Setting the chaperonin timer: A two-stroke, two-speed, protein machine

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In a study of the timing mechanism of the chaperonin nanomachine we show that the hemicycle time (HCT) is determined by the mean residence time (MRT) of GroES on the *cis* ring of GroEL. In turn, this is governed by allosteric interactions within the *trans* ring of GroEL. Ligands that enhance the R (relaxed) state (residual ADP, the product of the previous hemicycle, and K^+) extend the MRT and the HCT, whereas ligands that enhance the T (taut) state (unfolded substrate protein, SP) decrease the MRT and the HCT. In the absence of SP, the chaperonin machine idles in the resting state, but in the presence of SP it operates close to the speed limit, set by the rate of ATP hydrolysis by the *cis* ring. Thus, the conformational states of the *trans* ring largely control the speed of the complete chaperonin cycle.

allostery | GroEL | timing mechanism | GroES | FRET

Protein machines and their man-made, macroscopic counterparts share several common attributes, e.g., concerted, coordinated movements, cyclical operation, and energy transduction. These machines are seldom reversible because each cycle generally involves at least one irreversible step, e.g., the consumption of fuel. Often these machines operate at variable speed, a plethora of timing devices adjusting the cycle speed in response to demand.

An exemplary bipartite protein machine is the chaperonin system, typified by GroEL and GroES from *Escherichia coli*. GroEL is composed of 2 heptameric rings, stacked back to back, which, in the presence of GroES, operate out of phase with one another in the manner of a 2-stroke, reciprocating motor (1, 2). Driven by the hydrolysis of ATP, the chaperonin proteins function as a biological simulated annealing machine (3, 4), optimizing the folding of their substrate proteins (SPs) whose passage to biologically functional conformations is thus assured. A large body of literature dealing with many mechanistic and structural aspects has accumulated (for reviews, see refs. 5–11). However, despite this progress, surprisingly little is known about the timing mechanism of the chaperonin machine (however, see ref. 12). Here, we explore the location and operation of this timing device. We show that the timer is located on the *trans* ring and that it is regulated by allosteric transitions responsive to the nucleotides ADP and ATP, the potassium ion, and SPs.

The subunits of the chaperonin machine display 2 forms of cooperativity: positive cooperativity between the subunits of 1 ring and negativity cooperativity between the subunits of different rings. This is well described by the nested model of allostery, extensively applied to the chaperonin system by Horovitz and colleagues (8). Briefly, ATP is bound and hydrolyzed preferentially, but not exclusively, to the relaxed (**R**) conformation. The influence of K^+ is paradoxical. On one hand, it is needed for ATP hydrolysis in the steady state (13). However, at saturating concentrations of ATP, the rate of ATP hydrolysis is inversely proportional to the K^+ concentration: the higher the K^+ concentration, the lower the rate of ATP hydrolysis (14, 15). The third allosteric ligand, SP, binds preferentially to the taut (**T**) state. In the presence of saturating SP, the rate of ATP hydrolysis is stimulated 15- to 20-fold, and the inhibitory effects of K^+ at saturating ATP concentrations are largely suppressed.

At 30 °C, in the presence of the complete chaperonin machinery, GroEL and GroES, in the presence of saturating ATP and 100 mM K^+, but in the absence of SP, ATP is hydrolyzed at $\approx$0.5 min^{-1}. The chaperonin cycle, comprising 2 hemicycles, thus requires $\approx$2 min to complete. Under otherwise identical conditions, but in the presence of saturating SP, ATP is hydrolyzed at $\approx$9 min^{-1}. The hemicycle time (HCT) is thus reduced to $\approx$3–4 s.

In this article, we define the resting state of the chaperonin machine as the asymmetric complex comprising ($GroEL^{cis}$-[ADP]-[GroES]–$GroEL^{trans}$-ADP), where the square brackets indicate ligands that are not free to exchange with unbound ligand (1). We show that in the absence of SPs, the machinery spends almost all of the time in the resting state, i.e., the mean residence time (MRT) of the GroES bound to the *cis* ring of GroEL approaches the steady-state HCT ($\approx$60 s). In the absence of ADP on the *trans* ring, the MRT of *cis*-bound GroES is reduced 10,000-fold. In the presence of SPs, the MRT of *cis*-bound GroES is well beneath the steady-state HCT ($\approx$3–4 s). In the accompanying article (29), we show that in the presence of SP the chaperonin machinery operates in the steady state at very close to the speed limit, which is set by the rate at which fuel (ATP) can be consumed in the *cis* ring.

Results and Discussion

Dissociation of GroES from the *Cis* Ring. An important step in the chaperonin hemicycle is the dissociation of the ligands GroES and ADP from the *cis* ring in response to the binding of ATP to the *trans* ring (1, 16). Stopped-flow fluorescence energy transfer (FRET) is a useful method to follow this event (17, 18). Following Rye (17), we have inserted cysteines by directed mutagenesis of Glu-315 on the external surface of the apical domain of wild-type GroEL and at position 98 at the C terminus of GroES. GroEL E315C was modified with IAEDANS to varying degrees of saturation creating a FRET donor $GroEL^D$ (17). The FRET acceptor probe was introduced on Cys-98 of GroES by reaction with fluorescene-5-maleimide to create the FRET acceptor $GroES^A$ (17).

