

# GroEL/GroES cycling: ATP binds to an open ring before substrate protein favoring protein binding and production of the native state

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The GroEL/GroES reaction cycle involves steps of ATP and polypeptide binding to an open GroEL ring before the GroES encapsulation step that triggers productive folding in a sequestered chamber. The physiological order of addition of ATP and nonnative polypeptide, typically to the open *trans* ring of an asymmetrical GroEL/GroES/ADP complex, has been unknown, although there have been assumptions that polypeptide binds first, allowing subsequent ATP-mediated movement of the GroEL apical domains to exert an action of forceful unfolding on the nonnative polypeptide. Here, using fluorescence measurements, we show that the physiological order of addition is the opposite, involving rapid binding of ATP, accompanied by nearly as rapid apical domain movements, followed by slower binding of nonnative polypeptide. In order-of-addition experiments, approximately twice as much Rubisco activity was recovered when nonnative substrate protein was added after ATP compared with it being added before ATP, associated with twice as much Rubisco protein recovered with the chaperonin. Furthermore, the rate of Rubisco binding to an ATP-exposed ring was twice that observed in the absence of nucleotide. Finally, when both ATP and Rubisco were added simultaneously to a GroEL ring, simulating the physiological situation, the rate of Rubisco binding corresponded to that observed when ATP had been added first. We conclude that the physiological order, ATP binding before polypeptide, enables more efficient capture of nonnative substrate proteins, and thus allows greater recovery of the native state for any given round of the chaperonin cycle.

chaperonin | polypeptide binding | protein folding

The GroEL/GroES chaperonin system provides assistance to folding of a large number of proteins to the native state via 2 principal actions, one involving binding of nonnative protein in an open ring of GroEL through multivalent hydrophobic contacts formed between the nonnative protein and surrounding GroEL apical domains and the other involving folding occurring in the encapsulated hydrophilic chamber formed when ATP and the GroES “lid” are bound to the same ring as polypeptide (1–3). A number of studies have made it clear that the polypeptide binding step can rescue misfolded substrate proteins from kinetically trapped states that occur during folding (e.g., 4, 5), despite the lack of stable secondary structure in such conformations (6, 7). Such rescue has been associated with topological “stretching” of the substrate protein, as observed in a number of FRET studies (5, 8, 9). Substrate protein is released during large ATP/GroES-directed rigid body movements of the GroEL apical domains into the GroES-domed hydrophilic chamber, in which it proceeds to fold (10–12). As shown in a number of studies, the so-called “*cis*” chamber facilitates folding by preventing multi-molecular aggregation that could reduce both the rate of recovery of the native state (in those cases in which aggregation is reversible) and the extent of recovery (in those cases in which aggregation is irreversible), as occurs for substrate proteins folding in free solution (e.g., 13, 14).

Although the general trajectory of the reaction cycle has been understood for some time, there has remained an open question

concerning the order of arrival of the ligands, ATP and substrate protein, to the normal acceptor, the open ring of an asymmetrical GroEL/GroES/ADP complex. Does polypeptide arrive first? This has been assumed in a number of studies *in vitro*, where an order of addition has been programmed in which substrate is first incubated with such asymmetrical complexes, followed by addition of ATP (8, 9). In such studies, there have been observations that ATP addition produced additional stretching of nonnative protein, and it was suggested that this could constitute a necessary ATP-mediated “forced unfolding” step. Such a step would presumably result from the small rigid body elevation and counterclockwise twist of the apical domains of a GroEL ring that attend ATP binding to equatorial sites in the ring (15), preceding the large rigid body elevation and clockwise twist that accompany GroES binding. Yet, could it be that ATP binds first? If so, such forced unfolding would not likely be operative if the apical domains move on the same approximate time scale as ATP binding. Moreover, polypeptide association with apical domains that have been mobilized could potentially affect the efficiency of binding. Such an order of addition with ATP binding before substrate protein seems plausible considering previous rate measurements that indicate, on one hand, rapid binding of ATP to unliganded GroEL (16–18) and, on the other hand, relatively slow binding of such substrate proteins as MDH and Rubisco to unoccupied GroEL or to asymmetrical GroEL/GroES/ADP complexes, respectively (19, 20). Here, we have systematically investigated the relative rates of arrival of ATP and substrate protein ligands to both unliganded GroEL and asymmetrical GroEL/GroES/ADP complexes and find that the physiological order of arrival entails rapid ATP binding, producing nearly as rapid apical domain movement, followed by slower binding of substrate protein. Further, in order-of-addition experiments, the extent of recovery of the substrate protein Rubisco was greater with the physiological order of addition than with the opposite order, a consequence of more rapid substrate protein binding by ATP-mobilized apical domains.

