Single-molecule observation of protein–protein interactions in the chaperonin system

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We have analyzed the dynamics of the chaperonin (GroEL)–cochaperonin (GroES) interaction at the single-molecule level. In the presence of ATP and non-native protein, binding of GroES to the immobilized GroEL occurred at a rate that is consistent with bulk kinetics measurements. However, the release of GroES from GroEL occurred after a lag period (~3 s) that was not recognized in earlier bulk-phase studies. This observation suggests a new kinetic intermediate in the GroEL–GroES reaction pathway.

Existing approaches for studying protein–protein interactions include the two-hybrid systems, fluorescence resonance energy transfer, and conventional biochemical analysis[1,2]. Recently, techniques such as total internal reflection fluorescence microscopy and confocal microscopy have been used to investigate proteins at the single-molecule level[3–5]. Single-molecule imaging allows visualization of individual molecular interactions under physiological conditions and provides information that cannot be gained from conventional ensemble-averaged experiments.

Chaperonins are a class of molecular chaperones that promote protein folding in the cell. They are found in bacteria, chloroplasts, mitochondria, archaea, and the eukaryotic cytosol[6]. Chaperonins are essential and abundant proteins that form large toroid complexes. The best-characterized chaperonin is the *Escherichia coli* GroEL and its partner GroES, which function together as a complex molecular machine[6,7]. The double-toroid GroEL tetradecamer encapsulates non-native protein in the central cavity when capped by GroES in the presence of ATP (refs 8–12). The GroEL–GroES complex decays with a lifetime of 8–15 s by dissociation of GroES, followed by release of the trapped protein from the cavity[10,11,13–15]. We have constructed a system for observing the real-time GroEL–GroES interaction.

Results

Experimental design. Fluorescently labeled GroEL was immobilized on a glass surface and visualized by total internal reflection fluorescence microscopy, which only illuminates the region near the glass surface at a depth of ~150 nm in the medium[5] (Fig. 1A). GroES, labeled with a fluorophore of a different color, was then added to the cell. GroES that is not bound to GroEL cannot be observed because of rapid Brownian motion. Following addition of labeled GroES in the presence of ATP, we observed many individual molecules of GroES at the positions of GroEL, indicating GroES–GroEL binding.

To immobilize GroEL, we replaced Asp490, which is located on the outer surface of the equatorial domain[7], with cysteine. This mutant GroEL (termed EL490) was labeled with both biotin-maleimide and IC5-maleimide. The labeled EL490 (IC5-EL) behaved like wild-type GroEL by every measure examined, including steady-state ATP hydrolysis and assisted folding of rhodanese and green fluorescent protein (data not shown). The labeled GroEL molecules were immobilized on a glass surface through a biotin–streptavidin linker. Addition of 4 nM Cy3-labeled GroES (Cy3-ES), 2 mM ATP, and reduced lactalbumin as a non-native protein[16–18] produced many Cy3-fluorescent spots at the known positions of IC5-EL (Fig. 1B). The appearance of these spots was strictly dependent on the presence of ATP. A Quicktime movie of this is available as supplementary information in the Web Extras page of *Nature Biotechnology* Online.

Dynamics of the GroEL–GroES interaction. On average, 83% of the IC5-EL repeatedly bound Cy3-ES during 5 min of observation. Monitoring of a single GroEL molecule showed the repeated appearance and disappearance of Cy3 fluorescence, reflecting multiple rounds of binding and release of Cy3-ES to the immobilized GroEL (Fig. 1C). This on/off pattern was also seen at positions other than those of IC5-EL. This represented the binding of Cy3-ES to immobilized EL490 lacking the IC5 label: the relative population of the spots agreed with that predicted from the IC5-labeling yield, and the on/off patterns were indistinguishable from those at positions of IC5-EL. A histogram of the "off-time" for the binding (i.e., the duration of the off period) was well fit by a single exponential curve (Fig. 2A), indicating that the association event was a stochastic process. The association rate constant for GroES binding (k_{on}) was calculated to be 2.6×10^7 $M^{-1}s^{-1}$. The binding of Cy3-ES to GroEL was also monitored as the fluorescence change in a bulk-phase rapid-mixing experiment, and the results were simulated with $k_{on} = 3.9 \times 10^7$ $M^{-1}s^{-1}$ (Fig. 2A, inset). Good agreement of k_{on} values obtained by the two methods, as well as with values reported by others[11,19], suggested that the immobilization of GroEL did not perturb binding to GroES. Heptameric GroES has been reported to dissociate into monomers at low concentration[20]; we confirmed by gel-filtration high-performance liquid chromatography that Cy3-ES was stable as a heptamer at 4 nM under the conditions used for single-molecule analysis (data not shown).