The asymmetric complex ($GroEL^{cis}$-[ADP]-[GroES]–$GroEL^{trans}$-ADP), resting-state 1 (Fig. 1), to which the system

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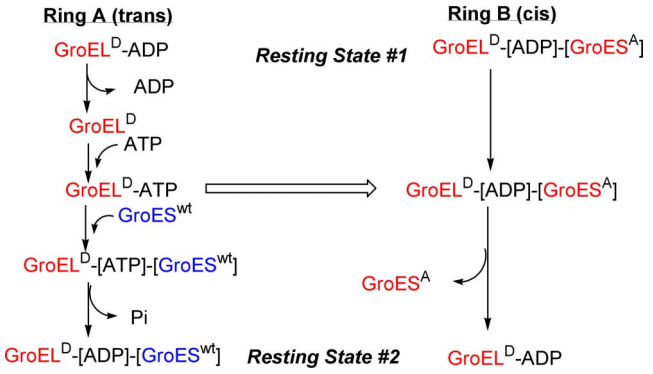

Fig. 1. In a single hemicycle, 7 of the 14 subunits hydrolyze ATP once, and the system progresses from resting-state 1, defined as the state to which the system reverts when the ATP is exhausted, to the reciprocal resting-state 2. In the dissociative FRET experiments, a FRET donor (D)—acceptor (A) pair GroEL^{D}-[ADP]-GroES^{A} was challenged with ATP and a 10-fold molar excess of wild-type GroES^{WT}. The experiment was initiated with the resting-state asymmetric complex containing ADP bound to the *trans* ring (i.e., GroEL^{D}-ADP) or asymmetric complex devoid of *trans* ring ADP (i.e., GroEL^{D}). Binding of ATP to the *trans* ring triggers ring-to-ring signaling ($\Rightarrow$) and the dissociation of the FRET acceptor GroES^{A} from the *cis* ring and permits the association of non-FRET GroES^{WT} with the *trans* ring, which, after ATP hydrolysis, leads to the formation of the reciprocal resting-state 2. Steady-state analysis of ATP hydrolysis by GroEL in the presence of GroES (30 °C, 100 mM K^+, and 1 mM ATP) gives a mean hemicycle time in the absence of SP of 61 s. Initiating with *trans* GroEL^{D}-ADP yields an MRT for the release of *cis* GroES of ≈50s, whereas initiating with *trans* GroEL yields an MRT of 6 ms.

reverts when ATP is exhausted, was prepared. The *cis* ring of the resting state is defined as GroEL^{D}-[ADP]-[GroES^{A}], where the square brackets around the ligands indicate that they are not free to exchange with unbound ligand, whereas the *trans* ring is defined as GroEL^{D}-ADP, where the absence of square brackets indicates that the ligand is free to exchange with unbound ligand. The resultant FRET complex was either used directly as the resting state (i.e., without removing the exchangeable ADP bound to the *trans* ring) or subjected to gel filtration that removes both the free ADP and the exchangeable ADP bound to the *trans* ring. When the FRET complex, with ADP bound to the *trans* ring, was mixed with ATP and a 10-fold excess of GroES^{WT}, a complex, multiphasic transition with a half-time of ≈50 s was observed in the FRET signal, attributable to the release of GroES^{A} from the *cis* ring (Fig. 2*A*, blue). This is similar in some respects to that reported, the differences being largely caused by the different conditions used: temperature, [K^+], and possibly the degree to which contaminating SP was removed. When repeated using GroEL^{WT} and His-tagged GroES^{His}, the discontinuous kinetics tracked the FRET signal (Fig. 2*A*), showing that neither the mutagenesis nor the introduction of the bulky FRET probes influenced the kinetics of *cis* GroES release. Because the kinetics are complex and multiphasic, we will refer to the half-time for release of *cis* GroES as the MRT. In a variation of the above experiment in which we use a 10-fold molar excess of GroES^{WT} over *cis*-bound GroES^{A} to minimize reassociation of the FRET pair (Fig. 2*A*, blue), we have also used a 10-fold molar excess of GroEL^{WT} over GroEL^{D} to accomplish the same end (Fig. 2*A*, green). The MRT of *cis*-bound GroES is slightly longer at ≈70 s with excess GroEL^{WT} than when measured with excess GroES^{WT}. The reason for this discrepancy is not obvious.

When free ADP and ADP bound to the *trans* ring of the resting-state complex was first removed by gel filtration before being challenged with ATP and excess GroES^{WT}, the *cis* GroES^{A} was released as before but on a dramatically abbreviated time scale (Fig. 2 *A*, red, and *B*, black). The MRT is reduced 10,000-fold from ≈50 s to ≈5 ms. Note that in the complete absence of *trans* ADP, some of the amplitude is lost in instrumental dead time (Fig. 2*B*, black). The transition is now kinetically much less complex, a double exponential with at least 1 of the phases being [ATP]-dependent, being sufficient to fit the data.

To explore this more fully we first removed the *trans* ring ADP by gel filtration as above, but then equilibrated the FRET complex with variable concentrations of ADP before challenging with ATP and excess GroES^{WT} as before. After equilibration of the *trans* ring with ADP the ensemble of FRET complexes responds to the challenge with ATP on 2 distinctly different time scales depending on whether or not the *trans* ring is populated with ADP (Fig. 2*B*). To accommodate the vastly different time scales, the results are displayed on a logarithmic time scale. The FRET complexes devoid of ADP on the *trans* ring respond rapidly with a MRT of ≈5 ms, whereas those with ADP bound to the *trans* ring respond much more slowly with a MRT measured in tens of seconds. The dissociation constant for the binding of ADP to the *trans* ring of the resting-state complex was determined to be ≈1.7 ± 0.4 μM from an analysis of the amplitudes of the fast phase (data not shown).

The response of the *trans* ring to the presence of ADP is not simply to partition the molecules into 2 populations, those with and without ADP bound to the *trans* ring. Higher concentrations of ADP, beyond that needed to populate the *trans* ring (10–15 μM) and suppress the fast phase, extend the MRT in a manner indicating competition between ADP and ATP for the unliganded *trans* ring (Fig. 2*C*).