## Results

**Rapid ATP Binding to GroEL as Revealed by Fluorescence Intensity Changes of GroEL F44C Labeled with Oregon Green 488.** To monitor binding of ATP by GroEL, we relied on a previously produced GroEL variant, F44C, containing a single cysteine in an otherwise “cysteine-zero” version of GroEL in which all 3 natural cysteines in the GroEL subunit (amino acids 138, 458, and 519) had been

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indicated by the ability of its encoding plasmid to rescue growth of a GroEL-deficient mutant and by the ability of the purified protein to carry out efficient ATP/GroES-dependent protein refolding in vitro (21). F44C was labeled with Oregon Green 488 (OG488)-maleimide to a level of  $\approx 70\%$  occupancy. It remained fully functional in mediating refolding of malate dehydrogenase (MDH) in vitro, exhibiting kinetics nearly identical to those of WT GroEL [supporting information (SI) Fig. S1A]. The steady-state rate of ATP turnover by the modified mutant, called EL44-OG, also resembled that of WT GroEL (Fig. S1B).

Fluorescence emission spectra of EL44-OG (excited at 496 nm) revealed that addition of ATP produced a large drop in steady-state fluorescence intensity ( $\approx 65\%$  in 500  $\mu\text{M}$  ATP), accompanied by a small blue shift of the emission maximum (Fig. 1B). By contrast, ADP produced a smaller drop of intensity, supporting that the  $\gamma$ -phosphate of ATP has a specific effect on the conformation of the 44-containing loop.

On stopped-flow mixing of 0.5 mM ATP with EL44-OG, a rapid drop of emission intensity was observed, with a curve that could be fit as the sum of 2 exponentials, one with a rate constant of  $\approx 100\text{ s}^{-1}$  and another with a rate constant of  $4\text{ s}^{-1}$  (Fig. 1C). The former rate indicates rapid interaction of ATP with unliganded GroEL and corresponds to rates reported earlier both for F44W (ref. 16;  $80 \pm 5\text{ s}^{-1}$ ) and for 2 other tryptophan-substituted versions of GroEL: Y485W, where a tryptophan was substituted elsewhere in the equatorial domain (ref. 18;  $123 \pm 2\text{ s}^{-1}$ ), and R231W (ref. 17;  $80\text{ s}^{-1}$ ), where a tryptophan was substituted into the cavity-facing aspect of the apical domain (GroEL is otherwise devoid of tryptophan). As expected, the rate of interaction of ATP with EL44-OG was dependent on ATP concentration (Fig. 1D), and a bimolecular rate constant of  $2 \times 10^5\text{ M}^{-1}\text{ s}^{-1}$  was determined for the faster phase. The slower kinetic phase, also dependent on ATP concentration, likely corresponds to the third fluorescent phase observed for GroEL's R231W and Y485W on ATP binding (17, 18). The possibility that this phase reflected ATP hydrolysis was excluded by employing a hydrolysis-defective D398A/EL44-OG chaperonin.

**Equally Rapid Fluorescence Change on ATP Binding to Open *Trans* Ring of GroEL/GroES/ADP Asymmetrical Complex.** Under physiological conditions, the normal acceptor state for ATP is the open *trans* ring of a GroEL/GroES/ADP asymmetrical complex. With stopped-flow mixing, we observed that the rate of ATP binding to the *trans* ring of EL44-OG/GroES/ADP complexes was similar to that to an unliganded GroEL ring (Fig. 1E, compare with Fig. 1F). Here, there was also ATP concentration dependence resembling that of unliganded EL44-OG (Fig. 1D).

