET assay (27), we first confirmed that binding of non-native Rubisco and ATP to the trans ring of an ADP bullet causes rapid (t1/2 ~ 0.5 s) dissociation
of GroES from the opposite ring. Under the same conditions, however, the release of ADP from the decaying cis complex is substantially slower, with a $t_{1/2}$ of 15–20 s (Fig. 7D). These observations strongly support the model we propose (Fig. 8). Furthermore, they suggest a central role for the dissociation of ADP in controlling the GroEL reaction cycle. A delay in ATP hydrolysis imposed by the slow release of ADP from the opposite ring also guarantees that ligand binding to the trans ring occurs much more rapidly than ADP is released, and ADP (Fig. 7) (27). Thus, because Rubisco binding to an open trans ring before ATP binds. A detailed kinetic study of GroES release from an ADP bullet in the presence of different concentrations of ADP and non-native substrate protein also strongly supports these conclusions.4

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