A single-molecule view of chaperonin cooperativity

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According to a survey in 2000 (1), 80% of genes in *Escherichia coli* code for proteins that can assemble into dimers, trimers, hexamers, or higher-order structures, including filaments. Adding to the complexity, many of these proteins have multiple binding sites for ligands such as ATP, GTP, small metabolites, and other proteins. Such multisubunit proteins represent major challenges and opportunities in developing new experimental approaches and theoretical framework needed to comprehend their interworking. In PNAS, Jiang et al. (2) take a major step toward this goal by devising a unique single-molecule technique to analyze the conformational states of a single multisubunit enzyme.

Consider a hexameric helicase made of six subunits, each subunit contributing one ATP binding site. One can measure how many ATPs are bound to the hexamer on average or how quickly ATP is consumed as a function of ATP concentration, gaining information on whether the subunits are independently operating or if they fire collectively or sequentially, that is, if they are cooperative (3). Because these values are typically averaged over a large number of molecules, our ability to distinguish among competing microscopic models is often limited. For instance, one can obtain three ATP molecules per hexamer on average by having all proteins occupied by three ATP molecules each or by having half the molecule fully occupied and the other half unoccupied. Ideally, one would like to obtain the full distribution of the ATP number in individual hexamers, but, so far, such distribution has been seen only in computational modeling.

Jiang et al. (2) provide just that by measuring the entire distribution of the number of nucleotides bound by a single multisubunit enzyme, the type 2 chaperonin called TriC. The mammalian TriC is made of two back-to-back rings, each ring consisting of eight different but related proteins. In all, there are 16 ATP binding sites in one TriC, but how many of these 16 binding sites are occupied by ATP? By using a fluorophore-labeled ADP (Cy3-ADP) and single-molecule confocal fluorescence microscopy, Jiang et al. (2) detect single TriC complexes and observe the stepwise decreases in the fluorescence intensity caused by photobleaching (Fig. 1). Single-molecule photobleaching analysis has been used previously to deduce the stoichiometry of protein complexes in vitro (4), in living cells (5), and in the native complexes freshly pulled down from cell extracts (6). For example, three photobleaching steps would indicate the presence of three proteins in a complex, assuming 100% efficiency of labeling with a fluorophore.