**Substrate Polypeptide Bound to the *Trans* Ring Does Not Affect Rate of ATP Binding.** In a number of in vitro studies, nonnative polypeptide has been first complexed to the open *trans* ring of an asymmetrical ADP GroEL/GroES complex before addition of ATP and excess GroES (8, 9). Under these conditions, ATP binding followed by binding of GroES is clearly able to encapsulate the nonnative polypeptide and direct productive folding. Is the rate of ATP binding in such a context affected by the bound substrate protein? To test this, nonnative MDH was bound to the asymmetrical EL44-OG/GroES/ADP complexes, and ATP was then added with stopped-flow mixing. Under these conditions, the rate of fluorescence intensity change was virtually identical to that of the EL44-OG/GroES/ADP complex in the absence of polypeptide (compare Fig. 1G with Fig. 1E). Thus, the presence of a nonnative substrate bound to the *trans* ring apical domains does not have any detectable effect on the rapid entry of ATP into the *trans* ring equatorial nucleotide binding pockets.

**Rapid Apical Domain Movement Accompanies ATP Binding.** When ATP binds rapidly to GroEL, does this cause significant adjustment

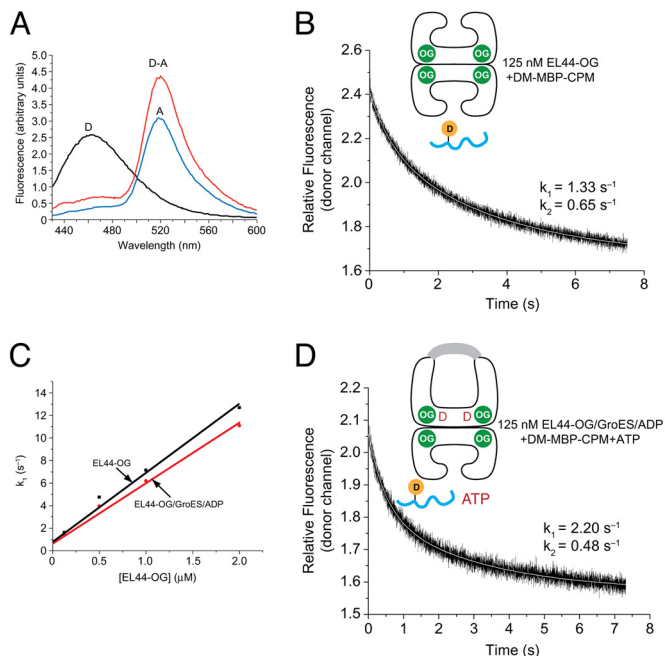
in the apical polypeptide binding domains on the same rapid time scale, or are the apical changes in response to ATP binding relatively slow, such that the effects of ATP binding must be considered to be occurring at a later time? The earlier study of R231W had already indicated that apical effects occurred rapidly with that mutant (17). In the present study as well, a fluorescent probe on the outside aspect of the apical domains, remote from the ATP binding site on the inside of the cylinder (GroEL E315C in a cysteine-zero background labeled with OG), showed a rapid change in fluorescence intensity, on the same time scale as ATP binding. For example, a rate constant of  $20\text{ s}^{-1}$  was observed for a GroEL315-OG/GroES/ADP asymmetrical complex at an ATP concentration of 150  $\mu\text{M}$  (Fig. 1H, compare with  $25\text{ s}^{-1}$  in Fig. 1E). Such apical changes occurred in the same ring to which ATP is bound, because analysis of an OG-modified single-ring version of E315C, SR315-OG, showed a similar rate of fluorescence intensity change (Fig. 1I). The rate of apical movement was also ATP concentration-dependent, with the rates paralleling those of ATP binding (Fig. S2, compare with Fig. 1D).