According to the current model of the GroEL–GroES reaction cycle[11], the immediate product of the ATP-triggered association of GroES to the GroEL–non-native protein complex is the *cis*-ATP complex (i.e., a GroEL–GroES complex with ATP bound at the *cis* ring (the GroES-bound ring of GroEL) and the substrate protein released in the *cis* cavity). The *cis*-ATP complex becomes the *cis*-

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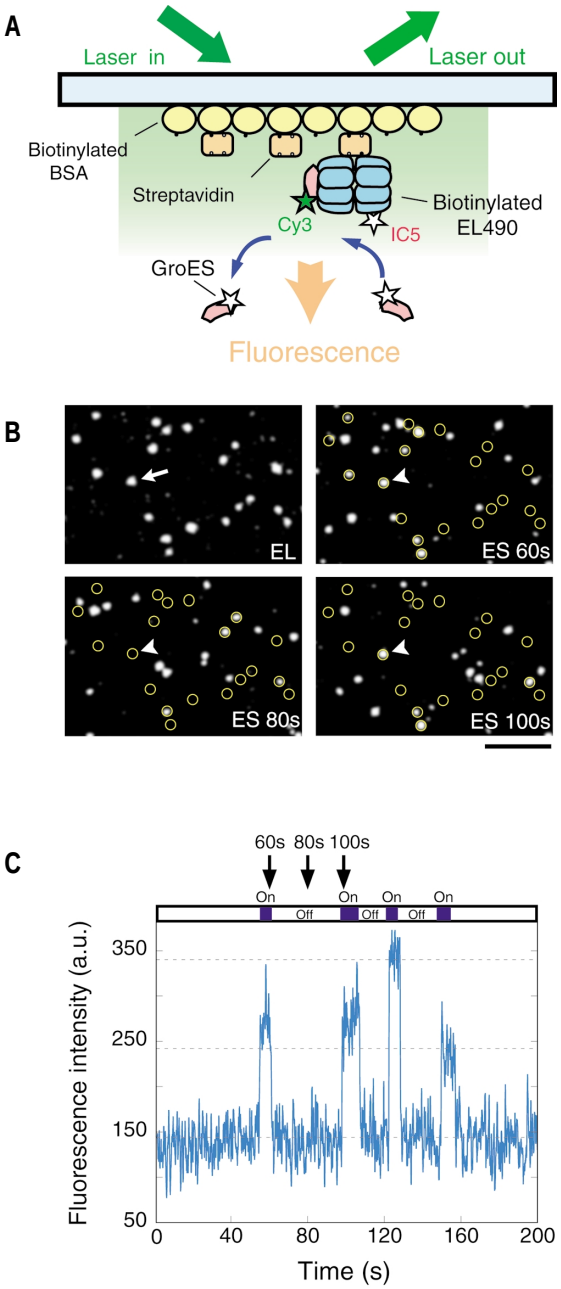