Association of GroES with the *Trans* Ring. Accompanying the dissociation of GroES from the *cis* ring is the association of GroES with the *trans* ring. To explore this associative step we have performed experiments reciprocal to those described above, the difference being that we start with a resting-state complex with a *cis* ring GroEL^{D}-[ADP]-[GroES^{WT}], a *trans* ring GroEL^{D}-ADP, and challenge with ATP and excess GroES^{A}. When the *trans* ring is initially populated with ADP, the resultant development of the FRET signal, attributable to the association of GroES^{A} with the *trans* ring of GroEL^{D}, is complex and multiphasic with the same half-time (≈50 s) as the dissociation of the *cis* GroES (Fig. 2*D*). However, when the *trans* ring of the resting-state complex is initially unliganded, the half-time for the associative step is again reduced to ≈5 ms, matching the half-time for the dissociation of *cis* GroES under identical conditions (Fig. 2*D*). As with the dissociation of *cis* GroES, under these conditions the kinetics of association of GroES with the *trans* ring become less complex; the data fit to a double exponential. One of the phases, with a rate constant of 11.4 ± 0.6 s^{-1}, is independent of [GroES^{A}]. The observed rate constant for the other phase is a linear function of [GroES^{A}], yielding the 2nd-order rate constant for the association (4.6×10^7 M^{-1} s^{-1}) that approaches the diffusional limit, as others have reported (18, 19).

No significance should be attached to the fact that the half-times for the dissociation of GroES^{A} from the *cis* ring and for the association of GroES^{A} with the *trans* ring are the same under both conditions. Much more important is the fact that the time scale on which both association and dissociation occur can be manipulated in parallel with one another over almost 4 orders of magnitude simply by removal of the ADP bound to the *trans* ring.

These results can be explained by Fig. 1. In the initial resting-state ADP, the product of the previous hemicycle is bound to the *trans* ring and in equilibrium with free ADP. Upon dissociation of this ADP (or upon its removal by gel filtration), the *trans* ring becomes unliganded. It is this state that is poised, when ATP binds to the *trans* ring, for the very rapid release of the *cis* GroES ($t_{1/2}$ ≈5 ms) or for the rapid association of GroES with the *trans* ring. Events leading up to this state, the dissoci-

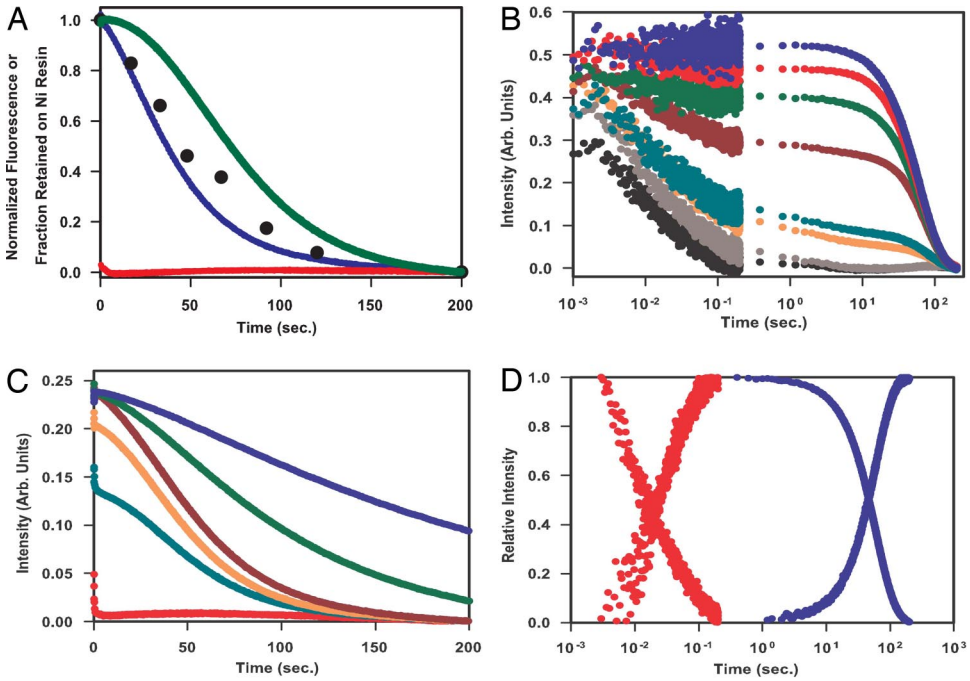

Fig. 2. Dissociation of GroES from the *cis* ring of GroEL upon addition of ATP to the *trans* ring of GroEL. (*A*) Dissociation of GroES^{A} from the *cis* ring of GroEL^{D} upon addition of ATP to the *trans* ring of GroEL^{D}, monitored continuously by the loss of the FRET signal (red and blue traces) accompanying the dissociation of the FRET pair GroEL^{D}–GroES^{A} or discontinuously by the dissociation of GroEL^{WT}–GroES^{His} using capture by nickel-chelate beads (black circles). The resting-state complex with (blue trace) or without (red trace) ADP bound to the *trans* ring was mixed with ATP and excess GroES^{WT} (blue) or excess GroEL^{WT} (green) to initiate the reaction. (*B*) Dissociation of GroES^{A} from the *cis* ring of GroEL^{D} upon addition of ATP to the *trans* ring of GroEL^{D}, after equilibrating 1 μM *trans* ring subunits with variable concentrations of ADP (black, 0 mM; gray, 1 μM; orange, 2 μM; teal, 3 μM; brown, 4 μM; green, 10 μM; red, 16 μM; blue, 20 μM). After mixing, [ATP] = 2 mM, [K^+] = 100 mM. Two datasets (0–0.2 s and 0–200 s are combined). Note the logarithmic time scale. (*C*) Dissociation of GroES^{A} from the *cis* ring of GroEL^{D} upon addition of ATP to the *trans* ring of GroEL^{D}, after equilibrating 1 μM *trans* ring subunits with variable concentrations of ADP (red, 0 μM; blue, 2 μM; orange, 4 μM; brown, 30 μM; green, 200 μM; and blue, 500 μM). After mixing, [ATP] = 250 μM, [K^+] = 100 mM. (*D*) Association of GroES^{A} (increasing FRET signal) with the *trans* ring of GroEL^{D} and dissociation of GroES^{A} (decreasing FRET signal) from the *cis* ring of GroEL^{D} upon binding of ATP to the trans ring. The conditions were identical in both cases. The associative reactions were initiated with a resting-state complex composed of GroEL^{D}–GroES^{WT} and challenged with ATP and excess GroES^{A}, whereas the dissociative experiments were initiated with a resting-state complex composed of GroEL^{D}–GroES^{A} and challenged with ATP and excess GroES^{WT}. The *trans* ring of the resting-state complexes was either populated with ADP (blue traces) or not (red traces).