**Relatively Slow Binding of Substrate Polypeptide as Measured by FRET.** To enable a comparison of the rate of binding nonnative substrate polypeptide with that of binding ATP, we next measured the rate of binding of substrate proteins to asymmetrical GroEL/GroES/ADP complexes using FRET. Three different substrate proteins were studied: MDH (33 kDa), Rubisco (51 kDa), and a double-mutant form of maltose binding protein (DM-MBP; 41 kDa). All 3 proteins behave as stringent substrate proteins at  $25^\circ\text{C}$ , requiring the presence of GroEL, GroES, and ATP to reach native form (3). In their absence, quantitative aggregation ensues. For monitoring binding, FRET was measured between substrate protein labeled with coumarin propyl maleimide (CPM; donor) on a cysteine residue (see *Materials and Methods*) and EL44-OG (acceptor). Exciting at the excitation maximum for CPM (384 nm), emission spectra were collected for the CPM (donor)-labeled substrates while bound to unlabeled GroEL (Fig. 2A, DM-MBP-CPM as an example, black trace), for EL44-OG complexed with unlabeled substrate (Fig. 2A, acceptor labeled, blue trace), or for complexes with CPM-labeled substrate bound to EL44-OG (Fig. 2A, red trace). For both DM-MBP-CPM and MDH-CPM, there was strong donor quenching on association with EL44-OG; at the same time, there was the appearance of a substantial acceptor signal.

On stopped-flow mixing of 125 nM DM-MBP-CPM with 125 nM EL44-OG, a drop of donor emission intensity was observed, with a curve that could be fit as the sum of 2 exponentials, one with a rate constant of  $1.33\text{ s}^{-1}$  and the other with a rate constant of  $0.65\text{ s}^{-1}$  (Fig. 2B). The faster rate ( $k_1$ ) was dependent on the concentration of chaperonin (Fig. 2C), but the slower one was not. At a GroEL concentration of 125 nM (Fig. 2B), the rate of substrate polypeptide binding ( $k_1 = 1.33\text{ s}^{-1}$ ) is 60-fold slower than the rate of ATP binding ( $100\text{ s}^{-1}$  at 500  $\mu\text{M}$  ATP; Fig. 1C). Although the rate of substrate binding would be predicted to be severalfold greater at the physiological GroEL concentration of 1–2  $\mu\text{M}$  (Fig. 2C), the rate of ATP binding would also likely be somewhat greater, considering that physiological ATP concentration is several millimolar (Fig. 1D). Thus, the rate of ATP arrival under physiological conditions is likely to be at least 10-fold greater than substrate arrival.

In a further test, the addition of ATP at the same time as fluorescently labeled DM-MBP had a reproducible effect of increasing the rate of DM-MBP binding (Fig. 2D and Table S2). In sum, the physiological order of addition to a *trans* ring appears to comprise rapid arrival of ATP followed by slower arrival of substrate protein. This order is opposite to that programmed in recent studies, where polypeptide was initially bound to the *trans* ring and ATP was then added (8, 9). Does the order of addition have any measurable effect on the refolding of the substrate





**Fig. 2.** Relatively slow binding of a nonnative substrate, DM-MBP, to unliganded GroEL and to the *trans* ring of the GroEL/GroES/ADP complex. (A) Emission spectrum of DM-MBP labeled with CPM (donor) while bound to GroEL (D, black trace), emission spectrum of EL44-OG (acceptor) while complexed with nonnative DM-MBP (A, blue trace), and emission spectrum of the complex of DM-MBP-CPM with EL44-OG (D-A, red trace), all excited at 384 nm, the excitation maximum for CPM. (B) Change in donor channel fluorescence on stopped-flow mixing of nonnative DM-MBP-CPM with EL44-OG. Blue line represents nonnative DM-MBP, and D in the yellow circle represents the CPM label. (C) Dependence of the faster rate constant for DM-MBP binding on GroEL concentration. Rate constants from experiments as in B at different concentrations of GroEL (black) or from similar experiments using the GroEL/GroES/ADP complex (red) are plotted vs. GroEL concentration, yielding straight lines with slopes (second-order rate constants) of  $6.11 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$  and  $5.36 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ , respectively. (D) Change in donor fluorescence on stopped-flow mixing of nonnative DM-MBP-CPM with the EL44-OG/GroES/ADP complex and 500 μM ATP. In multiple experiments ( $n = 6$ ), the rate of the faster phase was consistently slightly faster for the *trans* ring in the presence of ATP than in its absence:  $2.08 \pm 0.11$  vs.  $1.36 \pm 0.22$  (Table S2).

protein? To test this, we carried out order-of-addition experiments and measured recovery of native enzyme under essentially single-turnover conditions.