Figure 1. Single-molecule imaging of chaperonin GroEL–GroES dynamics. (A) Schematic drawing of the experiment. Biotin-, IC5-labeled GroEL mutant (IC5-EL) was immobilized on the glass surface through a biotinylated bovine serum albumin–streptavidin linker. The flow cell containing the immobilized GroEL was filled with Cy3-labeled GroES (Cy3-ES), ATP, reduced lactalbumin, and the oxygen scavenger system. Association and dissociation of Cy3-ES molecules with GroEL were visualized by total internal reflection fluorescence microscopy[5]. (B) Fluorescence images of single GroEL and GroES molecules. These images were generated from a 2 s interval of data. EL, immobilized IC5-EL. ES, Cy3-ES (time in seconds). Positions of IC5-EL are indicated by circles colored yellow. Arrowheads mark the positions of the IC5-EL indicated by the arrow in the first panel. Cy3-ES molecules observed outside of circles were those attached to GroEL molecules that were not fluorescently labeled. Calibration bar, 10 μm. The movie showing the binding-release events is available as supplementary material in the Web Extras page of *Nature Biotechnology* Online. (C) Time course of fluorescence intensity from Cy3-ES associating and dissociating with the single GroEL molecule indicated by the arrow in (B). The vertical arrows in (C) indicate the time when the fluorescence micrographs in (B) were taken. The purple-shaded bars in the upper panel indicate the period when Cy3-ES is associated with an IC5-EL. Broken lines indicate fluorescence intensities corresponding to background, single, and two fluorescent Cy3 dye molecules, respectively. Occasionally, brighter Cy3-ES molecules, corresponding to two Cy3 dyes, were attached and then released. These are most likely single GroES molecules labeled with two Cy3 dyes, rather than two independent GroES molecules, each of which contains single Cy3 dye, in that their relative population agreed with that predicted from the Cy3-labeling yield. Stepwise binding and release of two Cy3-ES molecules to GroEL, which indicates a formation of the "football" complex[13] (GroES–GroEL–GroES), were very rare events, suggesting that the asymmetric "bullet" complex (GroEL–GroES) is the dominant species.

ADP complex by hydrolysis of ATP, which then releases GroES upon binding of ATP to the other GroEL heptamer (the *trans* ring). The release of GroES from GroEL is predicted to be governed by a single rate constant (0.12 s^{-1})[11]. The rate-limiting step in this cycle is the hydrolysis of ATP on the *cis*-ATP complex, as shown in scheme (1) (refs 10,11,13–15).

$$[\text{GroEL–GroES}] \xrightarrow[k]{} [\text{GroEL}] + [\text{GroES}] \qquad (1)$$

If this model is correct, the histogram of the "on time" of GroES bound to GroEL in the presence of non-native protein should obey a single exponential curve. However, our single-molecule analysis gave an unexpected result: the histogram showed a maximum at ~5 s (Fig. 2B). In other words, the release of GroES from GroEL occurred after a lag period. This distribution cannot arise from a simple dissociation event but rather results from two sequential transitions. We therefore postulated the existence of a kinetic intermediate before the dissociation:

$$[\text{GroEL–GroES}] \xrightarrow[k]{} [\text{GroEL–GroES}]^* \xrightarrow[k']{} [\text{GroEL}] + [\text{GroES}] \qquad (2)$$

The single-molecule data were well fit by the equation deduced from scheme (2): $Ckk'\,[\exp(-kt) - \exp(-k't)]/(k'-k)$, where k = 0.34 s^{-1}, k' = 0.18 s^{-1}, and C = 882 (the number of total events). The lifetimes (τ, the reciprocal of the rate constant) of the two independent complexes were 3 s and 5 s, respectively, and the sum, 8 s, agreed well with the lifetime of 8–15 s of the GroEL–GroES complex estimated from the release of GroES in the bulk-phase kinetics[10,11,13,14,21]. We carried out a similar experiment using a much higher concentration of GroES (144 nM) and obtained similar values of k and k' (data not shown).