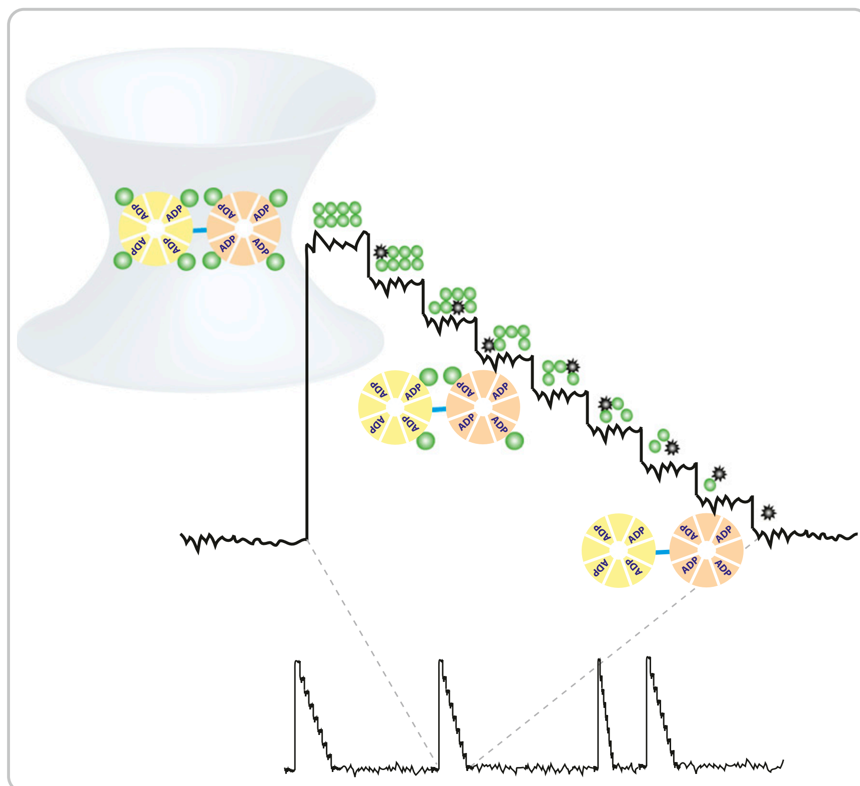

Fig. 1. One TriC multisubunit enzyme bound to Cy3-ADPs is captured in an electrokinetic trap. Upon laser illumination, Cy3 signal (black curves) decreases in a stepwise manner as illustrated via turning off of green spheres, reporting on the total number of ADPs bound per one TriC enzyme.

Because photobleaching analysis takes a few seconds under practical conditions, most previous single molecule fluorescence studies had to extend the observation time by tethering the molecule to a surface. However, tethering requires chemical modification of the molecule, and additional controls are required to make sure that there is no perturbation to function. Furthermore, tethering a multisubunit enzyme through multiple sites may interfere with its function by unnaturally constraining its conformations. Alternatively, one may use a 3D tracking system to continuously image a single particle during its diffusion (7–9), but this has not been achieved for single macromolecules free in solution as a result of rapid diffusion. Another method to avoid surface tethering is to confine the molecular motion to a small nanoscale container whose surface is inert to the molecule of interest (10, 11). A powerful and more sophisticated approach, taken in PNAS by Jiang et al. (2), is to confine the motion to pseudo-2D by using a thin slab of imaging chamber and then performing particle tracking in 2D (12). Instead of moving the laser beam to track the molecule, they applied electric field to nudge the molecule toward the laser excitation volume through electrokinetic force. This electrokinetic single-molecule trap was also used recently to study the dynamics of a photosynthetic antenna protein (13) and further refined to trap molecules smaller than 1 kDa (14).

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In their implementation, a TriC protein diffuses into the laser focal volume, which then switches on the feedback loop to keep the molecule in place. Fluorescence intensity drops in a stepwise fashion as a result of successive photobleaching of Cy3 molecules, and when the signal drops to the background level, the trap stops tracking and waits until another unsuspecting protein falls into the trap (Fig. 1). Another important feature of this scheme is its size dependence: because Cy3-ADP diffuses too quickly for the trap to follow, free Cy3-ADP released from TriC does not interfere with the enumeration of photobleaching steps. By using this approach, Jiang et al. (2) are able to determine how many ADPs remain bound to the TriC complex as a function of time, to determine the time course of ADP release, and more importantly, to compile the entire distribution of the number of ADPs bound to each TriC complex. The ADP number distribution is narrowly peaked at eight. Interestingly, as ADP dissociates from TriC over time, Jiang et al. (2) observe a decrease in the number of ADP-bound TriC molecules, but for those TriC molecules that still had any ADP, there are still eight ADPs. Therefore, ADP dissociation must be highly cooperative: when one of the eight ADP molecules has dissociated from the double octamer, the rest follow suit immediately.

The authors also incubate TriC with Cy3-ATP and AlFx at different nucleotide concentrations and determine the resulting ADP number distribution. ATP is quickly converted to ADP, and AlFx prevents ADP dissociation by forming a transition state analogue. Notably, at all ATP concentrations except the lowest, the ADP number distribution is again peaked at eight, and only the peak height decreases as ATP concentration decreases.

Standard models that can fit the average nucleotide occupancy vs. ATP concentration curve failed to account for the full distribution.

This observation suggests that (*i*) only half the ATP binding sites are stably occupied and (*ii*) ATP binding/hydrolysis is also highly cooperative. On this fact alone, TriC appears to be very similar to GroEL, which forms a back-to-back double heptamer and shows positive cooperativity within each ring and negative cooperativity between the rings (15). One possibility is that one octameric ring within a TriC complex is fully occupied by nucleotides whereas the other ring is empty. However, biochemical data presented in this study (2) and elsewhere indicate that each of the two rings contains four nucleotides each, a finding without a precedent in chaperonins.