ation of ADP or a change in the conformation of the *trans* ring that depends on the former, are several thousandfold slower.

Influence of Allosteric Ligands: Potassium Ion. In addition to the nucleotides ADP and ATP, which are known to bind more avidly to the **R** allosteric state (8), there are 2 additional allosteric ligands K^+ (13–15) and SP (20) that profoundly influence the chaperonin chronometer. The single ring variant of GroEL SR1 (21) is unencumbered by the complications arising from the negative cooperativity associated with the double-ringed wild type. A kinetic analysis of the influence of K^+ on the steady-state hydrolysis of ATP by the single-ring variant of GroEL SR1 (15) indicates that K^+ does not directly alter L, the equilibrium constant between the allosteric states **T** and **R**. Instead, increasing K^+ from 1 mM to 100 mM increases the ATP-binding affinity of both the **T** and **R** states by ≈30- to 50-fold. Because nucleotide binds more avidly to the **R** state, the consequence of increasing [K^+] in the presence of nucleotide is to favor the population of the **R** state. This interpretation is consistent with recent structural evidence that places K^+ near the nucleotide phosphate groups (22).

We have systematically explored the consequences of varying [K^+] on the dissociation of GroES from the *cis* ring and on the association of GroES with the *trans* ring, employing the resting-state complex with ADP bound to the *trans* ring, as well as the asymmetric complex with an unliganded *trans* ring (Fig. 3). The latter is the simpler case. Decreasing [K^+] from 100 mM to 1 mM results in a 500-fold increase in the MRT (Fig. 3*A*). In contrast, when the initial *trans* ring is populated with ADP, decreasing [K^+] from 100 mM to 1 mM results in a 100-fold decrease in the MRT (Fig. 3*B*). The same contrasting responses to variations in [K^+] can be observed if, instead, the association of GroES with the *trans* ring is monitored (15).

The contrasting effects of [K^+] suggest that it plays a double role and enable us to expand on our simple scheme and invoke specific allosteric conformations (Fig. 4). We assign the **R** conformation to the *trans* ring of the initial resting state to which ADP is bound. There is evidence that binding of GroES to the *cis* ring alters the allosteric constant L′ describing the **T** to **R** equilibrium of the *trans* ring in favor of the latter (23). This bound ADP is in equilibrium with free ligand. The consequence of increasing [K^+], increasing the affinity for ADP, is to displace the equilibrium to the left, thus increasing the MRT, which reflects the slow release of *trans* ADP (or a coupled conformational change).

$$^{trans}\text{GroEL}^{\mathbf{R}}\text{-ADP} \rightleftharpoons \text{ADP} + {}^{trans}\text{GroEL}^{\mathbf{R}} \qquad [1]$$

In the absence of *trans* ring nucleotide the *trans* ring is expected to adopt the **T** conformation. Under such conditions the allosteric constant L favors the **T** state, but because it is independent of [K^+], no effect on this equilibrium on varying [K^+] is expected.

$$^{trans}\text{GroEL}^{\mathbf{R}} \rightleftharpoons {}^{trans}\text{GroEL}^{\mathbf{T}} \qquad [2]$$

Considered together, these 2 steps make up the very slow process that is observed when one starts with the resting-state complex.

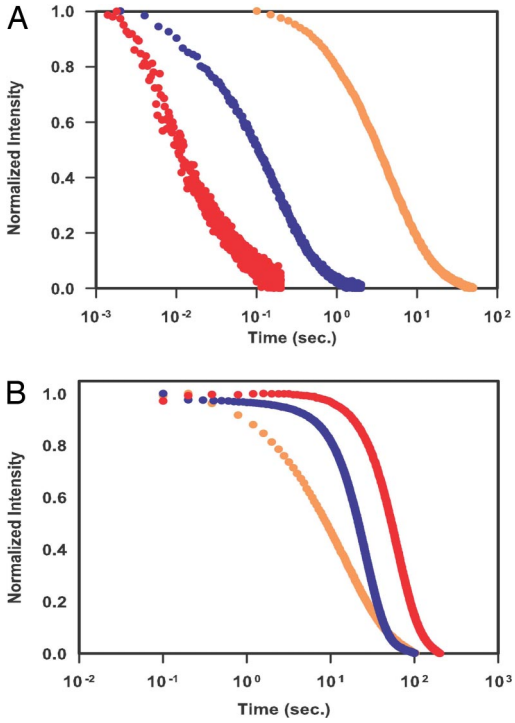

Fig. 3. Influence of varying [K^+] on the dissociation of GroES from the *cis* ring of GroEL. (*A*) Influence of varying [K^+] on the dissociation of $GroES^A$ from the *cis* ring of $GroEL^D$ upon binding of ATP to the ADP-free *trans* ring of $GroEL^D$ (red [K^+] = 100 mM, blue [K^+] = 10 mM, orange [K^+] = 1 mM). Other conditions were as in Fig. 2*B*. Note the logarithmic time scale. (*B*) Influence of varying [K^+] on the dissociation of $GroES^A$ from the *cis* ring of $GroEL^D$ upon binding of ATP to the *trans* ring of $GroEL^D$ that was preequilibrated with 30 μM ADP (red [K^+] = 100 mM, blue [K^+] = 10 mM, orange [K^+] = 1 mM). Other conditions were as in Fig. 2*B*. Note the logarithmic time scale.