Other than the detection of a kinetic intermediate, our observations were consistent with the current model of the GroEL–GroES reaction cycle[6]. (1) We studied a GroEL double mutant D398A/D490C (termed EL398) that can form the *cis*-ATP complex normally but hydrolyzes ATP very slowly (at ~2 % of the rate of wild-type GroEL; ref. 10). The number of fluorescent spots of Cy3-ES at the positions of IC5-labeled EL398 decreased only at the rate of photobleaching of Cy3 (τ > 200 s) (Fig. 3A), indicating that the complex is longer lived than the lifetime of Cy3. Thus, ATP hydrolysis by GroEL to form the *cis*-ADP complex is necessary for the release of GroES. (2) The *cis*-ADP complex, which we formed in bulk solution and immobilized on the glass surface, also had a lifetime longer than that of Cy3 photobleaching (data not shown), indicating that the *cis*-ADP complex is stable in the absence of ATP. (3) When ATP was provided to the *cis*-ADP complex by photolysis of caged ATP, ~85% of the Cy3-ES was released within ~1 s (data not shown). Thus, the *cis*-ADP complex decays rapidly in the presence of ATP. (4) We analyzed the effect of ATP concentration on the release of Cy3-ES at the steady state (Fig. 3A). Both k and k' remained unchanged at 2 mM and 50 μM ATP. At 20 μM ATP, k remained unchanged while k' slowed significantly, indicating that, at 20 μM ATP, ATP binding to the *trans* ring became the rate-limiting step in the release of GroES.

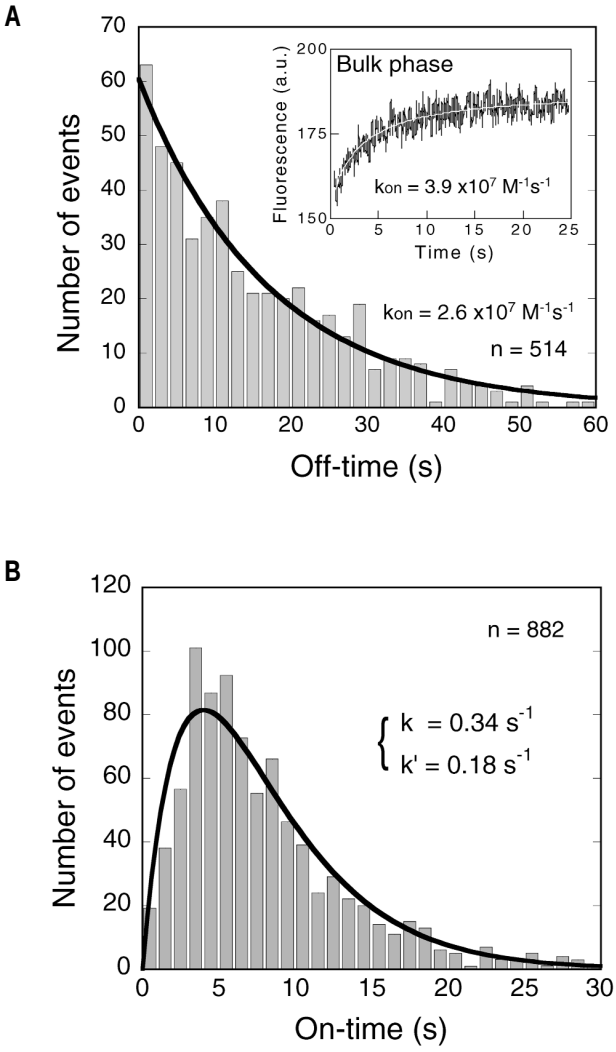

Figure 2. Statistical analysis of GroES association and dissociation with GroEL in the presence of reduced lactalbumin. (A) Histogram of the duration of "off time". The histogram data were fit by a single exponential function. (Inset) Binding of GroES to GroEL in the bulk phase. The change in the fluorescence of Cy3-ES induced by binding to GroEL was monitored after the addition of ATP at time 0. The association rate constant was obtained by fitting the following binding equation: $1/D(t) - 1/D(0) = kt$ where $D(t)$ is the concentration of unbound GroEL at time t, and k is the bimolecular rate constant for the association[11]. (B) Histogram of the duration of "on time". The solid line is the convolution of two exponentials: $Ckk'[\exp(-kt) - \exp(-k't)]/(k' - k)$ and was fit to the data by least-squares fitting. This formula is derived from the two-step reaction of scheme (2).