**More Extensive Recovery of the Native State When ATP Is Added First, Followed by Polypeptide, Compared with the Opposite Order.** An order-of-addition experiment was carried out using the single-ring version of GroEL, SR1, enabling “single-round” analysis. Polypeptide captured by SR1 is nearly quantitatively folded to native form, after binding of GroES, inside the long-lived *cis* cavity of the stable SR1/GroES complex (10, 11). Thus, SR1 could be incubated with ATP and nonnative polypeptide in either order, followed by addition of GroES, and the extent of recovery of native protein could be measured in relation both to the order of addition and to the interval between the additions (Fig. 3A). For these tests, GroES was added 2 sec after ATP/polypeptide to allow completion of the initial interactions. In an initial experiment, we reproducibly observed that when nonnative Rubisco was added first followed 2 sec later by ATP, the extent of Rubisco recovery in native form was only  $\approx 30\%$ . This compared with  $>60\%$  recovery when ATP was added first and with the  $\approx 70\%$  recovery observed when ATP and GroES were added to a preformed Rubisco-SR1 binary complex (produced

by a 10-min incubation of nonnative Rubisco with SR1). This suggests that in the context of a cycling reaction in which the *trans* ring of an acceptor GroEL/GroES/ADP complex is open and available for binding polypeptide for only  $\approx 1\text{--}2$  s before GroES binds, the mobilization of its apical domains by rapid ATP binding favors the binding of nonnative polypeptide. The opposite order, addition of polypeptide 2 s before ATP, appeared to be less favorable for binding, even though ATP-mobilized apical domains were subsequently available for 2 s before GroES addition. Notably, when the interval between the additions of Rubisco and ATP was increased to 4 s (Fig. 3A), the order-of-addition effect was relieved, with the kinetics and extent of recovery now equal to those of adding ATP first, presumably a function of more extensive polypeptide binding during the interval before ATP addition. Although the extent of recovery of Rubisco in the order-of-addition studies with a 2-s interval was significantly affected, the kinetics of recovery of the native state inside the stable SR1/GroES complexes were similar, consistent with an effect on substrate protein binding and not on the rate of folding in the encapsulated chamber.

**More Extensive Binding of Rubisco with ATP Added First.** To address the effect of the order of addition on substrate protein binding, we used <sup>35</sup>S-labeled Rubisco and measured the amount that became bound by SR1 during the order-of-addition incubations using gel filtration of the final product mixtures to separate the stable SR1/GroES/Rubisco complexes from unbound polypeptide. This revealed that  $\approx 10,000$  cpm <sup>35</sup>S-Rubisco had bound when 40,000 cpm <sup>35</sup>S-Rubisco (125 nM) was added 2 sec before ATP and that  $\approx 30,000$  cpm had bound when ATP was added 2 sec before Rubisco (Fig. 3B). The latter extent of binding was also achieved when SR1-Rubisco binary complexes formed over a period of 10 min were then incubated with ATP and GroES together (Fig. 3B). These measurements of the extent of substrate binding thus parallel the extent of recovery of enzymatic activity (compare Fig. 3B with Fig. 3A) and support the conclusion that in the context of a 2-s interval between the addition of ATP/polypeptide, the addition of ATP initially favors more efficient substrate protein binding.

#### Greater Rate of Rubisco Binding to an ATP-Exposed Chaperonin Ring.

The foregoing observation of greater extent of Rubisco binding in an order-of-addition experiment in which ATP is added initially suggests that the rate of Rubisco association with ATP-mobilized apical domains might be greater. To test this, an ATP hydrolysis-defective D398A version of SR1 with OG attached to a cysteine substituted at position 44 (in a C138A background) was used to report by FRET on the rate of binding of CPM-labeled Rubisco (Fig. 3C–E). We observed that the rate of Rubisco binding, carried out with the same conditions as the foregoing order-of-addition experiments, was 2-fold greater in the presence vs. absence of ATP (compare Fig. 3D with Fig. 3C). When ATP and nonnative Rubisco were added simultaneously, reflecting the physiological situation, the rate of Rubisco binding resembled that when ATP had been added before Rubisco (Fig. 3E, compare with Fig. 3D). These data support that Rubisco associates more rapidly with a ring whose apical domains have been ATP-mobilized and that, under physiological conditions in which both ATP and nonnative substrate are present, it is the ATP-mobilized apical domains that are operative in substrate binding.