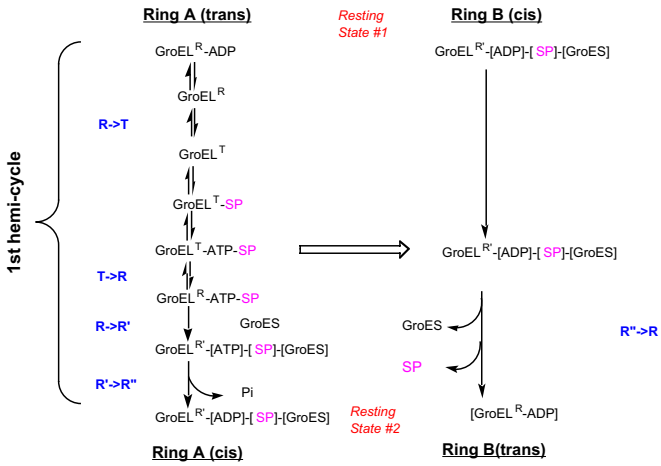

Fig. 4. Events in the chaperonin hemicycle. In a single cycle, all 14 subunits hydrolyze ATP once, and the system returns to the original resting-state 1. Because the 2 rings operate out of phase with one another, a single chaperonin cycle comprises 2 hemicycles and involves passage through the reciprocal resting-state 2. In resting-state 1, the *cis* ring B is that to which GroES is bound, whereas the other ring A is the *trans* ring. In the reciprocal resting-state 2, the roles are reversed. Ligands in the *cis* ring that do not exchange with unbound ligand are indicated with square brackets, e.g., [ADP]. These ligands become free to dissociate upon ring-to-ring signaling ($\Rightarrow$) triggered by the binding of ATP to the *trans* ring. It is proposed that a single cycle involves passage through 4 conformations (**T**, **R**, **R**′, and **R**″) and 5 transitions, e.g., $\mathbf{T} \rightarrow \mathbf{R}$ (blue). Because the chaperonin cycle operates, albeit slowly, in the absence of SP, the involvement of the latter is not mandatory. In the absence of added SP, the chaperonin cycle operates at 0.49 ± 0.2 min^{-1} (30 °C, 100 mM K^+, 1 mM ATP), giving a mean cycle time of 122 s. In the presence of SP, the mean cycle time is reduced to 7.0 s. Thus, the mean maximum time available for an encapsulated [SP] to fold is 3.5 s per hemicycle. Because the mean time for ATP hydrolysis is 2.0 s, it is evident that in the presence of SP, the chaperonin cycle operates close to the speed limit set by the rate at which ATP is hydrolyzed.

When the *trans* ring is free of ADP, i.e., in the **T** state, it is poised to undergo the very rapid reactions triggered by the binding of ATP. Increasing [K^+] increases the affinity for nucleotide, ATP in this instance, displacing the above equilibria to the right, thus decreasing the MRT (Fig. 3*A*).

$$^{trans}\mathrm{GroEL^{T}} + \mathrm{ATP} \rightleftharpoons {}^{trans}\mathrm{GroEL^{T}} - \mathrm{ATP} \qquad [3]$$

$$^{trans}\mathrm{GroEL^{T}} - \mathrm{ATP} \rightleftharpoons {}^{trans}\mathrm{GroEL^{R}}\text{-}\mathrm{ATP} \qquad [4]$$

Influence of Allosteric Ligands: Substrate Protein. The **T** state is known to bind SP with much greater avidity than the **R** state (8, 20) and can be thought of as the SP-receptive state. The involvement of $^{trans}\mathrm{GroEL^{T}}$ as an obligate intermediate in the nucleotide exchange process would thus fulfill one of the GroEL biological imperatives, the capture of SPs. It was already known from the first chaperonin FRET experiments (18) that addition of SPs enhanced the dissociation of GroES from the *cis* ring. These experiments were conducted by using a resting-state complex with ADP presumably occupying the *trans* ring. They did not address the conformational state of the *trans* ring. With this in mind, we systematically explored the consequences of varying [SP] on the dissociation of GroES from the *cis* ring and on the association of GroES with the *trans* ring, employing the resting-state complex with ADP bound to the *trans* ring, as well as the asymmetric complex with an unliganded *trans* ring (Fig. 5). For this purpose, we have used unfolded α-lactalbumin as an SP because it is unable to fold to its native state (20). In the first such experiments, the resting-state complex was equilibrated with varying [SP] before being challenged with ATP and excess $GroES^{WT}$ as before. The kinetics resulting from increasing [SP] (Fig. 5*A*) are complex; however, 2 points are evident. First, the MRT is decreased ≈1,000-fold as [SP] is increased in the preequilibration with the resting state, approaching the limit at 50 [α-lactalbumin]/[$GroEL_{14}$] seen in the absence of ADP (compare Figs. 2*C* and 5*A*). Second, as seen in Fig. 2*C*, where the population of the *trans* ring with ADP was varied, partitioning of the population of GroEL molecules into those that discharge the *cis* GroES very rapidly and those that do so much more slowly is apparent. This is particularly evident at intermediate [SP] (e.g., at 10[α-lactalbumin]/[$GroEL_{14}$]) (Fig. 5*A*, green).