The effect of the denatured proteins on chaperonin dynamics. In the above experiments, we used reduced lactalbumin as a model non-native protein. We also examined GroEL–GroES dynamics in the presence of other substrate proteins: denatured malate dehydrogenase[10,22,23], pepsin, which is unfolded at neutral pH[18,24], and "random" polypeptide RP3-42, which has an artificial sequence of 139 amino acid residues and no secondary structure[25,26]. In all cases, release of GroES occurred with similar kinetics (Fig. 3B). All the decay time courses were well fit by two exponentials, which corresponded to lifetimes of 7–8 s. The transitions represented by k and k' were nearly independent of the species of non-native protein. In contrast, when the non-native protein was removed from solution, the time course slowed significantly (Fig. 3B).

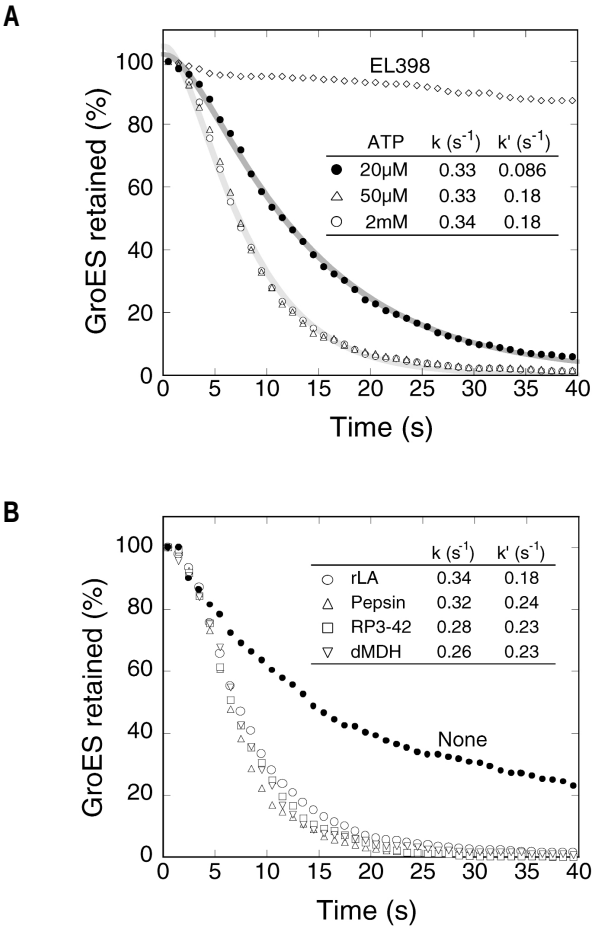

Figure 3. (A) Effect of ATP concentration on dissociation of GroES. The dissociation of Cy3-ES from IC5-EL in the presence of 20 μM, 50 μM, and 2 mM ATP. The percentage of GroES remaining at time t is obtained from the distribution of "on time" in Figure 2B, and is expressed by $N(on\text{-}time)$, the number of "on-time" events, as follows.

$$Percent\ of\ GroES\ retained\ at\ time\ t\ = \frac{[100 \times \sum_{on\text{-}time=t}^{\infty} N(on\text{-}time)]}{[\sum_{on\text{-}time=0}^{\infty} N(on\text{-}time)]}$$

It is fitted by the following equation: $C[k'\exp(-kt) - k\exp(-k't)]/(k - k')$. The rate constants are shown in the inset table. Dissociation of Cy3-ES from an ATPase-deficient GroEL mutant (EL398) is also shown (◇). EL398-bound Cy3-ES complexes decreased at the rate of photobleaching of Cy3, indicating that the complex is more stable than the lifetime of Cy3. (B) Dissociation of Cy3-ES from EL490 in the absence (●) or the presence of non-native proteins; reduced lactalbumin (rLA; ○), pepsin denatured in the neutral pH buffer (△), an artificial nonstructured polypeptide RP3-42 (□), and denatured malate dehydrogenase (dMDH; ▽). Rate constants are shown in the inset table. A trap GroEL mutant (GroEL-N265A), which traps non-native protein perpetually but does not bind GroES in the presence of ATP (refs 19,37), was included in a solution, to remove other contaminating non-native proteins such as those included for the oxygen scavenger system (●).