#### Discussion

**Physiological Order of Binding to an Open Acceptor Ring Involves Rapid ATP Binding Followed by Nonnative Substrate Protein, Producing More Efficient Capture of Substrate Protein.** The kinetic studies presented here with both unliganded GroEL and asymmetrical GroEL/GroES/ADP acceptor complexes indicate that ATP binds rapidly to an open ring, at rates of  $>100 \text{ s}^{-1}$  at physiological



## Two Distinct ATP Actions During Production of a Folding-Active Ring.

In summary, the present observations indicate that the physiological sequence of the chaperonin reaction cycle involves ATP action at 2 steps in the formation of a folding-active complex. Initially, on rapid ATP binding, fast mobilization of the apical domains occurs ( $\approx 100 \text{ s}^{-1}$ ), entailing their release from each other and producing a small degree of elevation and counter-clockwise twist. Next, substrate protein binds ( $\approx 5\text{--}10 \text{ s}^{-1}$ ), with, as shown here, the mobilized state of the apical domains accelerating association. Then, in a step that must be slower than substrate binding (ref. 23;  $1\text{--}2 \text{ s}^{-1}$ ), GroES collides with the mobilized apical domains via its mobile loops and triggers the large subsequent apical domain movements ( $60^\circ$  elevation and  $120^\circ$  clockwise twist) that release the bound substrate protein into the domed folding chamber. These latter movements require the presence of ATP so that its binding energy can provide the force necessary for releasing substrate protein (23). Thus, in effect, ATP binding to the open *trans* ring of an asymmetrical complex supports 2 distinct events in this ring, separated by the binding of nonnative polypeptide, which lead to the formation of a folding-active complex and the initiation of folding.

## Materials and Methods

**Proteins and Assays.** Proteins were expressed, purified, and assayed as in *SI Materials and Methods*.

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**Formation of Asymmetrical GroEL/GroES Complexes.** In all experiments, asymmetrical GroEL/GroES/ADP complexes were formed by incubating  $7 \mu\text{M}$  EL44-OG with  $8 \mu\text{M}$  GroES and  $250 \mu\text{M}$  ATP in  $50 \text{ mM}$  Tris-HCl (pH 7.5),  $50 \text{ mM}$  KCl,  $10 \text{ mM}$   $\text{MgCl}_2$ , and  $1 \text{ mM}$  DTT for 10 min at  $25^\circ\text{C}$ . Before loading into the stopped-flow syringe, the complex was diluted 25-fold in the same buffer, giving a residual ADP concentration of  $10 \mu\text{M}$ .

**Fluorescence Labeling.** The cysteine-substituted GroEL and SR1 complexes were labeled with OG488, and MDH and Rubisco were labeled with 7-diethylamino-3-(4'-maleimidylphenyl)-4-methyl coumarin (CPM) using standard labeling conditions as described in *SI Materials and Methods*.

**Stopped-Flow Fluorescence.** Labeled DM-MBP was unfolded in  $3 \text{ M}$  guanidine-HCl, and labeled Rubisco was unfolded in  $5 \text{ M}$  urea and  $10 \text{ mM}$  HCl, before loading into the stopped-flow syringe. Both substrates were diluted 25-fold on mixing with the various GroEL complexes. Concentrations given are the final ones after mixing. For all fluorescence experiments using OG488, the fluorophore was excited at  $496 \text{ nm}$  and emission was measured using a color separation filter ( $510\text{--}570 \text{ nm}$ ). For substrate binding FRET experiments, the CPM donor was excited at  $384 \text{ nm}$  and the emission was recorded by using a cutoff filter ( $410 \text{ nm}$ ) and a band-pass filter ( $360\text{--}500 \text{ nm}$ ). Traces are the sum of the data from 10 individual mixes. Fitting of experimental data was performed with Origin (OriginLab).

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