Next, the resting-state complex was challenged with a solution of ATP and excess $GroES^{WT}$ containing variable [SP]. The results [Fig. 5*B*] show a decrease in the MRT with increasing [SP]. However, the very rapid initial phase that is essentially complete by 100 ms in the preequilibration experiment (Fig. 5*A*) is entirely missing (Fig. 5*B*). Reciprocal experiments monitoring the association of GroES with the *trans* ring showed the same acceleration in response to added SP (data not shown).

The effects of added [SP] equilibrated with the asymmetric complex lacking ADP on the *trans* ring (i.e., $^{trans}\mathrm{GroEL^{T}}$) contrast with the above results and are smaller in magnitude (Fig. 5*C*). Whereas the addition of SP to a *trans* ring containing bound ADP accelerated the release of the *cis* GroES upon challenge with ATP, the opposite effect is observed when SP is equilibrated with the *trans* ring that is free of ADP. A 4- to 5-fold increase in the MRT of *cis* GroES was observed. The kinetic data are well described by a double exponential. However, the system is much more complex because both rate constants and amplitudes depend on [SP], [ATP], and [K^+] (data not shown).

These allosteric effects of SP are in accord with the conclu-

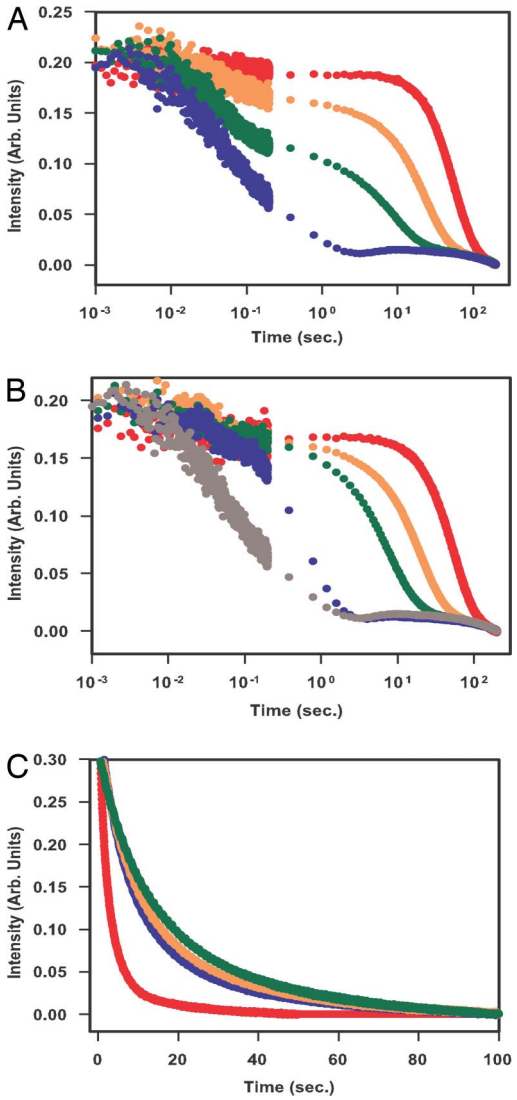

Fig. 5. Influence of substrate protein on the dissociation of GroES from the *cis* ring of GroEL on binding of ATP to the *trans* ring of GroEL. (*A*) Influence of increasing [α-lactalbumin] on the dissociation of $GroES^{A}$ from the *cis* ring of $GroEL^{D}$ upon binding of ATP to the *trans* ring of $GroEL^{D}$ that had been preequilibrated with 30 μM ADP and after [α-lactalbumin] (red, 0 μM; pink, 1.16 μM; green, 2.9 μM; blue, 7.25 μM; orange, 14.5 μM). The concentration of *trans* rings before mixing was 0.29 μM ($[K^+]$, 100 mM, [ATP] after mixing 2 mM). Two datasets (0–0.2 s) and (0–200 s) are combined. (*B*) Influence of increasing [α-lactalbumin] on the dissociation of $GroES^{A}$ from the *cis* ring of $GroEL^{D}$ upon binding of ATP to the *trans* ring of $GroEL^{D}$ that had been preequilibrated with 30 μM ADP but without α-lactalbumin, which was introduced along with the ATP challenge. After mixing, the final concentrations were [α-lactalbumin] orange, 0 μM; blue, 0.58 μM; red, 1.45 μM; green, 7.25 μM; pink, 14.5 μM; [*trans* rings], 0.145 μM; $[K^+]$, 100 mM; [ATP], 2 mM; $GroES^{WT}/GroES^{A}$ after mixing, 10. Two datasets (0–0.2 s and 0–200 s) are combined. (*C*) Influence of increasing [α-lactalbumin] on the dissociation of $GroES^{A}$ from the *cis* ring of $GroEL^{D}$ upon binding of ATP to the ADP-free *trans* ring of $GroEL^{D}$ that had been preequilibrated without ADP and the following [α-lactalbumin] (red, 0 μM; blue, 1.45 μM; green, 2.9 μM; black, 7.25 μM). The concentration of *trans* rings before mixing was 0.29 μM; $[K^+]$, 10 mM; [ATP] after mixing, 50 μM; $GroES^{WT}/GroES^{A}$ after mixing, 10.

sions reached above. Although K^+ has no effect on the allosteric constant L in the absence of nucleotide, SP, which binds preferentially to the **T** state, displaces the **R** to **T** equilibrium to the right.

$$^{trans}GroEL^{R}\text{-SP} \rightleftharpoons {}^{trans}GroEL^{wt}\text{-SP} \qquad [5]$$

Consequently, equilibrating the resting-state complex with increasing [SP] shifts the equilibrium toward the **T** state and enhances the population of *trans* rings devoid of ADP. These are poised to undergo rapid release of *cis* GroES when challenged with ATP. When SP presented to the *trans* ring of the resting state along with the challenge ATP, no superfast phase is evident, but instead the rate of the slow phase is accelerated.