In both experiments, single-molecule analysis reveals the full distribution, not just the average nucleotide occupancy, therefore providing a much more stringent test for possible models of intersubunit cooperativity. Indeed, standard models that can fit the average nucleotide occupancy vs. ATP concentration curve failed to account for the full distribution, demonstrating a discriminating power of the richer data set. The authors also present a working model that posits that ATP hydrolysis within an octameric ring is triggered when four ATP binding sites are occupied but not when there are fewer or more ATPs.

So what is next? There is no doubt the same approach can be readily used to investigate many other multimeric ATPases because the enzymes need not be modified for surface tethering or fluorescent labeling. It will also be exciting to determine the nucleotide occupancy while the enzyme is turning over its substrates, which will be misfolded vs. nascent proteins for TriC. In this study by Jiang et al. (2), the investigators have to use a size-exclusion column to remove free Cy3-ATP molecules before loading the sample to the single molecule trap, introducing a delay of approximately 10 min before the measurement. Otherwise, background fluorescence from Cy3-ATP in solution would have been too high for single-molecule detection. A method to limit the effective excitation volume, for example, by using a convex lens (16) or stimulated emission depletion of excited fluorophores (17), may be pursued in combination with the electrokinetic single-molecule trap to allow a similar analysis but at much higher ATP concentrations.

1. Goodsell DS, Olson AJ (2000) Structural symmetry and protein function. *Annu Rev Biophys Biomol Struct* 29: 105–153.
2. Jiang Y, et al. (2011) Sensing cooperativity in ATP hydrolysis for single multisubunit enzymes in solution. *Proc Natl Acad Sci USA*, 10.1073/pnas.1112244108.
3. Liao JC, Jeong YJ, Kim DE, Patel SS, Oster G (2005) Mechanochemistry of t7 DNA helicase. *J Mol Biol* 350: 452–475.
4. Shu D, Zhang H, Jin J, Guo P (2007) Counting of six pRNAs of phi29 DNA-packaging motor with customized single-molecule dual-view system. *EMBO J* 26: 527–537.
5. Ulbrich MH, Isacoff EY (2007) Subunit counting in membrane-bound proteins. *Nat Methods* 4:319–321.
6. Jain A, et al. (2011) Probing cellular protein complexes using single-molecule pull-down. *Nature* 473: 484–488.
7. Levi V, Ruan Q, Gratton E (2005) 3-D particle tracking in a two-photon microscope: application to the study of molecular dynamics in cells. *Biophys J* 88:2919–2928.
8. Cang H, Wong CM, Xu CS, Rizvi AH, Yang H (2006) Confocal three dimensional tracking of a single nanoparticle with concurrent spectroscopic readouts. *Appl Phys Lett* 88:223901.
9. Berglund A, Mabuchi H (2005) Tracking-FCS: Fluorescence correlation spectroscopy of individual particles. *Opt Express* 13:8069–8082.
10. Boukobza E, Sonnenfeld A, Haran G (2001) Immobilization in surface-tethered lipid vesicles as a new tool for single biomolecule spectroscopy. *J Phys Chem B* 105:12165–12170.
11. Cisse I, Okumus B, Joo C, Ha T (2007) Fueling protein DNA interactions inside porous nanocontainers. *Proc Natl Acad Sci USA* 104:12646–12650.
12. Cohen AE, Moerner WE (2006) Suppressing Brownian motion of individual biomolecules in solution. *Proc Natl Acad Sci USA* 103:4362–4365.
13. Goldsmith RH, Moerner WE (2010) Watching conformational- and photo-dynamics of single fluorescent proteins in solution. *Nat Chem* 2:179–186.
14. Fields AP, Cohen AE (2011) Electrokinetic trapping at the one nanometer limit. *Proc Natl Acad Sci USA* 108: 8937–8942.
15. Yifrach O, Horovitz A (1995) Nested cooperativity in the ATPase activity of the oligomeric chaperonin GroEL. *Biochemistry* 34:5303–5308.
16. Leslie SR, Fields AP, Cohen AE (2010) Convex lens-induced confinement for imaging single molecules. *Anal Chem* 82:6224–6229.
17. Hell SW, Wichmann J (1994) Breaking the diffraction resolution limit by stimulated emission: Stimulated-emission-depletion fluorescence microscopy. *Opt Lett* 19:780–782.