Discussion

The fact that all nonnative proteins tested yielded similar values for k and k' is further support for the existence of a kinetic intermediate in the GroEL–GroES reaction pathway. Several previous studies support the existence of the putative intermediate. First, a delay in GroES release was observed by atomic force microscopy[27]. Second, a

kinetic study of the nucleotide-induced allosteric transition of GroEL suggested an intermediate state of GroEL that binds both non-native protein and GroES (ref. 28). Third, analysis of the fluorescence anisotropy of the polypeptide captured by GroEL showed that, when ATP and GroES were added, the polypeptide underwent an initial transition (τ ~1.5 s) from the restricted state to the flexible state, followed by a steady decrease in flexibility, reflecting further folding[10,15]. Finally, analysis of a GroEL mutant (C138W) showed the existence of a ternary intermediate complex in which GroES caps the GroEL cavity but folding of arrested substrate protein is halted and the ATPase activity is blocked[29]. Taken together, these previous reports and our single-molecule observations support the existence of a kinetic intermediate in the GroEL–GroES reaction cycle.

In summary, our single-molecule imaging of individual GroEL–GroES complexes has shown that the release of GroES from GroEL occurs after a lag period of ~3 s. Several reports on the observation of single protein molecules by fluorescence microscopy have been published recently[27,30–35]; here this technique was adapted to study single protein–protein interactions. Our approach should be useful for future studies of the dynamic behavior of chaperonins and other protein–protein interactions.

Experimental protocol

Proteins and reagents. Bovine serum albumin, apo α-lactalbumin (type III), pepsin, bovine mitochondrial rhodanese, glucose oxidase, and catalase were obtained from Sigma (St. Louis, MO). Streptavidin was from Molecular Probes (Eugene, OR). Porcine heart malate dehydrogenase was from Roche. Random peptide RP3-42 was a gift from Dr. K. Aoki. The GroEL mutants were produced by site-directed mutagenesis using the Kunkel method[36]. EL490 was overexpressed in *E. coli* and purified as described[12,19]. Purified protein was stored as a 65% saturated ammonium sulfate suspension until use. For the labeling, EL490 was pretreated with 5 mM dithiothreitol and was purified by gel filtration on a Sephadex G-25 column equilibrated with buffer A (25 mM HEPES-KOH, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$). EL490 was labeled with IC5 maleimide (Dojindo Laboratories, Kumamoto, Japan) for 30 min at room temperature. Following IC5 labeling, biotin-$PEAC_5$-maleimide (Dojindo Laboratories) was added to 1.3-fold molar excess (relative to tetradecamer) and incubated 1 h more. We confirmed by spectral analysis that intrinsic cysteine residues remained predominantly unlabeled in these experimental conditions. The extent of labeling was determined by absorption spectroscopy. The labeled EL490 (IC5-EL) was separated from unreacted reagents by gel filtration. The molar ratio of IC5 to the EL490 (tetradecamer) was 0.5–1.3 throughout the study. Biotinylation was confirmed by western blotting using streptavidin–alkaliphosphatase conjugate. GroES were overexpressed in *E. coli* and purified as described[12]. GroES was labeled with Cy3-NHS (Fluorolink Cy3 Monofunctional Dye; Amersham-Pharmacia, Buckinghamshire, UK) in buffer A containing sodium bicarbonate to raise the pH (~8.5). Labeling resulted in a stoichiometry of 0.8–1.2 Cy3 dye molecules per GroES heptamer. Protein concentration was expressed as the oligomer (GroEL, tetradecamer; GroES, heptamer) throughout the study.