We suggest that the binding of ATP to $^{trans}GroEL^{T}$ is associated with 3 events. First, the binding of ATP to $^{trans}GroEL^{T}$ constitutes the ring-to-ring signal that commits the *cis* GroEL ring to the release of GroES. A similar scenario has been proposed on the basis of a cryo-EM analysis (24). The results of cross-linking experiments in which the *trans* GroEL ring is locked in the **T** state indicate that the binding of ATP is necessary and sufficient to trigger the release of the *cis* ligands (G. Curien and J.P.G., unpublished data). The next events, the **T** to **R** transition in the *trans* ring and the binding of GroES, occur subsequent to commitment. In this scenario, the symmetrical, football-shaped complex is at best a transient, but not obligatory, intermediate. As shown in Fig. 2*D*, the association and dissociation of GroES occur on the same time scale, allowing little opportunity for the accumulation of a complex with GroES bound to both GroEL rings.

Third, the counterclockwise twisting of the apical domain with respect to the equatorial domain brings about the dispersal of the SP-binding sites that are located in the apical domain (25). The presence of SP bound to multiple sites in the apical domain might provide mechanical resistance to the counterclockwise twisting motion that accompanies the **T** to **R** transition, with the consequence that the rate of release of *cis* GroES is measurably decreased (Fig. 5*C*). This implies that mechanical force or "work" is imparted to the SP as a consequence of the movement of the apical domain, a conclusion that others have reached (26, 27).

***Trans* Ring as a Variable Timer.** We set out to understand the timing of each of the major events in the cycle (nucleotide exchange, the association and dissociation of GroES and ATP hydrolysis) in relation to the steady-state operation of the system and to determine the mechanism by which allosteric ligands such as SP and K^+ influence the timing mechanism. Several important points emerge from this work. First, the *trans* ring controls the timing mechanism, largely by governing the steps leading to the formation of the SP and ATP acceptor state $^{trans}GroEL^{T}$. These are the steps that are most profoundly influenced by SP and K^+. Subsequent steps (the binding of ATP, the release of *cis* GroES, the association of GroES with the *trans* ring, and the hydrolysis of ATP by the newly formed *cis* ring) all occur on a time scales that are from 10 to 10^4 faster.

In a single chaperonin cycle, all 14 subunits hydrolyze ATP once, and the system returns to the original resting-state 1 (Fig. 4). Because the 2 rings operate out of phase with one another, a single chaperonin cycle comprises 2 hemicycles and involves passage through the reciprocal resting-state 2. In resting-state 1, the *cis* ring B is that to which GroES is bound, whereas the other ring A is the *trans* ring. In the reciprocal resting-state 2, the roles are reversed. Ligands bound to the *cis* ring are unable to dissociate until ATP binds to the *trans* ring. The chaperonin cycle operates in the absence of SP at a steady-state rate of ATP hydrolysis of $0.49 \pm 0.02\ min^{-1}$ (30 °C, 100 mM K^+, 1 mM ATP) giving a mean hemicycle time of 61 s. The MRT of *cis* GroES under identical conditions, measured in the stopped-flow FRET experiments, is ≈50 s or slightly longer if excess $GroEL^{WT}$ is used (Fig. 2*B*). Thus, in the absence of SP the system spends most of the time in the quiescent state preceding the SP and ATP acceptor state $^{trans}GroEL^{T}$.

The picture of the chaperonins that emerges from our work is that of a machine equipped with a timer, the *trans* ring, poised

to respond to the appearance of SP but otherwise idling in a quiescent state. We note that Nature's design of this 2-speed protein machine minimizes the hydrolysis of ATP in the absence of SP. However, it maximizes the number of turnovers and minimizes the residence time available to the encapsulated SP to reach the native state, design principles well suited to the operation of an iterative annealing device (28).

Materials and Methods

Construction of GroEL^{E315C} and GroES^{98C}. Following refs. 17 and 18, the mutants were prepared as described in supporting information (SI) *Materials and Methods*. Note that we have inserted the E315C mutation into a wild-type background rather than into a cysteine-free background as in ref. 17 because in our hands cysteine-free GroEL is much less stable than the wild type.

Purification of GroEL, GroES, and GroES^{His}. Details are provided in *SI Materials and Methods*.

Labeling GroEL^{E315C} and GroES^{98C}. A slight modification of the method described by Rye *et al*. (18) was used to label GroEL^{E315C} with IAEDANS and GroES^{98C} with fluorescein-5-maleimide; details are provided in *SI Materials and Methods*. GroEL^{E315C} labeled to varying extents (40–90%) with IAEDANS was used. Note, however, that the kinetics of *cis* GroES from labeled GroEL^{E315C} release are independent of the extent of labeling (J.P.G., unpublished data) and are the same as the kinetics of *cis* GroES^{His} release from completely unlabeled GroEL^{WT} (Fig. 2*A*).

Stopped-Flow Measurements of FRET. All FRET measurements were carried out on an Applied Photophysics SX18 MV-R stopped-flow apparatus. The instrument was configured with a 20-μL flow cell with a pathlength of 2 mm and a 530-nm emission cut-off filter. The monochrometer slits were set to a width of $\approx$9 mm. The apparatus was thermostatted at 30 °C. The excitation wavelength used for all measurements was 336 nm. Details are provided in *SI Materials and Methods*.

Measuring the Kinetics of *Cis* GroES Release with GroES^{His}. The kinetic data of *cis* GroES release were independently confirmed by measuring the exchange of GroES^{His} for GroES^{WT} after ATP addition. This experiment was designed to reproduce the conditions of the stopped-flow experiments as closely as possible, to confirm that the presence of bulky hydrophobic fluorescent probes on GroEL did not influence the behavior of GroEL. Experimental details are provided in *SI Materials and Methods*.

Preparation of Unfolded α-Lactalbumin. Unfolded α-lactalbumin was prepared according to Yifrach and Horovitz (20) with modifications.

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1. Todd MJ, Viitanen PV, Lorimer GH (1994) Dynamics of the chaperonin ATPase cycle: Implications for facilitated protein folding. *Science* 265:659–666.
2. Lorimer GH (1997) Protein Folding: Folding with a two-stroke motor. *Nature* 388:720–721.
3. Kirkpatrick S, Gelatt CD, Vecchi MP (1983) Optimization by simulated annealing. *Science* 220:671–680.
4. Todd MJ, Lorimer GH, Thirumalai D (1996) Chaperonin-facilitated protein folding: Optimization of rate and yield by an iterative annealing mechanism. *Proc Natl Acad Sci USA* 93:4030–4035.
5. Thirumalai D, Lorimer GH (2001) Chaperonin-mediated protein folding. *Annu Rev Biophys Biomol Struct* 30:245–269.
6. Lin Z, Rye HS (2006) GroEL-mediated protein folding: Making the impossible, possible. *Crit Rev Biochem Mol Biol* 41:211–239.
7. Horwich AL, Farr GW, Fenton WA (2006) GroEL–GroES-mediated protein folding. *Chem Rev* 106:1917–1930.
8. Horovitz A, Fridmann Y, Kafri G, Yifrach O (2001) Allostery in chaperonins. *J Struct Biol* 135:104–114.
9. Taguchi H (2005) Chaperonin GroEL meets the substrate protein as a ''load'' of the rings. *J Biochem* 137:543–549.
10. Xu Z, Sigler PB (1998) GroEL/ES: Structure and function of a two-stroke folding machine. *J Struct Biol* 124:129–141.
11. Saibil HR, Horwich AL, Fenton WA (2001) Allostery and protein substrate conformational change during GroEL/GroES-mediated protein folding. *Adv Protein Chem* 59:45–72.
12. Ueno T, Taguchi H, Tadakuma H, Yoshida M, Funatsu T (2004) GroEL mediates protein folding with a two-successive timer mechanism. *Mol Cell* 14:423–434.
13. Viitanen PV, *et al*. (1990) Chaperonin-facilitated refolding of ribulosebisphosphate carboxylase and ATP hydrolysis by chaperonin 60 (groEL) are K^+ dependent. *Biochemistry* 29:5665–5671.
14. Widjaja L (2002) Allosteric control of GroEL by ATP: Effects of monovalent and divalent cations. MS thesis (Univ of Maryland), pp 40–46.
15. Gresham JS (2004) Allostery and GroEL: Exploring the tenets of nested cooperativity. PhD thesis (Univ of Maryland), pp 69–78.
16. Weissman JS, Kashi Y, Fenton WA, Horwich AL (1994) GroEL-mediated protein folding proceeds by multiple rounds of binding and release of nonnative forms. *Cell* 78:693–702.
17. Rye HS (2001) Application of fluorescence resonance energy transfer to the GroEL–GroES chaperonin reaction. *Methods* 24:278–288.
18. Rye HS, *et al*. (1999) GroEL–GroES cycling: ATP and nonnative polypeptide direct alternation of folding-active rings. *Cell* 97:325–338.
19. Burston SG, Ranson NA, Clarke AR (1995) The origins and consequences of asymmetry in the chaperonin reaction cycle. *J Mol Biol* 249:138–152.
20. Yifrach O, Horovitz A (1996) Allosteric control by ATP of nonfolded protein binding to GroEL. *J Mol Biol* 255:356–361.
21. Weissman JS, *et al*. (1995) Mechanism of GroEL action: Productive release of polypeptide from a sequestered position under GroES. *Cell* 83:577–587.
22. Wang J, Boisvert DC (2003) Structural basis for GroEL-assisted protein folding from the crystal structure of (GroEL–KMgATP at 2.0 Å resolution. *J Mol Biol* 327:843–855.
23. Inbar E, Horovitz A (1997) GroES promotes the T to R transition of the GroEL ring distal to GroES in the GroEL–GroES complex. *Biochemistry* 36:12276–12281.
24. Ranson NA, *et al*. (2006) Allosteric signaling of ATP hydrolysis in GroEL–GroES complexes. *Nat Struct Mol Biol* 13:147–152.
25. Ranson NA, *et al*. (2001) ATP-bound states of GroEL captured by cryoelectron microscopy. *Cell* 107:869–879.
26. Motojima F, Chaudhry C, Fenton WA, Farr GW, Horwich AL (2004) Substrate polypeptide presents a load on the apical domains of the chaperonin GroEL. *Proc Natl Acad Sci USA* 101:15005–15012.
27. Lin Z, Madan D, Rye HS (2008) GroEL stimulates protein folding through forced unfolding. *Nat Struct Mol Biol* 15:303–311.
28. Betancourt MR, Thirumalai D (1999) Exploring the kinetic requirements for enhancement of protein folding rates in the GroEL cavity. *J Mol Biol* 287:627–644.
29. Grason JP, Gresham JS, Widjaja L, Wehri SC, Lorimer GH (2008) Setting the chaperonin timer: The effects of K^+ and substrate protein on ATP hydrolysis. *Proc Natl Acad Sci USA* 105:17334–17338.