Single-molecule imaging of GroEL–GroES dynamics. A flow cell, shown schematically in Figure 1A, was made from a glass slide and coverslip with two slivers of film 50 μm thick acting as spacers. To immobilize the IC5-EL, 43 μM biotinylated bovine serum albumin in buffer A was infused into the cell. After washing with buffer A, 17 μM streptavidin and then biotinylated IC5-EL were successively flowed into the cell. Excess amounts of streptavidin or the IC5-EL were removed by washing with buffer A. The flow cell containing IC5-EL was filled with buffer A containing 4 nM Cy3-ES, the oxygen scavenger system (25 mM glucose, 2.5 μM glucose oxidase, 10 nM catalase, 10 mM dithiothreitol), 75 μM reduced lactalbumin, and, unless otherwise stated, 2 mM ATP. Binding and release of individual Cy3-ES molecules to IC5-EL were visualized by total internal reflection fluorescence microscopy. Bound IC5-EL molecules were illuminated with a He-Ne laser (1.0 mW, 632.8 nm). Cy3-ES molecules were illuminated with a green solid-state laser (2.8 mW, 532 nm, μ-Green Model 4601, Uniphase, San Jose, CA). Images were taken by an SIT camera (C2400-08, Hamamatsu Photonics, Shizuoka, Japan) coupled to an image intensifier (VS4-1845, Video Scope International, Sterling, VA) and recorded on videotapes for subsequent analysis. At least two fields of images were recorded for 5 min for each assay, and statistical analysis was made from 10 independent assays. Images were digitally captured at video rate (30 frames/s), and the fluorescence intensities of individual molecules were measured. The positions of IC5-EL were marked using a program specifically developed to interface with the Halcon image processor (MVTec Software GmbH, Munich, Germany). The fluorescence of surface-adsorbed IC5-EL disappeared in either a one-step or a two-step process, as expected for the photobleaching reactions of one- or two-dye labeled molecules, respectively. A statistical analysis of the photobleaching characteristics of individual spots was consistent with the random incorporation of the dyes to GroEL, reflecting that single GroEL molecules were immobilized on the surface. The duration of GroEL–GroES binding was determined after nine-frame averaging by marking the binding and dissociation events of GroES molecules. Nonspecific binding of Cy3-ES to the glass surface was observed as a flickering spot with a short duration (<1 s) and occurred randomly at all positions throughout the observation field. For each analysis, the nonspecific binding of Cy3-ES around the position of GroEL (21% of the total binding events) was subtracted.

Measurement of the GroES dissociation rate. The dissociation rate of GroES from the *cis*-ADP complex upon ATP binding was measured as follows. The *cis*-ADP complex was made by mixing 1.4 μM EL490, 2.8 μM Cy3-ES, and 2 mM ADP in buffer A. After 10 min incubation, it was infused into a flow cell and *cis*-ADP complex was attached to glass surface via streptavidin as described above. The solution was exchanged to buffer A containing 1 mM caged ATP and oxygen scavenger system, and the positions of the *cis*-ADP complex were marked by visualizing EL490 and Cy3-ES. ATP was released by epifluorescence illumination of UV light for 250 ms with a 100 W mercury light source (U-MWU & IX-FLA, Olympus, Tokyo, Japan). Approximately 40% of caged ATP was split to produce ATP under the experimental conditions. Fluorescence spots of Cy3-ES before and after ATP release were recorded on videotape, and the dissociation rate was determined from the decrease in the number of fluorescent spots.

Measurement of bulk-phase GroES–GroEL binding. A 4 μl aliquot of ATP was rapidly injected (0.6 mM final concentration) into 1.2 ml of buffer A containing 5 mM dithiothreitol, 10 nM GroEL(D398A) (ref. 10), 10 nM Cy3-ES, and 75 μM reduced lactalbumin at 25°C. We observed an increase in Cy3-ES fluorescence (excitation at 545 nm, emission at 563 nm) induced by binding to GroEL, which was monitored continuously with a fluorometer (F-4500, Hitachi, Tokyo, Japan). The dead time of the measurement was ~0.4 s.

Note: Supplementary information can be found on the Nature Biotechnology *website in Web Extras (http://biotech.nature.com/web_extras).*

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